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*J Immunol* published online 16 March 2012
http://www.jimmunol.org/content/early/2012/03/16/jimmunol.1100718

Supplementary Material http://www.jimmunol.org/content/suppl/2012/03/16/jimmunol.1100718

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Vigorous Response of Human Innate Functioning IgM Memory B Cells upon Infection by Neisseria gonorrhoeae

Nancy S. Y. So,* Mario A. Ostrowski, † and Scott D. Gray-Owen*

Neisseria gonorrhoeae, the cause of the sexually transmitted infection gonorrhea, elicits low levels of specific Ig that decline rapidly after the bacteria are cleared. Reinfection with the same serovar can occur, and prior gonococcal infection does not alter the Ig response upon subsequent exposure, suggesting that protective immunity is not induced. The mucosal Ig response apparent during gonorrhea does not correlate with that observed systemically, leading to a suggestion that it is locally generated. In considering whether N. gonorrhoeae directly influences B cells, we observed that gonococcal infection prolonged viability of primary human B cells in vitro and elicited robust activation and vigorous proliferative responses in the absence of T cells. Furthermore, we observed the specific expansion of IgD^+CD27^+ B cells in response to gonococcal infection. These cells are innate in function, conferring protection against diverse microbes by producing low-affinity, broadly reactive IgM without inducing classical immunologic memory. Although gonococcal infection of B cells produced small amounts of gonococcal-specific IgM, IgM specific for irrelevant Ags were also produced, suggesting a broad, polyspecific Ig response. The gonococci were effectively bound and engulfed by B cells. TLR9-inhibitory CpGs blocked B cell responses, indicating that intracellular bacterial degradation allows for innate immune detection within the phagolysosome. To our knowledge, this is the first report of a bacterial pathogen having specific affinity for the human IgM memory B cells, driving their potent activation and polyclonal Ig response. This unfocused T-independent response explains the localized Ig response that occurs, despite an absence of immunologic memory elicited during gonorrhea. The Journal of Immunology, 2012, 188: 000–000.

N. gonorrhoeae (gonococci) is a human-specific pathogen that causes the sexually transmitted infection gonorrhea and is a major cause of morbidity. The rate of gonorrhea infection is increasing worldwide, with a recent estimate of 88 million new infections per year (1). Gonorrhea results from overwhelming inflammation, producing purulent discharge at the site of infection. The majority of women infected with N. gonorrhoeae are clinically asymptomatic (2). Left untreated, this can progress to serious complications including ectopic pregnancies because of fallopian tube scarring, sterility, or acquiredblindness because of gonococcal conjunctivitis in children born to infected mothers (3). Once infection is detected, gonorrhea can be effectively treated with antibiotics; however, antibiotic resistance is increasing at an alarming rate, and gonococcal infections may soon become untreatable (4, 5). Gonococcal coinfection can also increase viral shedding in HIV patients (6), further heightening the urgency to control the spread of this important pathogen.

Received for publication March 11, 2011. Accepted for publication February 15, 2012.

This work was supported by Canadian Institute of Health Research Operating Grant 414775.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CEA, carcinoembryonic Ag; CEACAM, carcinoembryonic Ag-related cell adhesion molecule; HSPG, heparan sulphate proteoglycan; iCpG, inhibitory CpG ODN TTAGGG; KLH, keyhole limpet hemocyanin; MAMP, microbial-associated molecular pattern; MOI, multiplicity of infection; ODN, oligodeoxynucleotide; OpaM13A/C19, carcinoembryonic Ag-related cell adhesion molecule-specific Opa; OpaHSPG, heparan sulfate proteoglycan-specific Opa; TT, tetanus toxoid.

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male and female genital tract is distinct from that of other mucosal surfaces in that IgG predominates rather than IgA (17). Although the levels of total mucosal IgG were slightly lower in gonococcal-infected versus uninfected women, total mucosal IgM increased in those with gonorrhoea (7). Gonococcal-specific IgM was present in 59% of cervical mucus samples obtained from women with gonorrhoea but were undetectable in samples obtained from individuals who were uninfected (18). Curiously, the Ig response in serum did not reflect that found in cervical mucus or vaginal washes, suggesting that Ig production was localized rather than being a systemic response (7). The production of IgM in local secretions did not require active gonococcal infection, because antibiotic therapy to eliminate the infection correlated with a decrease in gonococcal-specific IgM in cervical mucus samples in all women who were treated (18). Puzzlingly, the antigenococcal response was not greater against the infecting strain, because it frequently cross-reacted equally or, in some cases, better with the prototypical but antigenically distinct N. gonorrhoeae MS11 (7).

Such clinical data prompted us to consider that, in addition to suppressing T cell responses (12), the gonococci may directly affect B cell function. Previous work suggested that gonococcal infection kills human B cells in a CEACAM1-dependent manner (19); however, this does not explain the elevated Ig levels during N. gonorrhoeae infection. Alternatively, because the production of IgM by human B cells does not require supplemental signaling from T cells (20, 21), it is enticing to consider that gonococcal infection could directly induce the production of IgM, thereby explaining the clinical observations.

N. gonorrhoeae is strictly a human pathogen, with several virulence factors that show exquisite host specificity. As such, no mouse model of infection exists that accurately mimics the progression of gonococcal disease in the human host. Furthermore, taking into account the intrinsic differences between human and mouse B cells [e.g., TLR4 signaling induces strong responses in murine B cells but is not detected in human B cells (20, 22)], we developed in vitro infection protocols using purified primary human B cells. Unexpectedly, and in stark contrast to parallel protocols developed in vitro infection protocols using purified primary murine B cells but is not detected in human B cells (20, 22], we observed that N. gonorrhoeae strains 2061 and 2066 were isolated from male urethra. Immunoblot analysis indicates that strain 2061 does not express any Opa protein, N2066 expresses at least one Opa adhesin, and neither strain expresses pili. These clinical strains were obtained from a sexually transmitted disease clinic in the district of Pumwani in Nairobi, Kenya, and were a gift from Dr. F.A. Plummer (University of Manitoba, Winnipeg, MB, Canada). Neisseria species were grown from frozen stocks on GC agar (Difco) supplemented with 1% (v/v) IsoVitalex (BBL Microbiology Systems) at 37°C in a 5% CO2 atmosphere with humidity. A binocular microscope was used for daily selection of gonococcal colony opacity phenotypes for the MS11 strains. Opa protein expression was routinely monitored by immunoblotting using the Opa cross-reactive mAb 4B12/ C11 (25), provided by Prof. M. Achtman (Environmental Research Institute, University College Cork, Cork, Ireland).

E. coli (DH5α) was grown from frozen stocks in aerated Luria–Burtani broth overnight at 37°C. Liquid cultures were subcultured onto Luria–Burtani agar prior to infection assays.

Infection of lymphocytes

Freshly isolated human B cells were infected with a multiplicity of infection (MOI) of 10 bacteria per cell, unless otherwise indicated. Gentamicin (Biochip) was routinely added to infected samples 3 h after bacterial addition to prevent overgrowth. For E. coli, gentamicin was added immediately upon infection to prevent bacterial overgrowth of the B cell cultures. Although the gentamicin effectively inhibited bacterial growth, it did not otherwise affect observed B cell responses.

Confocal microscopy

B cells were infected with the indicated N. gonorrhoeae and E. coli strains at an MOI of 10 for durations between 1.5 and 44 h. Acid-washed glass coverslips were coated with mouse mAb specific for human CD19 (clone HB19; eBioscience) to capture B cells for microscopy. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), and gonococci were detected using a polyclonal rabbit anti-gonococcal rabbit polyclonal serum (UTR01), followed by Alexa 488-conjugated secondary Ab (Invitrogen-Molecular Probes) after permeabilization of mammalian cell membranes using 0.4% Triton X-100 (Sigma-Aldrich). E. coli was detected using the purified polyclonal goat Ab ab25823 (Abcam), followed by a goat-specific secondary Ab in a process similar to the gonococcal staining protocol. Where indicated, bacteria were prelabeled using Texas Red succinimidyl ester (Molecular Probes) rather than using anti-Opa. B cells were stained using Fab′(1) of biotinylated goat anti-human IgM, IgG, and IgA (Jackson ImmunoResearch Laboratories), followed by streptavidin-Alexa 488 (Molecular Probes).

To examine whether primary human B cells were able to engulf whole bacteria, cells were infected with the indicated N. gonorrhoeae strains at an MOI of 10 for 3 h. Acid-washed coverslips coated with the human CD19-specific Ab clone HIB19 were used to capture B cells prior to fixation. Extracellular gonococci were stained first, using a polyclonal rabbit anti-gonococcal serum (UTR01), followed by anti-ribb IgG Alexa 647 (Molecular Probes). Mammalian cell membranes were permeabilized using 0.4% Triton X-100 (Sigma-Aldrich), allowing for total gonococci to be stained using the polyclonal rabbit antigonococcal serum (UTR01), followed by anti-ribb IgG Alexa 488 (Molecular Probes).

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IgG, IgM, or IgA (Jackson ImmunoResearch Laboratories). CD27 and IgD immunostaining was performed on live cells, whereas all other immunostaining was performed on fixed cells. CompBeads (BD Biosciences) were used for fluorescent compensation. All fixed cells were fixed prior to flow analysis.

To identify the B cell subpopulations associated with gonococci or *E. coli*, live bacteria were labeled with Alexa 647 succinimidyl ester ( Molecular Probes) and then used to infect purified B cells at an MOI of 2 bacteria per cell for 3 h before treatment with gentamicin. Bacterial association to B cell subpopulations was visualized by flow cytometry.

For viability, activation, and proliferation assays, infected cells and uninstructed controls were fixed at time points indicated in the figure legends. Where indicated, 16 μM staurosporine (Sigma-Aldrich) was added to uninstructed cells to induce B cell death as a control for viability experiments. Forward- and side-scatter data and annexin V-PE (BD Biosciences) staining were used to quantify cell death. Cellular activation was quantified using CD69-PE (clone FUN-1; BD Biosciences) and forward- versus side-scatter data, because activated cells increase in forward scatter compared with cells at rest.

To examine proliferation, B cells were infected overnight with bacteria or were left uninstructed, at which point BrdU (Sigma-Aldrich) was added to the culture media. BrdU incorporation was quantified using monoclonal mouse anti-BrdU (clone IU-4; Invitrogen-Caltag Laboratories), which was directly conjugated to biotin using Zenon Mouse IgG Labeling Kit ( Molecular Probes). Streptavidin-allophycocyanin (Jackson ImmunoResearch Laboratories) was then used to visualize the Ab for detection by flow cytometry. To identify and quantify populations of cells undergoing proliferation at the time of fixation, Ki67-Alexa 647 (clone B56; BD Biosciences) was used. To confirm the B cell response, and to assess whether gonococci associated with CD27–IgD+ B cells with the following reagents (all from InvivoGen): Pam3CSK4 at 2.5 μg/mL, FSL1 at 1 μg/mL, and CpG ODN2006 at 5 μg/mL for 3 d, at which time they were stained for activation marker CD69-PE (clone FUN-1; BD Biosciences) or nuclear proliferation Ag, Ki67-Alexa 647 (clone B56, BD Biosciences), and B cell subpopulation markers CD27 and IgD as described above.

To examine the role of TLR9 signaling in *N. gonorrhoeae* infection of B cells, inhibitory CpG oligodeoxynucleotide (ODN) TTAAGG (iCpG; InvivoGen) was used, which functions as a dominant-negative TLR9 ligand. B cells were either left untreated/uninfected, treated with CpG ODN2006 (InvivoGen) at 0.65 nmol/mL or infected with *N. gonorrhoeae* strain N302 Opa+ at an MOI of 10. Cell cultures were then treated with either CpG or ODN TTAAGG control (cCpG; InvivoGen), a control ODN that possess similar sequence to the iCpG but without the ability to inhibit TLR9 signaling, both at 10-fold molar concentrations of stimulating CpG ODN2006 for 3 d, at which time cells were stained for Ki67-Alexa 647 (clone B56; BD Biosciences) and B cell subpopulation markers CD27 and IgD as already described previously.

**ELISA**

All ELISAs were read at 450 nm using a 1420 Victor*2* (PerkinElmer) plate reader, unless otherwise indicated. Total Ig in cell culture supernatant was quantified by IgM, IgG, or IgA ELISA kits (Zetametric), used according to manufacturer’s instructions.

To monitor Ag-specific Ig production, 96-well Maxisorp plates (Nunc) were coated with either heat-killed Opa gonococci (N302), keyhole limpet hemocyanin (KLH; Sigma-Aldrich), or tetanus toxoid (TT; List Biological Labs) resuspended in pH 7.4 PBS (Wisent). The plates were dried under a vacuum and stored at 4°C until use. Five percent BSA (Bioshop) in PBS was used to block wells, and 0.05% Tween 20 (Sigma-Aldrich) in PBS was used as wash buffer. Culture supernatants from uninfected cells or those infected with either gonococci or *E. coli* were diluted using 1% BSA in 0.05% Tween 20.

For KLH and TT ELISAs, biotinylated F(ab)’2 fragments of goat anti-human IgM, IgG, and IgA (Jackson ImmunoResearch Laboratories) were used separately to determine the amount of each class of Ig produced in response to bacterial infection. After incubation with streptavidin HRP Ultra (Sigma-Aldrich), 3′,3′,5′-Tetramethylbenzidine (KPL) was used as the colorimetric reagent for HRP activity.

For *N. gonorrhoeae* plates, F(ab)’2 fragments of goat anti-human IgG, IgM, and IgA directly conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories) were used separately to determine the amount of each class of Ig present in culture supernatants. BluePhos (KPL) was used as the developing reagent. These ELISAs were read at 595 nm.

**Gentamicin protection assay**

To examine the viability of engulfed intracellular gonococci, B cells were infected with an MOI of 50 with the indicated *N. gonorrhoeae* strains for 1.5 h. Infection cultures were then treated with and maintained in 100 μg/ml gentamicin (Biohop) to kill any extracellular (nonengulfed) gonococci. At 2.5 and 6 h postinfection, cells were washed and concentrated in PBS containing 1 mM MgCl₂ and 0.5 mM CaCl₂ using Transwells with a 3-μm pore size to retain the cells (Corning). One-percent saponin (Sigma-Aldrich) was added to lyse mammalian membranes. Serial dilutions of the lysis mixture were plated on GC agar (Difco) supplemented with 1% (v/v) IsoVitalex (BBL Microbiology Systems) and incubated at 37°C in a 5% CO₂ atmosphere with humidity for at least 48 h before enumeration of bacterial colonies.

**Statistics**

Differences between experimental conditions were determined to be significant when *p* < 0.05 by one-way ANOVA with a Dunnett’s posttest, calculated by Prism 5.0 (GraphPad).

**Results**

*N. gonorrhoeae* associates with human peripheral blood B cells

*N. gonorrhoeae* adheres directly to primary human CD4⁺ T cells in an Opa protein-dependent manner, allowing effective suppression of T cell activation (13). To establish whether *N. gonorrhoeae* also interacts with primary human B cells, we exposed freshly purified cells to isogenic strains of gonococci expressing either no Opa adhesin, Opa₅₃₋₅₅, or Opa₁₋₇₅₋₇₉, and then monitored their association with the B cells over time using confocal microscopy. Cellular association with the prototypical Gram-negative bacterium *E. coli* was also examined to assess the general propensity of B cells to bind bacteria. To confirm that bacteria were associating with B cells and not a copurified cell type, the infected cells were stained for BCR, as depicted in Fig. 1A. We then quantified the percentage of B cells that were associated with at least one bacterium. As illustrated in Fig. 1B, substantially more B cells were associated with gonococci than *E. coli*, regardless of which neisserial adhesin was expressed. However, unexpectedly, even after 44 h of infection, some B cells did not associate with gonococci, suggesting that binding may be restricted to a specific subpopulation of cells. Moreover, no recruitment of BCR to sites of bacterial attachment was evident, indicating that this association is independent of BCR clustering, a strategy used by other bacteria (26).

Although Fig. 1B quantifies the percentage of B cells with at least one bacterium bound, this does not illustrate how many bacteria associate with each cell. We quantified the number of bacteria per cell (Fig. 1C) and observed that most infected B cells are associated with one to five bacteria per cell. The ability of gonococci to associate with cells is largely Opa-independent (compare Opa⁻ gonococci), although Opa expression did have some influence on bacterial association with the lymphocytes in some donors (e.g., compare donors 02L and 07L).

Gonococcal infection inhibits B cell death and promotes prolonged B cell viability

Previous work suggested that in the presence of potent stimulation, infection of primary human B cells with *N. gonorrhoeae* caused B cell death (19). This could be explained either by a direct cytotoxic effect of the gonococci or by *N. gonorrhoeae* contributing to activation-induced cell death. We explored whether *N. gonorrhoeae* infection affected the viability of freshly purified primary human B cells by monitoring changes in cell morphology and membrane integrity during infection experiments. Loss of membrane integrity is an early event of cell death and results in a decrease in forward scatter and increase in side scatter, reflecting corresponding changes in cell size and granularity, respectively (Fig. 2A). Annexin V binding to phosphatidylinerine exposed on a depolarized cell membrane is also a well-established method to measure cell viability (27). To confirm the positioning of the live
and dead populations within flow cytometry plots, B cell death was induced by treating cells with staurosporine. Comparison between gated populations in Fig. 2A with annexin V staining profiles of these gates in Fig. 2B verify the positioning of live and dead cells.

Infections using a range of MOIs for both \textit{N. gonorrhoeae} and \textit{E. coli} were conducted in pilot experiments to assess their effect on B cell viability. Infection with \textit{E. coli} at an MOI of >10 resulted in rapid B cell death (Supplemental Fig. 1A). Although a similar effect was not apparent with \textit{N. gonorrhoeae} (Supplemental Fig. 1B), we selected an MOI of 10 for our standard assay system because it allowed us to compare the effects of gonococcal infection with \textit{E. coli} over time.

Primary human B cells are notoriously difficult to maintain in viable culture. However, for each individual donor (as indicated by different symbols within each plot), the percentage of viable cells are significantly increased upon infection with 10 gonococci/cell relative to those remaining uninfected, as indicated by forward and side scatter (Fig. 2C). Cells exposed to \textit{E. coli} at the same MOI were morphologically indistinguishable from the uninfected controls, whereas \textit{N. gonorrhoeae} infection consistently boosted B cell viability. Moreover, the percentage of gonococci-infected B cells labeled with soluble annexin V was lower than cells either left uninfected or exposed to \textit{E. coli} (Fig. 2D). These results confirm that the protective effect of \textit{N. gonorrhoeae} is not a general response to bacteria. It is pertinent to note that, although \textit{N. gonorrhoeae} association with primary human B cells. (A) B cells were infected with a MOI of 10 of either Texas Red-labeled \textit{N. gonorrhoeae} expressing no Opa adhesin, Opa\textsubscript{HSPG}, or Opa\textsubscript{CEA}, or Texas Red-labeled \textit{E. coli} for 6 h and then visualized by confocal microscopy. Scale bar, 5 \(\mu\text{m}\). (B) B cells were infected as described in (A) but using unlabeled bacteria that were stained postinfection, and bacterial association was assessed over a time course by confocal microscopy. (C) The total number of bacteria associated per cell was quantified 3 h postinfection, considering only cells that have at least one bacteria bound (excluding cells that did not associate with bacteria). Data for two representative donors are shown. DIC, differential interference contrast.
Infection of primary human B cells with gonococci clearly increased viability of the B cells, it did not protect B cells from apoptotic death induced upon treatment with staurosporine (data not shown). When considered together, these data indicate that *N. gonorrhoeae* infection supports the survival of primary human B cells and that this effect occurs regardless of Opa protein expression, suggesting that it is not CEACAM1 dependent.

*N. gonorrhoeae* elicits robust B cell activation

During flow cytometric analysis, we consistently observed a striking increase in B cell size and granularity upon exposure to *N. gonorrhoeae*, characteristic of a blasting phenotype that is induced by BCR cross-linking (Fig. 3A, 3B). Flow cytometry for one representative donor is illustrated in Fig. 3B, displaying robust B cell blasting in response to either BCR cross-linking or infection with *N. gonorrhoeae* while not occurring in cells left uninfected or infected with *E. coli*. To compare multiple donors, the proportion of blasting cells in each sample was normalized against the percent of total live cells, the gating of which is reflected in Fig. 3B. As evident in Fig. 3C, the proportion of blasting cells is significantly higher in *N. gonorrhoeae*-infected but not *E. coli*-infected cells compared with the uninfected samples. This effect is consistent among all donors examined and occurs regardless of Opa protein expression.

Because blasting is usually indicative of cellular activation, we measured the expression of activation-induced costimulatory molecule CD86 on cells infected with *N. gonorrhoeae*. Fig. 3D illustrates that gonococcal infection, but not *E. coli* infection, induces significantly more cells to express CD86 than when cells were left uninfected. Combined, these results demonstrate a potent activation response that is specific to the gonococci.

*N. gonorrhoeae* alone can provide the necessary signals to induce proliferation of B cells in a T-independent manner

If cells receive the appropriate combination of signals, they will not only become activated but will also proliferate. To ascertain whether simple exposure to *N. gonorrhoeae* was sufficient to promote B cell proliferation, BrdU incorporation was used to observe genome replication. Fig. 4A illustrates that infection with gonococci promotes significantly increased BrdU incorporation by B cells, compared with uninfected cells. Proliferation of B cells infected with *E. coli* tended to reflect that of uninfected cells, although the extent varied slightly between donors (Fig. 4B); this again illustrates that B cell proliferation is not a general response to microbial-associated molecular pattern (MAMP) molecules or other effects of bacterial infection. However, kinetic analysis of infection for all donors shows progressive B cell accumulation of BrdU in response to infection with gonococci, consistent with proliferation as a specific and reproducible response to *N. gonorrhoeae* (Fig. 4B).

Gonococcal interaction with B cell populations is specific and favors binding to IgM memory B cells

Because it was clear that some but not all B cells associated with the gonococci (Fig. 1B, 1C), we considered whether cellular responses to infection correlated with the action of a specific B cell subset. In human peripheral blood, naïve, IgM memory, and switched memory B cells can be differentiated by the combined expression of CD27, CD10, and IgD. Fig. 5A illustrates that gonococcal infection, but not *E. coli* infection, induces significantly more cells to express CD86 than when cells were left uninfected. Combined, these results demonstrate a potent activation response that is specific to the gonococci.

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FIGURE 3. A robust activation response is observed by primary human B cells infected with *N. gonorrhoeae* but not with *E. coli*. All B cell cultures were infected for 3 d with either gonococci or *E. coli* at an MOI of 10 prior to cellular activation analysis. (A) B cells activated for 3 d with 10 μg/ml BCR cross-linker (anti-BCR) displayed a typical blasting phenotype, with an increase in cell size, as reflected in the shifting of the live cells along the forward-scatter axis. Note that the live gate encompasses both blasting and non-blasting viable cells. Representative of two independent experiments with different donors. (B) Blasting cells were observed upon infection with *N. gonorrhoeae* but not in uninfected or *E. coli*-infected cells. Compare the anti–BCR-activated cells in the live gate with *N. gonorrhoeae* or *E. coli*-infected cells. Representative of at least six independent experiments with different donors. (C) Quantification of the percent of cells that acquired a blasting phenotype after 3 d of infection with *N. gonorrhoeae* or *E. coli*. The percentage of blasting cells was obtained by taking the percentage of blasting cells (blasting gate) divided by percentage of total live cells (live gate). (D) Expression of costimulatory receptor and activation marker CD86 on B cells that were infected with either gonococci or *E. coli*. Bars indicate mean. Asterisks indicate a comparison of costimulatory receptor and activation marker CD86 on B cells that were infected with either gonococci or *E. coli*. Bars indicate mean. Asterisks indicate a comparison between uninfected and infected cells. *p < 0.05, **p < 0.001. Data points corresponding to the same individual donor are represented by the same symbol within each plot.

memory cell lineage, whereas IgD is primarily expressed on naive cells only.

Freshly purified IgD<sup>+</sup>CD27<sup>−</sup> (naive) B cells primarily express surface IgM (Fig. 5B). IgD<sup>+</sup>CD27<sup>+</sup> (IgM memory) B cells also consistently express surface IgM; however, IgG- and IgA-expressing cells also exist within this population (Fig. 5B), suggesting that these cells have recently switched Ig classes (29). Most IgD<sup>−</sup>CD27<sup>+</sup> (switched memory) B cells have undergone Ig class switching to express surface IgG or IgA (Fig. 5B). Although the relative proportion of B cells that represent each subpopulation differed slightly between donors, the average proportion of each subpopulation in freshly purified uninfected PBMC preparations was 57% naive (IgD<sup>+</sup>CD27<sup>−</sup>), 26% IgM memory (IgD<sup>−</sup>CD27<sup>+</sup>), and 11% switched memory (IgD<sup>−</sup>CD27<sup>+</sup>) B cells (data not shown).

To identify which populations of B cells were associating with *N. gonorrhoeae*, we prepared live fluorescently labeled *N. gonorrhoeae* or *E. coli* and used these to infect purified B cells for 3 h with a low MOI of 2 bacteria per cell. Although *E. coli* binding to all peripheral B cell subsets was very low, gonococci displayed substantial binding to naive, IgM memory, and switched memory populations of B cells (Fig. 5C). Comparable binding by the three isogenic gonococcal strains suggests that this association is not strictly dependent on receptors for either Opa variant, although the ability of the B cell subpopulations to bind gonococci expressing these different adhesins is not equal (Fig. 5D). Surprisingly, we observed that the simple expression of Opa adhesin did not translate into higher levels of bacterial binding, because Opa<sub>2</sub> expression of gonococci associated less with the B cells than did the strain that expresses no adhesin (Opa<sub>−</sub>; Fig. 5D). In general, both IgM memory and switched memory subsets display a greater affinity for gonococci than do naive cells, but the gonococci had a particular affinity for the IgD<sup>−</sup>CD27<sup>+</sup> IgM memory B cells (Fig. 5C, 5D). Strikingly, up to 80% of these IgM memory B cells were associated with the gonococci. Although it is possible that, in rare instances, some gonococcal recognition could be mediated through the BCR, it is clear that this is not the primary mechanism of binding because it is unreasonable to assume this large proportion of B cells are *N. gonorrhoeae* specific in all donors examined. The lack of BCR involvement is also consistent with the fact that we observed no BCR recruitment to the bound bacteria during our immunofluorescence-based studies (Fig. 1A).

There was little association of any B cell subpopulations with *E. coli*, consistent with the interaction being unique to the gonococci.

IgM memory B cell population expands in response to infection with *N. gonorrhoeae*

Considering that B cells proliferate in response to gonococcal infection (Fig. 4), we examined whether one or more B cell subset(s) were specifically induced. After 5 d of infection, the IgD<sup>+</sup>CD27<sup>+</sup> IgM memory cell population had clearly expanded in response to gonococci, but not to *E. coli*, in all donors (Fig. 6). This increase in the IgM memory cells was reflected by a tendency toward a reduction in the naive and, less so, switched memory B cell populations. However, in contrast to the significant increase in IgM memory cells, the apparent changes in naive and switched memory cells were not statistically significant (Fig. 6B). Therefore, despite variability in the proportion of each of the B cell subpopulations between individuals, there is a consistent expansion of the IgM memory B cell subset in response to gonococci. Moreover, the different levels of bacterial association conferred by
any B cell subpopulation (Fig. 7). The potent IgD^+CD27^+ IgM memory cell response thus appears to be an intrinsic feature of *N. gonorrhoeae* rather than being a strain-specific effect.

**Polyreactive IgM is produced by B cells infected with *N. gonorrhoeae***

Although naive B cells require AgR signaling for the production of Ig, IgM memory B cells have the potential to produce Ab in a T-independent manner, both in the presence and absence of Ag receptor signaling (30). For example, Cpg DNA has been shown to induce Ig production by the IgM memory B cells, even in the absence of BCR signals (30). This prompted us to examine whether Ig production could be elicited by *N. gonorrhoeae* infection of purified primary human B cells. As depicted in Fig. 8A, IgG, IgM, and IgA classes of Ab were all induced in significant amounts upon infection with gonococci relative to uninfected cells. This effect was not apparent upon infection with *E. coli*, indicating that it is not a generalized response to bacteria or bacterial-derived products such as endotoxin or other MAMPs. IgM is the principal class of Ig elicited in response to *N. gonorrhoeae*, with ~20-fold induction versus the uninfected controls (Fig. 5A). Neither Opa expression nor the relative level of association by the isogenic neisserial strains with B cells (Fig. 5C, 5D) correlated with total Ig titers induced or the relative proportion of individual Ig classes (Fig. 8A). Considering that the study population was not believed to have high-risk sexual behaviors and are therefore not uniformly exposed to *N. gonorrhoeae*, it is unlikely that this is a result of conventional memory recall responses.

To examine whether gonococcal infection produced *N. gonorrhoeae*-specific Ab, we prepared ELISA plates coated with the Opa^− strain of gonococci. Using this strain to measure Ig responses against all three isogenic *N. gonorrhoeae* strains eliminates potential skewing of results caused by responses to the strongly immunogenic Opa proteins (31). *N. gonorrhoeae* infection elicited a small increase in gonococci-specific IgM (Fig. 8B), whereas no detectable gonococci-specific IgG or IgA was evident (data not shown). Importantly, the baseline amount of *N. gonorrhoeae*-reactive Ig in *E. coli*-infected samples closely reflected that in uninfected samples, indicating that the observed increase in Ig required exposure to the gonococci.

The relatively low increase in *N. gonorrhoeae*-specific Ig did not reflect the significant responses apparent when total Ig was measured (Fig. 8A). To assess the specificity of the Ig produced, we performed ELISAs to monitor the production of Abs specific for other, nonneisserial Ags upon B cell infection. For this, we measured Ig specific for a recall Ag, TT, as well as an irrelevant Ag, KLH. Although little TT-specific Ig was apparent in uninfected samples, significant increases in IgM specific for TT were apparent in the culture supernatants of all *N. gonorrhoeae*-infected cells (Fig. 8C) and the purified B cells from 5 of 10 donors produced TT-specific IgG upon exposure to the gonococci (data not shown). Considering that a recall response to TT would be expected to primarily be IgG, this implies that the *N. gonorrhoeae*-induced TT-specific IgM observed is not a conventional memory response. TT-specific Abs were not detected in *E. coli*-infected samples (Fig. 8C), again indicating that this is not a generalized response to bacteria-derived products. B cells from the majority of donors (90%) also produced IgM reactive with KLH following infection with gonococci but not *E. coli* (Fig. 8D), even though we would not expect any of the volunteer blood donors to have been exposed to this Ag previously. When combined, these results indicate that *N. gonorrhoeae* infection of primary B cells results in broad, polyclonal activation and differentiation of IgM-bearing B cells rather than a focused, clonal response to the gonococci.
B cell responses to N. gonorrhoeae infection involve TLR9

Because all strains of N. gonorrhoeae examined produced robust proliferation in the IgM memory population of B cells, we explored the possibility that gonococci were being detected by innate immune receptors, resulting in downstream proliferation and differentiation of B cells. It has already been established that TLR expression varies between human peripheral B cell subpopulations, such that naive cells express few TLRs, each at very low (to undetectable) levels when they are present, whereas both IgM memory and switched memory subsets can express high levels of TLR6, 7, 9, and 10 (20). Neisseria species are known to constitutively express outer membrane porins that can be detected by complexes of TLR2 and TLR1 (32, 33), in addition to possessing a lipo-oligosaccharide that is recognized by TLR4 (34). In this study, we systematically examined primary human B cellular responses to defined TLR agonists. To assess functional TLR responses in the B cell subsets, we examined a panel of specific TLR agonists—Pam3CSK4 (a synthetic triacylated lipoprotein that signals through TLR2/1), FSL1 (a synthetic diacylated lipoprotein that signals through TLR2/6), E. coli K12 LPS (TLR4), and CpG ODN2006 (TLR9)—for their ability to cause activation and proliferation. Surprisingly, when considered in the context of TLR expression-based studies (20), naive cell activation was significantly increased in response to the TLR2/6 agonist FSL1 and TLR9 agonist CpG ODN2006 (Fig. 9A). Naive cells were weakly activated in response to treatment with TLR2/1 agonist Pam3CSK4; however, this difference did not reach statistical significance (Fig. 9A). CD27+ memory B cells responded to Pam3CSK4, FSL1, and CpG

FIGURE 5. Three populations of B cells are found in human peripheral blood, and although gonococci are able to bind to all of them, N. gonorrhoeae association is strongest with IgM memory B cells. (A) The populations of B cells found in human peripheral blood can be differentiated by the combined expression profiles of CD27 and IgD. We show freshly isolated B cells from one representative donor can be separated into the three B cell populations found in human peripheral blood: naive (IgD+,CD27-), IgM memory (IgD+,CD27+), and switched memory (IgD-,CD27+). Representative of four independent experiments with different donors. (B) Expression of IgM, IgG, or IgA was examined in uninfected cells by gating on the B cell subpopulations. Solid line, IgG; dotted line, IgA; and dashed line, IgM. Representative of four independent experiments with different donors. (C) B cells were infected with a low MOI of 2 for 3 h to examine bacterial binding patterns. The percentage of bacteria bound to each population of cells was determined by flow cytometric analysis. (D) The results in (C) are regraphed to allow for ease of comparison, examining how the different cell populations associate with the different strains of N. gonorrhoeae or E. coli. Bars indicate mean. Asterisks indicate a comparison between gonococcal and E. coli-infected cells. **p < 0.01, ***p < 0.001. Data points corresponding to the same individual donor are represented by the same symbol within each plot.
ODN2006 with strong and significant increases in cellular activation (Fig. 9A). Treatment with LPS did not induce any response in any of the B cell populations (Fig. 9A, 9B), confirming previously published reports that TLR4 is not expressed in high enough levels in any human B cell subset to facilitate signaling (20, 35).

Although naive cells expressed the CD86 activation marker upon treatment with some TLR agonists, proliferation was not observed in these cells in response to any of the TLR agonists tested (Fig. 9B). Both IgM memory as well as switched memory B cells underwent strong proliferation responses to CpG ODN2006. Although both memory cell populations did increase proliferation in response to treatment with Pam3CSK4 and FSL1 compared with untreated cells, the difference did not reach statistical significance (Fig. 9B).

Treatment with all TLR agonists examined resulted in proliferation by both IgM memory and switched memory subsets to varying degrees; however, treatment with TLR9 induced by far the strongest response within both populations (Fig. 9B). Others have shown that treatment with CpG ODN2006, either alone or in combination with other cytokines, induces only IgM class Ab from IgM memory cells (suggesting that it cannot induce class switching), whereas switched memory cells predominately produce either IgG or IgA Ab (20). Because we observed that N. gonorrhoeae infection induces the specific expansion of IgM memory B cells...
(Fig. 6B) and that the majority of Ig induced is IgM (Fig. 8A), we considered whether TLR9 signaling might also contribute to the gonococcal-induced IgM memory B cell response.

A dominant-negative CpG-containing oligonucleotide that inhibits TLR9 signaling (iCpG) has been shown to compete with the stimulatory effects of other CpG-containing DNA in both primary murine and primary human immune cells (36, 37). We took advantage of this reagent to examine whether TLR9 contributes to the gonococcal-induced induction of B cell proliferation. Although the iCpG had little effect on uninfected B cells, this inhibitor had a pronounced effect on B cells infected with N. gonorrhoeae, as shown in Fig. 9A and B.

FIGURE 8. Primary B cells produce polyclonal IgM, including pathogen-specific Ab, in response to infection with N. gonorrhoeae, an effect that is not observed with E. coli infection. (A) Five days postinfection, cell-free supernatants taken from B cell cultures infected with MOI of 10 bacteria per cell were analyzed for total Ig production by ELISA. (B–D) ELISA plates were coated with Opa− gonococci, TT, or KLH to examine the Ag specificity of the Ig produced upon infection. After 5 d of infection, supernatants were applied to these plates to examine the production of Ig specific for gonococci (B), to determine whether pathogen-specific Ig is produced, TT (C), to examine whether polyclonal activation of B cells includes activation of switched memory cells, and keyhole limpet hemocyanin (D), to determine if Ig specific for irrelevant Ags is produced. All three classes of Ig were tested. All cells from donors examined produced IgM that recognized TT, whereas 9 of 10 donors produced IgM specific for KLH and 7 of 9 donors produced IgM specific for gonococci. All data points are graphed as a ratio of Ig produced relative to Ig levels present in cells left uninfected. Bars indicate mean. Asterisks indicate a comparison between uninfected and infected cells. *p < 0.05, **p < 0.01, ***p < 0.001. Data points corresponding to the same individual donor are represented by the same symbol within each plot.

FIGURE 9. TLR9 signaling is induced upon N. gonorrhoeae infection of primary human B cells. (A and B) B cells were treated with 2.5 μg/ml Pam3CSK4, 1 μg/ml FSL1, 1 μg/ml LPS, and 5 μg/ml CpG ODN2006, infected with MOI:10 of N. gonorrhoeae strain N302, or left untreated for 3 d before analysis of CD86 for cellular activation (A) and Ki67 for proliferation (B) was conducted by flow cytometry, differentiating between B cell subpopulations by staining for IgD and CD27. (C) B cells were either left untreated, infected with MOI:10 of N. gonorrhoeae strain N302, or treated with 5 μg/ml CpG ODN2006. iCpG was added to inhibit TLR9 signaling, and in parallel wells, a control for iCpG was added (cCpG). After 3 d of incubation, cells were stained for B cell subpopulations and proliferation by Ki67. The relative percent of Ki67-positive cells was obtained by taking the ratio between proliferation observed in iCpG-treated cells versus cCpG-treated cells. Bars indicate mean. Asterisks indicate a comparison between uninfected and infected cells. *p < 0.05, **p < 0.01, ***p < 0.001. Data points corresponding to the same individual donor are represented by the same symbol within each plot.
inhibitor effectively reduced the proportion of actively proliferating cells in response to the synthetic CpG-containing oligonucleotide ODN2006 and to intact *N. gonorrhoeae* by mean values of 54.9 and 35.6%, respectively (Fig. 9C); this effect was consistent with cells from all donors tested. Thus, TLR9 contributes, at least in part, to B cell responses induced by *N. gonorrhoeae* infection.

*N. gonorrhoeae* can be engulfed and subsequently killed by primary human B cells

TLR9, unlike other TLRs, is not expressed on the cell surface but is found within endolysosomes (38). Although B cells will endocytose soluble Ag (39), their ability to engulf intact bacteria is largely unexplored. CD4+ T cells do not engulf adherent *N. gonorrhoeae* (13). However, we considered the possibility that B cells might have the capacity to engulf and kill whole gonococci, effectively delivering the CpG-containing bacterial DNA directly to intracellular TLR9. As depicted in Fig. 10A, primary human B cells are able to engulf whole gonococci, as determined by differential staining of total and extracellular gonococci.

To determine the fate of the internalized bacteria, a gentamicin protection assay was used to quantify viable intracellular gonococci. This assay takes advantage of the fact that gentamicin does not permeate mammalian membranes, so engulfed bacteria are protected from the antibiotic. B cells were infected with the specified strain of gonococci for 90 min before the addition of gentamicin for 1 h. Viable intracellular gonococci are observed at this point (2.5 h postinfection), supporting the microscopic evidence that the bacteria were being internalized. However, when the infection was allowed to persist for an additional 3.5 h (for a total of 6 h), the vast majority of intracellular gonococci is dead (Fig. 10B). Taken together, these results suggest that the high-affinity binding of *N. gonorrhoeae* to human B cells leads to engulfment of whole bacteria. Once internalized, the bacteria die and lyse, delivering a large bolus of bacterial DNA directly to intracellular lysosomal compartments where it can interact with TLR9 (Fig. 11).

**Discussion**

*N. gonorrhoeae* has a curious niche, such that the pathogen seems to penetrate and persist within the subepithelial space (9); this site is rich in nutrients but also patrolled by resident sentinel immune cells (16, 40) that the bacteria must contend with. Leukocytes, including B cells, accumulate in the endocervical mucosa during both symptomatic and asymptomatic gonococcal infections (16),

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**FIGURE 10.** B cells are able to engulf and kill whole gonococci. (A) B cells were infected with MOI:50 of indicated strains of *N. gonorrhoeae* for 3 h, stained for extracellular gonococci first, and then permeabilized to stain for total gonococci. Intracellular *N. gonorrhoeae* are single-color stained. Scale bars, 5 μm. (B) B cells were infected with the indicated strain of gonococci for 90 min and then treated with gentamicin to kill any extracellular bacteria. At 2.5 and 6 h postinfection, cells were washed, lysed, and then plated for growth on GC agar. Bars indicate mean. Data points corresponding to the same individual donor are represented by the same symbol within each plot. DIC, differential interference contrast.

**FIGURE 11.** Proposed model describing how *N. gonorrhoeae* infection effectively prevents the production of a protective immune response in infected individuals. (A) Upon vaccination or infection with microbes that elicit T-dependent B cell responses, activated T cells can provide co-stimulatory signaling to B cells. This is required for clonal expansion of B cells and the generation of a highly specific Ig response against the Ag/microbe. (B) Activation and proliferation of CD4+ T cells is inhibited by infection with *N. gonorrhoeae* through the clinically relevant engagement of the coinhibitory receptor CEACAM1 by the neisserial Opa proteins. This prevents T cell and B cell collaboration, abrogating the production of a T cell-dependent memory response. However, B cells are able to respond to *N. gonorrhoeae* infection in a T-independent manner, an effect relying in part on *N. gonorrhoeae* engulfment allowing direct delivery of neisserial DNA to intracellular TLR9. This potently activates IgM memory cells to produce a polyclonal IgM response which includes Ig that may bind to the gonococci but also to irrelevant (nongonococcal) Ags. Cumulatively, this allows suboptimal control of the current infection and precludes development of a classical memory response upon re-exposure to the gonococci.
making it likely that infecting *N. gonorrhoeae* come into direct contact with B cells at the site of infection as well as in the draining lymph nodes. When considering this, it is surprising that the Ab response elicited by *N. gonorrhoeae* is poor compared with other mucosal infections and does not elicit a classical immunologic memory response that would otherwise protect upon subsequent exposure (7). B cells are APCs and express many pattern recognition molecules capable of detecting bacterial-derived products (20, 41–44). Although this suggests that B cells should recognize all bacteria equally, our results unexpectedly reveal that *N. gonorrhoeae* has a specific tropism for the IgM memory subset of human B cells that elicits both their proliferation and a potent but broadly reactive T cell-independent Ig response.

Past reports indicate that infection by a variety of bacteria can cause B cell death (45, 46), yet we observed that *N. gonorrhoeae* infection instead supports primary human B cell viability and promotes their proliferation, even decreasing cell death that normally occurs during in vitro culture of freshly isolated human B cells. In other cell types, both protection from (47, 48) and promotion of (49) cell death have been observed upon infection with *N. gonorrhoeae*. Although the majority of studies examining this effect have focused on epithelial cells, it is becoming clear that infection with *N. gonorrhoeae*, more often than not, inhibits apoptosis (50). Previous work using preactivated human B cells suggested that infection with CEACAM1-binding gonococci results in B cell death (19), but we did not observe similar responses using freshly purified primary human B cells. On the basis of our findings described in this article, those previous observations may reflect cell death attributable to the overwhelming stimulation applied to the cells (gonococci as well as cytokines), rather than a direct effect of the bacteria itself. Although specific conclusions regarding the survival signals elicited by the gonococci await further molecular studies, we propose that the protection from cell death observed in our assays stems, in part, from the innate nature of the lymphocytes that are responding. The IgM memory B cells have a lower activation threshold than that of naïve cells (51), suggesting that they have evolved to respond by rapid activation, proliferation, and differentiation in response to microbes.

We observe that B cell infection with *N. gonorrhoeae* elicits an impressive production of mainly IgM, which recognizes both immunologically relevant (*N. gonorrhoeae*) and heterologous (TT and KLH) Ags. The production of Ngo-specific IgM in response to gonococcal infection in the absence of T cell help would be expected if it was a specific BCR-dependent response, but our observations indicate that *N. gonorrhoeae* recognition by B cells is not due to the BCR. However, IgM specific for TT and KLH, heterologous Ags consisting of single subunit proteins that contain fewer potential epitopes than do the intact bacteria, was produced in greater amounts upon infection with *N. gonorrhoeae* than was IgM specific for the infecting pathogen. This both highlights that the T-independent Ig response is broadly polyspecific and makes it intriguing to consider that the gonococci may have a mechanism by which to allow the selective expansion of heterologous, nonneisserial Ig.

IgG and IgA production by B cells infected with *N. gonorrhoeae* is significantly higher than the levels produced in response to infection with *E. coli* (Fig. 7B). This suggests that either IgG and IgA can arise from the IgM memory cells (via class switch recombination) or that switched memory cells, in addition to the IgM memory cells, are differentiating into Ab-secreting cells in response to the gonococci. We consider it plausible that the switched memory cells may benefit from T-independent Neisseria-induced “bystander” activation signals that originate from the IgM memory B cells, which would at least partially explain the switched memory response. Bernasconi et al. demonstrate bystander activation of switched memory B cells, wherein microenvironments produced upon activation of classical Ag-specific memory cells supports the activation of noncognate switched memory cells in a T-dependent manner (30). Although previously undescribed, the possibility that IgM memory B cells could play a role in the bystander activation of switched memory cells is enticing because it would suggest that these cells could polyclonally activate conventional memory B cells at first sign of infection.

It is unreasonable to expect that the large proportion of B cells that we observe respond to *N. gonorrhoeae* are specific for neisserial Ags, especially considering our observation that it is primarily the IgM memory subset that vigorously responds to infection by a pathogen that is uncommon in the study population. Neisserial activation of B cells does not occur via BCR clustering, such as that which occurs with the *Moraxella catarrhalis* superantigen *Moraxella* IgD-binding protein (26). To date, bacterial products such as capsular polysaccharide vaccines (28, 52), and purified CpG DNA (30) have been shown to expand the population of human peripheral blood IgM memory B cells. Interestingly, *N. gonorrhoeae* does not express a polysaccharide capsule, confirming that stimulation of this subset does not require antigenic signaling by these structures. As such, to our knowledge, this is the first report indicating the direct induction of polyclonal proliferation and IgM production by the human IgM memory B cell subpopulation in response to a bacterium.

*N. gonorrhoeae* is a complex organism, with many components that have the potential to interact with host cell receptors and cause immune activation, especially on APCs such as B cells. Although it has been established that the IgM memory subset expresses various TLRs (20), their TLR functionality had not, to our knowledge, been systematically explored. Although neisserial endotoxin might be considered an obvious candidate for eliciting the B cell response when considering its stimulatory effect on a variety of other immune cells (53–55), we did not observe a TLR4 response in any of the human B cell subsets described in this article (Fig. 9A, 9B).

Considering that the IgM memory B cells had a particular affinity for *N. gonorrhoeae* but not *E. coli*, which was used as a prototypical Gram-negative bacterial control to account for generalized MAMP responses, and that these cells also displayed a potent response to a synthetic CpG oligonucleotide agonist, TLR9 activation following *N. gonorrhoeae* engulfment seemed a likely explanation for the observed switched memory B cell response. Consistent with this premise, gonococcal-induced B cell proliferation was lower upon treatment with TLR9 antagonist iCpG (Fig. 9C). Why the iCpG-mediated inhibition was incomplete remains unclear. It may simply not be sufficient to fully compete with a bolus of CpG-containing DNA released upon neisserial lysis within the phagosome. Alternatively, B cell recognition of other neisserial-specific factors may also contribute to this response. Interesting to consider in this context is the fact that neisserial outer membrane-expressed porins have been shown to strongly induce both activation and proliferation by human and murine B cells through interaction with surface-expressed TLR2 and TLR1 (33, 56), particularly because IgM memory express sufficient TLR2 and TLR1 to respond to purified Pam3CSK4 (Fig. 9A, 9B).

It is becoming increasingly clear that TLR9 is centrally important in the induction of immune cell responses to the pathogenic Neisseria *sp.* (37, 57–59). In B cells, TLR9 is highly expressed relative to other TLRs (20), but it is only found in intracellular
compartments (38) rather than at the cell surface. Important in this context has been the demonstration that the delivery of bacterial DNA directly to TLR9-containing compartments is absolutely required for B cell responses, because they are poor at engulfing exogenous DNA (60). B cells are surprisingly effective at engulfing whole gonococci (Fig. 10A, 10B), allowing for the delivery of gonococcal DNA to TLR9, whereas engulfment of E. coli was rarely observed (data not shown). This difference may result from the combination of the gonococci’s tropism for B cells, increasing the time spent in contact with the cells, and/or the larger size of E. coli hindering the engulfment of these bacteria.

IgM memory B cells represent the first line of defense by the adaptive immune response because of their natural (not requiring antigen stimulation) production of low affinity but polyreactive IgM, the majority of which express germline-encoded receptors that can undergo somatic hypermutation (61). Some of this “natural” IgM presumably functions by opsonizing encapsulated microbes in a T-independent manner is plainly apparent, it has been recently suggested that murine innate B-1 B cells may possess a form of “memory” that is distinct from the conventional T-dependent response (72). In humans, an analysis of female sex workers from Kenya showed the induction of an incomplete, but serotype-specific, immunologic memory response following repeated N. gonorrhoeae infections over years of high-incidence exposure (73). Although these women could be very gradually acquiring immunity by traditional T-dependent mechanisms, it is tempting to propose the possibility that this memory response results from the repeated N. gonorrhoeae-dependent expansion of human IgM memory B cells. Further in vivo studies will be required to test this postulate.

In this report, we establish that the human-specific pathogen N. gonorrhoeae elicits a vigorous innate B cell response. Considering that other bacteria, including E. coli used in this study and Listeria monocytogenes (45) and Francisella tularensis (46) in previous work, do not elicit a similar response, this appears to be a specific effect of the gonococci rather than being a prototypical immune response. Indeed, it is intriguing to speculate that N. gonorrhoeae may promote the specific expansion of IgM memory B cells to amplify the production of nonspecific Ig in a manner that negatively affects specific immunity in infected individuals. We propose that, in the early stages of infection, circulating IgM memory B cells may come into contact with the gonococci, either at the site of infection or in the draining lymph nodes surrounding the urogenital tract. This interaction promotes the engulfment of whole gonococci by the B cells, which may contribute to the clearance of the pathogen, but also allows for the direct detection of bacterial products by innate receptors including TLR9. This results in a vigorous T-independent proliferation, expanding the IgM memory B cell population, and the induction of an acute, localized, effector response in the form of low-affinity, polyclonal IgM (see model, Fig. 11). As the infection progresses, the gonococci may attach to CD4+ T cells, an interaction mediated by their Opa adhesins binding to the coinhibitory receptor CEA- CAM1, because the vast majority of clinical neisserial isolates have the ability to bind CEA CAM1 (15). In contrast to B cells, T cells do not engulf the gonococci; this facilitates the inhibitory effect of CEA CAM1-mediated phosphatase-dependent signaling at the cell surface, thereby preventing T cell activation and clonal expansion in response to TCR engagement (12, 13). Consequently, the absence of effective T cell help precludes the production of high-affinity class-switched Ab, but the T cell-independent production of innate IgM production is unaffected. The result is a broadly specific and thereby weakly effective humoral response to gonococcal infection that may assist in clearing the current infection but, in the context of an unguided B cell response, provides no conventional immunologic memory responses upon subsequent exposure to N. gonorrhoeae.

Such a model, involving the suppression of CD4+ T cells while activating the innate B cell response, is in agreement with clinical observations indicating a modest increase in total IgM levels localized within the mucosa during natural gonococcal infection, the unexpectedly rapid decline in N. gonorrhoeae-specific Ig once the infection is cleared, and the absence of immunological memory upon subsequent exposure to these bacteria (7, 18). In the context of the remarkable antigenic variability of this pathogen, such direct subversion of the protective immune response explains the ongoing persistence of this highly evolved human pathogen.

Acknowledgments

We thank the volunteer blood donors, without whom our study would not have been possible. We thank Dr. Chao Wang and Shannon McCaw for technical assistance in completing this work and Drs. Alberto Martin and Michael J.H. Ratcliffe for insightful comments and critical reading of the manuscript.
Disclosures
The authors have no financial conflicts of interest.

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