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Experimental vaccine induces Th1-driven immune responses and resistance to *Neisseria gonorrhoeae* infection in a murine model

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Female mice were immunized intravaginally with gonococcal outer membrane vesicles (OMVs) plus microencapsulated interleukin-12 (IL-12), and challenged using an established model of genital infection with *Neisseria gonorrhoeae*. Whereas sham-immunized and control animals cleared the infection in 10–13 days, those immunized with OMV plus IL-12 cleared infection with homologous gonococcal strains in 6–9 days. Significant protection was also seen after challenge with antigenically distinct strains of *N. gonorrhoeae*, and protective anamnestic immunity persisted for at least 6 months after immunization. Serum and vaginal immunoglobulin G (IgG) and IgA antibodies were generated against antigens expressed by homologous and heterologous strains. Iliac lymph node $CD4^+$ T cells secreted interferon-γ (IFNγ), but not IL-4, in response to immunization, and produced IL-17 in response to challenge regardless of immunization. Antigens recognized by immunized mouse serum included several shared between gonococcal strains, including two identified by immunoproteomics approaches as elongation factor-Tu (EF-Tu) and PotF3. Experiments with immunodeficient mice showed that protective immunity depended upon IFNγ and B cells, presumably to generate antibodies. The results demonstrated that immunity to gonococcal infection can be induced by immunization with a nonliving gonococcal antigen, and suggest that efforts to develop a human vaccine should focus on strategies to generate type 1 T helper cell (Th1)-driven immune responses in the genital tract.

INTRODUCTION

Neisseria gonorrhoeae is a well-adapted and exclusively human pathogen that causes the sexually transmitted infection, gonorrhea. The most recent estimates based on data collected in 2012 indicate an annual incidence of 78 million new cases worldwide.[1] The reported incidence in the United States is >350,000 cases per year,[2] although the real incidence is believed to exceed 800,000 cases per year.[3] In the absence of a vaccine, control depends upon effective screening and diagnosis followed by prompt antibiotic treatment, which are not available in all parts of the world. However, *N. gonorrhoeae* has rapidly developed resistance to all classes of antibiotics that have been deployed against it, including most recently fluoroquinolones and extended-spectrum cephalosporins, giving rise to fears that gonorrhea might become untreatable.[4,5] Infection usually presents as a mucopurulent discharge, cervicitis in women and urethritis in men, but >50% of infections in women may be clinically inapparent.[6] Men typically become aware of infection within a few days, but it is increasingly recognized that asymptomatic infection can also occur in men. Women bear the greater burden of morbidity, as if left untreated gonorrhea can ascend to the upper reproductive tract and cause salpingitis, leading to tubal scarring, infertility, pelvic inflammatory disease, and increased risk for ectopic pregnancy that can be life-threatening. In men, untreated infection can progress to prostatitis and epididymitis.

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Newborns delivered through an infected birth canal can acquire eye infections that lead to blindness. In both sexes, *N. gonorrhoeae* can invade systemically, giving rise to disseminated gonococcal infection with septic arthritis and dermatitis being the most common manifestations. In addition, untreated gonorrhea enhances the transmission and acquisition of HIV by up to fivefold.[7]

The emergence of multiple drug-resistant strains of *N. gonorrhoeae* has led the World Health Organization and the US Centers for Disease Control and Prevention to call for new approaches to treatment and renewed efforts at vaccine development.[8] Previous attempts to develop a vaccine have come to nothing.[9] A small-scale trial of a killed whole-cell vaccine in Alaska in the 1970s was unsuccessful.[10] A major effort to develop a vaccine based on gonococcal pilus succeeded in inducing protective antibody responses against strains bearing antigenically similar pili, but the extensive variability of the pilin protein among naturally occurring *N. gonorrhoeae* strains rendered this vaccine totally ineffective in a field trial.[11] A more recent effort was made to develop a vaccine based on gonococcal porin, the major outer membrane protein,[12] but plans for a clinical trial were apparently abandoned.

Vaccine efforts are complicated by the extensive antigenic variability of *N. gonorrhoeae*, in which most major surface antigens, including lipooligosaccharide (LOS), porin, pilin, and the opacity proteins (Opa), are subject to phase-variable expression (LOS, Opa, pilus), allelic variation (porin, Opa), or recombinatorial expression (pilin) (reviewed in refs 9,13). In addition, it is widely recognized that gonorrhea can be acquired repeatedly with little or no evidence of effective immunity being developed as a result of therapeutic recovery from infection. Anecdotal evidence going back to the early twentieth century indicates that gonorrhea may ultimately be self-limiting, suggesting that an infected person might eventually develop a sufficient immune response to eliminate the pathogen, but ethical considerations prohibit prospective investigation of this. Furthermore, with the possible exception of chimpanzees, there are no animal models that closely mimic human infection and disease to permit vaccine studies that can be readily translated into clinical trials.[14]

However, a small animal model of genital tract gonococcal infection has been established, using estradiol-treated female mice,[15,16] and at present this remains the only model in which the response of an intact mammalian immune system to genital gonococcal infection can be prospectively studied and manipulated. In this model, infection is maintained for ~10–20 days (depending on various experimental factors) but is eventually eliminated possibly because *N. gonorrhoeae* is not adapted to colonize mice. Nevertheless, this period of infection provides an opportunity to evaluate host immune responses and to test strategies of immunization that inhibit infection and lead to accelerated clearance. Notably, infection does not result in specific serum or local genital antibody responses.[16,17] Moreover, although some strains of mice (especially BALB/c, but not C57BL/6) develop a neutrophil infiltrate into the vagina within a few days, there is no evidence for the induction of adaptive type 1 or type 2 T helper (Th1, Th2) cell responses.[16–19] In contrast, a Th17 response occurs with the production of interleukin (IL)-17 and IL-22 (ref. 19) which upregulate the secretion of innate antimicrobial proteins by epithelial cells and the recruitment of neutrophils. Abrogation of IL-17-mediated responses with neutralizing antibody or in IL-17 receptor-knockout mice results in diminished neutrophil influx and prolonged infection, suggesting that innate defense mechanisms contribute to clearance.[19] Furthermore, if mice are allowed to recover from infection and then reinfected, the course of infection is exactly the same as in age-matched control mice, and there is no evidence for recall of any anamnestic immune response, either elevated antibodies or enhanced Th1, Th2, or Th17 cellular responses.[16,17] These findings are reminiscent of the human immune response to uncomplicated gonococcal infection that similarly is minimal with respect to both antibody and T cell-mediated responses, regardless of history of prior infection.[20,21] IL-17 is reported to be elevated in humans infected with *N. gonorrhoeae*.[22,23]

Further study of the responses to genital gonococcal infection in mice demonstrated that *N. gonorrhoeae* upregulates the production of the immunosuppressive cytokines, transforming growth factor-β (TGFβ) and IL-10.[24,25] Counteracting these cytokines with neutralizing antibodies allows the emergence of Th1-driven responses including anti-gonococcal immunoglobulin G (IgG) and IgA antibodies in serum and vaginal secretions, establishment of immune memory, and accelerated clearance of inection.[24,25] Subsequent reinfection of such mice without further anti-TGFβ or anti-IL-10 treatment resulted in resistance to reinfection, and the recall of antibody responses to higher levels as well as Th1 (and Th17) cellular responses. These findings imply that *N. gonorrhoeae* suppresses adaptive immune responses, and that reversal of the induced immunosuppression enables the development of protective immunity. We have further developed this approach to counter-manipulating the ability of *N. gonorrhoeae* to suppress adaptive immune responses that would eliminate it, by treating gonococcus-infected mice intravaginally (i.vag.) with IL-12 encapsulated in microspheres (IL-12/ms).[26] This too permits the development of protective immunity that not only accelerates clearance of the existing infection, but also generates resistance to repeated infection. Because this treatment in effect converts the infection into a live vaccine, we hypothesized that microencapsulated IL-12 would serve as an adjuvant for a locally administered nonliving gonococcal vaccine. To test this hypothesis, we have immunized mice i.vag. with a gonococcal outer membrane vesicle (OMV) preparation administered with or without IL-12/ms. OMVs were selected as a vaccine immunogen because they contain most of the surface antigens in native conformation, not denatured by heat or chemical inactivation. Moreover, meningococcal vaccines have been successfully developed based on OMV preparations from *Neisseria meningitidis*.[27] The results demonstrate the generation of a Th1-driven, antibody-dependent, protective immune response that persists for at least several months and is effective against antigenically diverse strains of *N. gonorrhoeae*.

RESULTS

Intravaginal immunization of mice with gonococcal OMV plus IL-12/ms accelerates clearance of challenge infection with *N. gonorrhoeae*

Groups of 8 female BALB/c mice were immunized i.vag. with gonococcal OMV (strain FA1090; 40 μg protein) plus IL-12/ms (1 μg IL-12), or with OMV plus control (blank) ms; two additional control groups were sham-immunized with IL-12/ms or with blank ms alone. Immunizations were repeated 1 week and 2 weeks later, and all mice were challenged after a further 2 weeks by i.vag. instillation of *N. gonorrhoeae* FA1090 (5×10^6 colony-forming units (CFUs)). Control (sham-immunized) mice, or mice immunized with OMV plus blank ms cleared the infection commencing at day 7 after challenge and were all cleared by day 15; median days of clearance were 10–13. There was no significant difference in the clearance rates between these three control groups (**Figure 1a**). However, mice immunized with OMV plus IL-12/ms cleared the infection beginning at day 6 and were all cleared by day 9; median clearance was 7.5 days compared with 12 days in mice immunized with OMV plus blank ms ($P < 0.01$, Kaplan–Meier; **Supplementary Table S1 online** and **Figure 1a**). This experiment was repeated twice more with similar results (see **Supplementary Table S1** and **Supplementary Figure S1**). Further examples of replication of this finding can be seen in subsequent experiments reported below, for example, **Figures 1d, 4** and **5a,c** (**Figure 5b,d,e** for other gonococcal strains), and **Figure 7a,b,f,g** (with C57BL/6 mice).

Serum and vaginal wash samples collected after clearance (at termination, day 15 after inoculation) were assayed for antibodies against intact gonococci (FA1090) by enzyme-linked immunosorbent assay (ELISA). This showed that mice immunized with OMV plus IL-12/ms had developed the highest levels of vaginal and serum IgG and IgA antibodies, whereas those mice immunized with OMV plus blank ms developed much lower levels of these antibodies (**Figure 1b**). Mice that were unimmunized and sham-infected showed no antibodies detectable above assay background at the starting dilutions, and mice immunized with blank ms alone and infected also did not develop detectable antibodies (**Figure 1b**). Iliac lymph node (ILN) mononuclear cells collected at the same time were stained for surface CD4 expression and for intracellular cytokines, and analyzed by flow cytometry. This revealed that only mice immunized with OMV plus IL-12/ms generated $CD4^+/IFN\gamma^+$ (and $CD8^+/IFN\gamma^+$) T cells, whereas no mice developed significant numbers of $CD4^+/IL\text{-}4^+$ T cells (**Figure 1c**; see also **Supplementary Figure S2**). However, all mice that were infected with *N. gonorrhoeae* regardless of immunization developed $CD4^+/IL\text{-}17^+$ T cells (**Figure 1c** and **Supplementary Figure S2**), as observed previously.[19,26] These findings are all consistent with the results of our previous experiments in which mice that were first infected with *N. gonorrhoeae* and then treated i.vag. with IL-12/ms cleared the infection significantly faster than infected mice that were untreated or treated with blank ms, and that IL-12/ms-treated mice developed serum and vaginal IgG and IgA anti-gonococcal antibodies, as well as interferon (IFN)γ-secreting Th1 cells in the draining ILN.[26]

Further experiments were performed to determine the minimum number of immunizations required to induce immune resistance to infection. A single dose of OMV plus IL-12/ms given i.vag. did not consistently generate resistance to challenge, but two doses of OMV (40 μg protein) plus IL-12/ms (1 μg IL-12) given at an interval of 2 weeks were sufficient to induce similar resistance to infection; median clearance was 8 days (**Figure 1d** and **Supplementary Table S1**). In addition, control immunization with OMV prepared from nontypeable *Haemophilus influenzae* (NTHI) plus IL-12/ms failed to induce resistance to *N. gonorrhoeae*; median clearance was 13 days (**Figure 1d** and **Supplementary Table S1**). This induced antibodies to NTHI but not to *N. gonorrhoeae* (**Supplementary Figure S3a,b**) and generated IFNγ-producing $CD4^+$ and $CD8^+$ cells in the ILN (**Supplementary Figure S3c**).

Intravaginal immunization with gonococcal OMV plus IL-12/ms induces persistent gonococcus-specific antibody responses and Th1 cellular responses

To characterize the local and systemic immune responses after immunization with 1, 2, or 3 doses of gonococcal OMV plus IL-12/ms and before challenge, serum, vaginal washes, and ILNs were collected from immunized and control mice 2 weeks after the last immunization. Serum anti-gonococcal IgM antibodies were at low levels with little difference between experimental groups. IgA and IgG antibodies were not detectable above background in vaginal wash or serum samples of mice given blank ms alone. Intravaginal immunization with three doses of gonococcal OMV plus blank ms elevated vaginal and serum anti-gonococcal IgA and IgG antibodies but to a lesser degree than immunization with OMV plus IL-12/ms (**Figure 2a**). In contrast, immunization with one dose of OMV plus IL-12/ms induced low levels of anti-gonococcal antibodies in both serum and vaginal wash; a second dose elevated antibody production, but no further elevation was seen after three doses (**Figure 2a**). Antibodies appeared to be specific for *N. gonorrhoeae*, as they were not detected above control levels against *Escherichia coli* or NTHI. Assay of IgG subclass antibodies in both vaginal wash and serum showed a predominance of IgG2a, with lesser amounts of IgG1 and IgG2b and low levels of IgG3 (**Supplementary Figure S4**). The production of anti-gonococcal IgA and IgG antibodies in vaginal wash and serum peaked at 3 months after immunization with two doses of OMV plus IL-12/ms, and were still detectable at 6 months after immunization (**Figure 2b,c**).

Flow cytometric analysis of ILN cells revealed increased numbers of $IFN\gamma^+/CD4^+$ and $IFN\gamma^+/CD8^+$ T cells from mice immunized with OMV plus IL-12/ms compared with those from control mice (**Figure 3a**). As observed with the antibody responses, one immunization was sufficient to induce IFNγ production, and it was further elevated by two immunizations; three doses did not further increase it. In

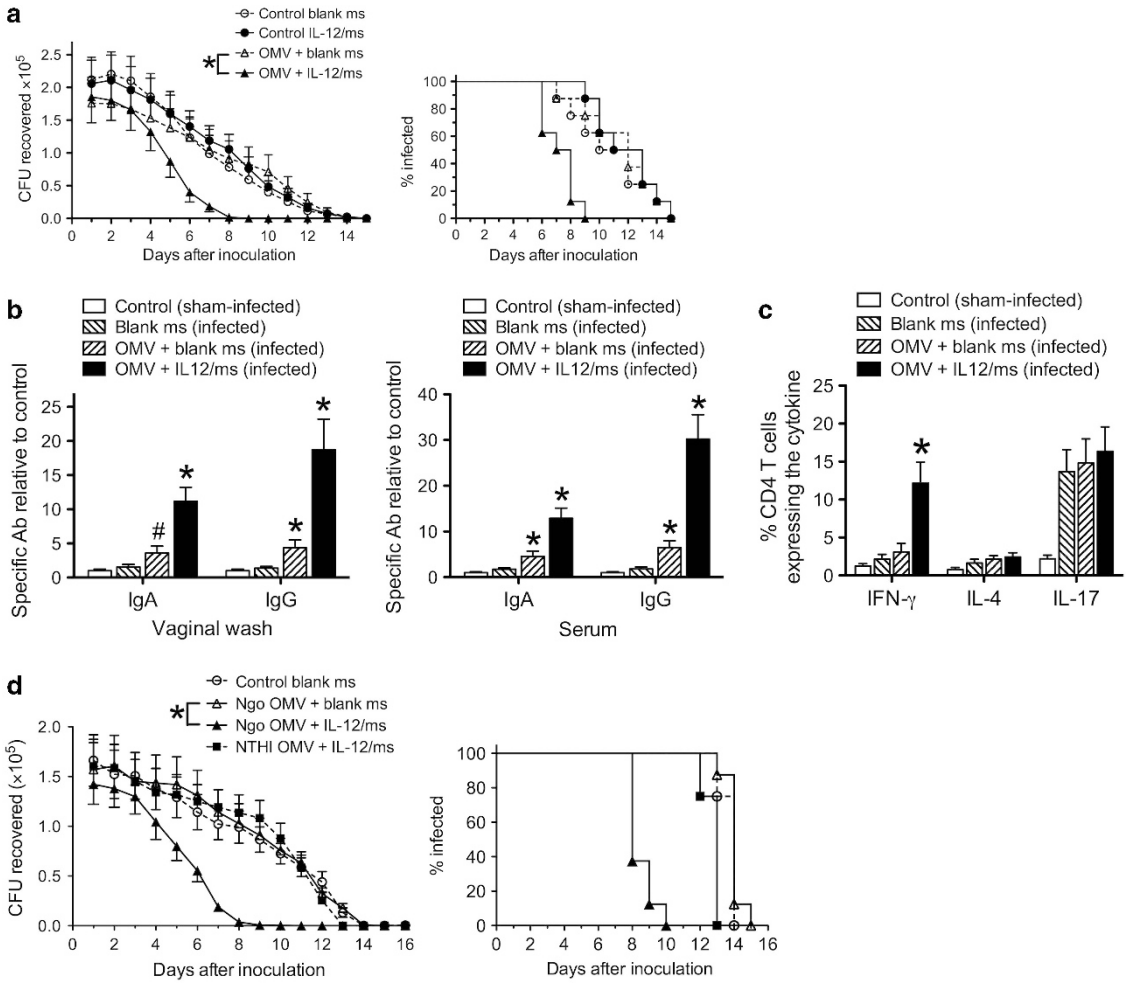

Figure 1 Intravaginal (I.vag) immunization with gonococcal outer membrane vesicle (OMV) plus interleukin-12 encapsulated in microspheres (IL-12/ms) induced resistance to genital infection with *Neisseria gonorrhoeae*, and generated an immune response. (**a**) Mice were immunized 3 times at 7-day intervals with OMV (40 μg protein) from strain FA1090 plus control (blank) ms or IL-12/ms (1 μg IL-12); control mice were sham-immunized with either blank ms, or with IL-12/ms alone. At 2 weeks after the last immunization, all mice were challenged by i.vag. inoculation with *N. gonorrhoeae* strain FA1090 (5×10^6 colony-forming units (CFUs), and infection was monitored by vaginal swabbing and plating. Left panel: recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^*P < 0.01$ (analysis of variance (ANOVA)); right panel: % of animals remaining infected at each time point, $P < 0.01$ (Kaplan–Meier analysis, log-rank test, OMV plus IL-12/ms vs. OMV plus blank ms). (**b**) Vaginal wash (left) and serum (right) antibodies against strain FA1090 in samples collected after termination (day 15), shown as mean ± s.e.m., $N = 5$ samples; $^{\#}P < 0.05$, $^*P < 0.01$, Student's *t*-test. (**c**) Intracellular cytokine staining in $CD4^+$ cells recovered from iliac lymph node (ILN) at termination (day 15), shown as mean ± s.e.m., $N = 3$ samples, % of $CD4^+$ staining for each cytokine; $^*P < 0.01$ Student's *t*-test. (**d**) Mice were immunized twice at a 14-day interval with gonococcal (Ngo) OMV (40 μg protein) plus blank ms or IL-12/ms (1 μg IL-12); control mice were sham-immunized with blank ms alone or with nontypeable *Haemophilus influenzae* (NTHI) OMV (40 μg protein) plus IL-12/ms (1 μg IL-12). After 2 weeks, all mice were challenged with *N. gonorrhoeae* FA1090 (5×10^6 CFUs). Left panel: recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^*P < 0.01$ (ANOVA, gonococcal OMV plus IL-12/ms vs. gonococcal OMV plus blank ms); right panel: % of animals remaining infected at each time point, $P < 0.0001$ (Kaplan–Meier analysis, log-rank test, gonococcal OMV plus IL-12/ms vs. gonococcal OMV plus blank ms).

contrast, immunization with OMV plus IL-12/ms did not significantly increase the numbers of $IL\text{-}4^+/CD4^+$ and $IL\text{-}17^+/CD4^+$ T cells relative to controls (**Figure 3a**). To determine whether the induced $IFN\gamma^+/CD4^+$ (and $IFN\gamma^+/CD8^+$) T cells were specific for gonococcal antigens, $CD4^+$ cells isolated from ILNs were preloaded with carboxymethyl fluorescein succinimide ester (CFSE), cultured *in vitro* for 3 days in the presence of antigen-presenting cells with gonococcal OMV or without stimulation as controls, and their proliferation was assessed by flow cytometry. $CD4^+$ cells from the ILN of immunized mice proliferated significantly more, and produced significantly more IFNγ, in response to stimulation *in vitro*, than the cells from control mice (**Supplementary Figure S5a**). No production of IL-4 was seen, but IL-17 was generated by $CD4^+$ T cells cultured with gonococcal OMV, regardless of immunization, similar to what was observed previously with murine cells stimulated with gonococcal antigen *in vitro* [24] (**Supplementary Figure S5a**). IFNγ production by ILN $CD4^+$ T cells remained elevated, although at slowly declining levels, for 4 months after immunization (**Figure 3b**).

In addition, vaginas were excised from killed mice 3 days after the last immunization and RNA was extracted from the whole tissue. Reverse transcriptase-PCR analysis showed that, in comparison with controls, immunization with gonococcal

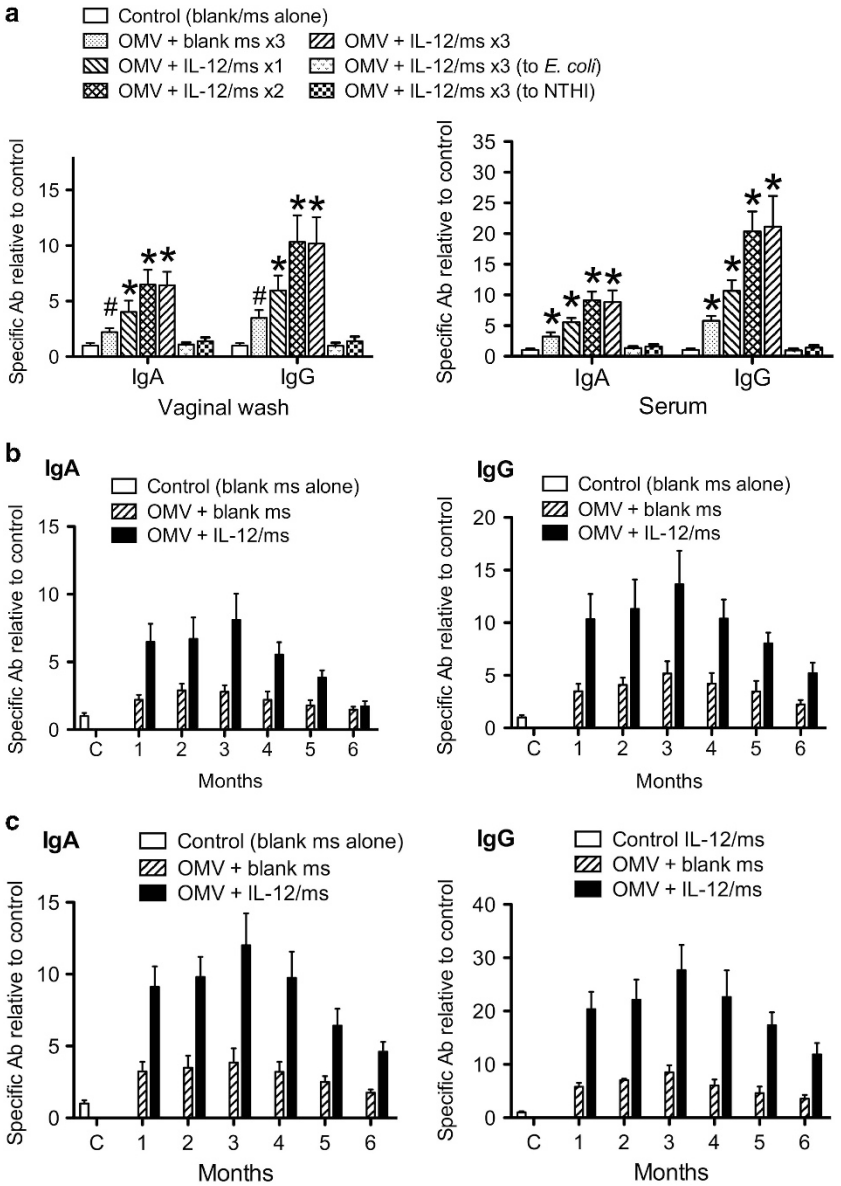

Figure 2 Antibody responses generated by immunization with gonococcal outer membrane vesicle (OMV) plus interleukin-12 encapsulated in microspheres (IL-12/ms) before gonococcal challenge. (**a**) Vaginal wash (left panel) and serum (right panel) antibodies assayed by enzyme-linked immunosorbent assay (ELISA) 2 weeks after the last immunization with 1, 2, or 3 doses of gonococcal OMV (40 μg protein) plus IL-12/ms (1 μg IL-12). Control samples were obtained from mice sham-immunized with blank ms (3 doses); additional mice were immunized 3 × with gonococcal OMV plus blank ms. Data shown as mean ± s.e.m., $N = 5$ samples, $^{\#}P < 0.05$, $^{*}P < 0.01$ relative to control samples (analysis of variance (ANOVA)). Duration of vaginal wash (**b**) and serum (**c**) antibodies in mice immunized with two doses of FA1090 OMV plus blank ms or IL-12/ms; data shown as mean ± s.e.m., $N = 5$ samples; C, control samples from unimmunized mice.

OMV plus IL-12/ms significantly enhanced the expression of mRNA for IFNγ but not for IL-4 or IL-17 (**Supplementary Figure S5b**). IFNγ mRNA expression in vaginal tissue, and production of IFNγ by ILN $CD4^+$ cells, remained elevated for up to 2 months following i.vag. immunization with gonococcal OMV plus IL-12/ms (**Supplementary Figure S5c**). These findings support the cytokine expression results obtained with cells from the draining ILN.

Duration of vaccine-induced resistance to infection

To evaluate the duration of immune resistance, groups of eight mice were immunized with gonococcal OMV plus IL-12/ms and were challenged with the same strain (FA1090) of *N. gonorrhoeae* at 2, 4, or 6 months after immunization. Compared with age-matched controls that were either sham-immunized or immunized with OMV plus blank ms, mice immunized with OMV plus IL-12/ms were resistant to *N. gonorrhoeae* infection when challenged at 2 or 4 months after immunization; median clearance in controls was 11–11.5 days vs. 7 days in immunized mice (**Supplementary Table S1**). Similar results were obtained in a replicate experiment when mice were challenged 6 months after immunization; median clearance in controls was 9.5–10 days vs. 6.5 days in mice immunized with OMV plus IL-12/ms (**Figure 4** and

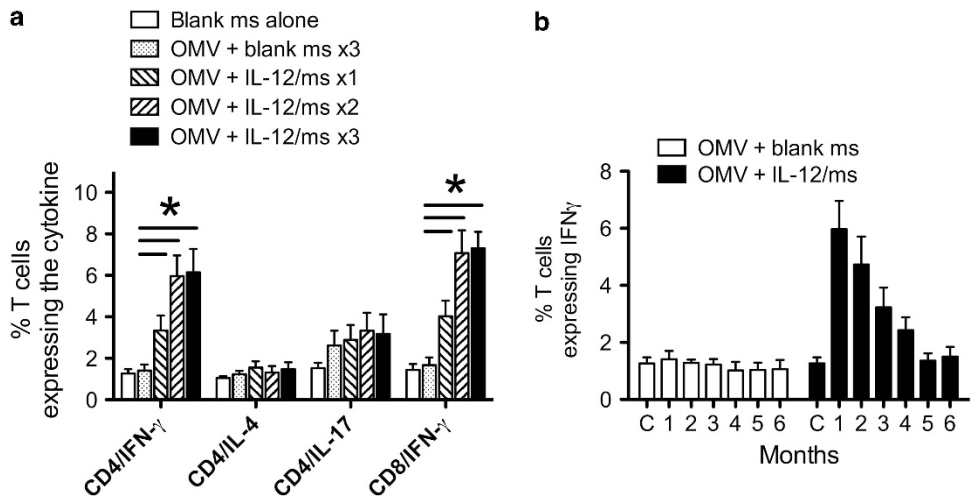

Figure 3 (**a**) T-cell cytokine responses in iliac lymph node (ILN) cells induced by immunization with gonococcal outer membrane vesicle (OMV) plus interleukin-12 encapsulated in microspheres (IL-12/ms) 2 weeks after the last immunization with 1, 2, or 3 immunizations with gonococcal OMV (40 μg protein) plus IL-12/ms (1 μg IL-12). Control ILNs were obtained from mice sham-immunized with blank ms (3 doses) and additional mice were immunized 3 × with gonococcal OMV plus blank ms. Data shown as mean ± s.e.m., $N = 3$ samples, % of $CD4^+$ or $CD8^+$ cells staining for each cytokine. *$P < 0.01$ (Student's *t*-test) comparing immunization with IL-12/ms with blank ms. (**b**) Duration of interferon-γ (IFNγ) responses in $CD4^+$ ILN cells 1–6 months after two immunizations with gonococcal OMV plus IL-12/ms or with OMV plus blank ms. Data shown as mean ± s.e.m., $N = 3$ samples, % of $CD4^+$ cells staining for IFNγ; C, control ILN from unimmunized mice.

Supplementary Table S1). After termination, anti-gonococcal antibodies were detected in serum and vaginal washes (**Supplementary Figure S6a,b**). IFNγ (but not IL-4-)-secreting $CD4^+$ T cells were present in ILN (**Supplementary Figure S6c**). Notably, the antibody and IFNγ responses detected after challenge were higher than those before challenge (compare with **Figures 2b,c** and **3b**) implying recall of memory. As observed previously, IL-17-secreting T cells were always found after challenge with *N. gonorrhoeae*, regardless of immunization (**Supplementary Figure S6c**). Longer time periods were not evaluated because mice become increasingly resistant to gonococcal infection as they age.

Immunization induces resistance to heterologous strains of *N. gonorrhoeae*

An important consideration for any vaccine is that it should be effective against different strains of the pathogen, as well as those antigenically homologous to the immunizing strain. *N. gonorrhoeae* is well known for its antigenic variability involving most of its surface antigens through multiple molecular mechanisms. We therefore determined whether i.vag. immunization with one strain of gonococcal OMV would be effective against challenge with other strains to a similar extent as challenge with the same strain. At first, mice (8 per group) were immunized i.vag. with OMV prepared from strain FA1090 plus IL-12/ms or blank ms, and were challenged 1 month later with *N. gonorrhoeae* strains FA1090 or MS11 (5×10^6 CFUs). Immunization with FA1090 OMV induced resistance to challenge with either FA1090 or MS11 to similar extents (**Figure 5a** and **Supplementary Table S2**). After challenge and clearance, antibodies were elevated to similar levels against MS11 and the Th1 responses indicated by $IFN\gamma^+/CD4^+$ T cells in ILNs were similarly enhanced (**Supplementary Figure S7a,b,c**). In a reciprocal manner, immunization with MS11 OMV (plus IL-12/ms) induced resistance to challenge with strain FA1090 (**Figure 5b** and **Supplementary Table S2**).

Gonococcal strains FA1090 and MS11 both possess porin of the same major type (PorB.1B), although of different subtypes.[28,29] Therefore, to determine whether the major porin type is integral to immune resistance, further experiments were performed with strain FA19 (PorB.1A). Immunization with FA1090 OMV (plus IL-12/ms) induced resistance to challenge with FA19 (**Figure 5c** and **Supplementary Table S2**). Antibody responses assayed at termination showed crossreactivity against FA19, and $IFN\gamma^+/CD4^+$ T cells in ILNs were elevated (**Supplementary Figure S7d,e,f**). Reciprocally, immunization with FA19 OMV induced resistance to challenge with strain FA1090 (**Figure 5d** and **Supplementary Table S2**). Other immunization and challenge combinations (i.e., MS11 against FA19, and vice versa) similarly showed similar cross-resistance (**Supplementary Table S2**).

N. gonorrhoeae strains FA1090, MS11, and FA19 have been widely used in many laboratories and extensively subcultured since their original isolation. As a result, it is possible that they have acquired mutations and become altered in some of their characteristics. Therefore, we also challenged immunized mice with novel clinical strains that have been minimally passaged *in vitro* since their isolation.[30] Mice immunized with FA1090 OMV plus IL-12/ms were also resistant to challenge with clinical isolates GC68 (a PorB.1B strain; **Figure 5e** and **Supplementary Table S2**) and GC69 (PorB.1A; **Supplementary Table S2**).

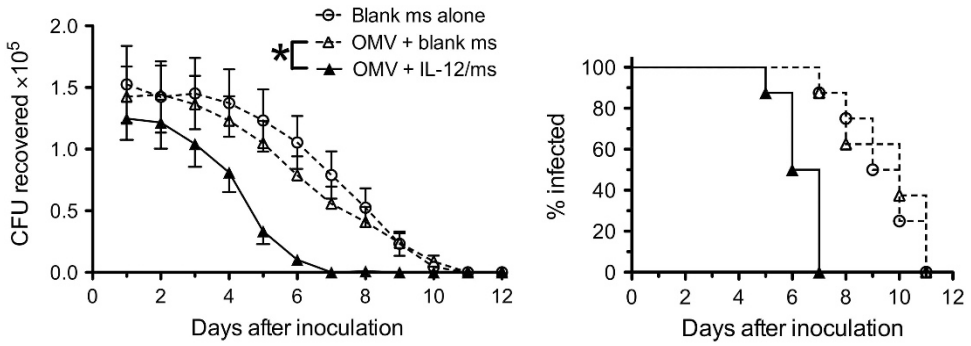

Figure 4 Resistance to gonococcal (FA1090) challenge persisted for at least 6 months after immunization with two doses of gonococcal (FA1090) outer membrane vesicle (OMV) plus interleukin-12 encapsulated in microspheres (IL-12/ms). Left panel: recovery (colony-forming units (CFUs)) of *Neisseria gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^*P < 0.01$ (analysis of variance (ANOVA), gonococcal OMV plus IL-12/ms vs. gonococcal OMV plus blank ms); right panel: % of animals remaining infected at each time point, $P < 0.001$ (Kaplan–Meier analysis, log-rank test, gonococcal OMV plus IL-12/ms vs. gonococcal OMV plus blank ms).

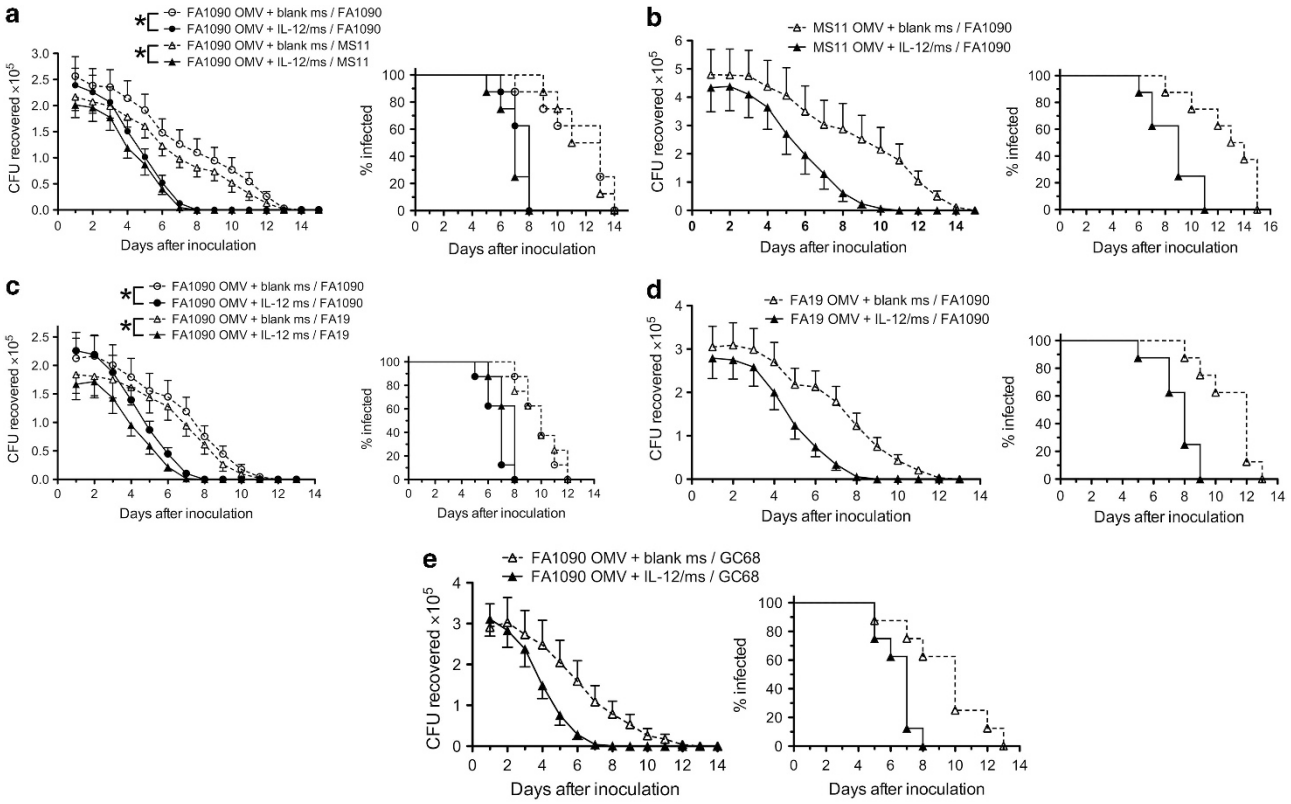

Figure 5 Resistance to heterologous gonococcal challenge. (**a**) At 1 month after immunization with FA1090 outer membrane vesicle (OMV) plus interleukin-12 encapsulated in microspheres (IL-12/ms) or blank ms, mice were challenged with *Neisseria gonorrhoeae* strain FA1090 (homologous challenge) or strain MS11 (heterologous challenge). Left panel: recovery (colony-forming units (CFUs)) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^*P < 0.001$ (analysis of variance (ANOVA), for comparisons shown); right panel: % of animals remaining infected at each time point, $P < 0.02$ for FA1090 challenge, IL-12/ms vs. blank ms; $P < 0.001$ for MS11 challenge, IL-12/ms vs. blank ms (Kaplan–Meier analysis, log-rank test). (**b**) Mice immunized with MS11 OMV were resistant to challenge with *N. gonorrhoeae* FA1090. Left panel: recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $P < 0.01$ (ANOVA); right panel: % of animals remaining infected at each time point), $P < 0.01$ (Kaplan–Meier analysis, log-rank test). (**c**) Mice immunized with FA1090 OMV were resistant to challenge with *N. gonorrhoeae* FA19. Left panel: recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^*P < 0.01$ (ANOVA, for comparisons shown); right panel: % of animals remaining infected at each time point, $P < 0.01$, IL-12/ms vs. blank ms for FA1090 challenge; $P < 0.0001$, IL-12/ms vs. blank ms for FA19 challenge (Kaplan–Meier analysis, log-rank test), $N = 8$ mice. (**d**) Mice immunized with FA19 OMV were resistant to challenge with *N. gonorrhoeae* FA1090. Left panel: recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $P < 0.01$ (ANOVA); right panel: % of animals remaining infected at each time point), $P < 0.01$ (Kaplan–Meier analysis, log-rank test). (**e**) Mice immunized with FA1090 OMV were resistant to challenge with clinical isolate GC68. Left panel: recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $P < 0.01$ (ANOVA); right panel: % of animals remaining infected at each time point), $P < 0.01$ (Kaplan–Meier analysis, log-rank test).

Antigens targeted by immunization-induced antibodies

When examined by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the protein profiles of FA1090, MS11, and FA19 OMV were similar, but with apparent quantitative as well as qualitative variations (**Figure 6a**). This was expected based on a previous study using

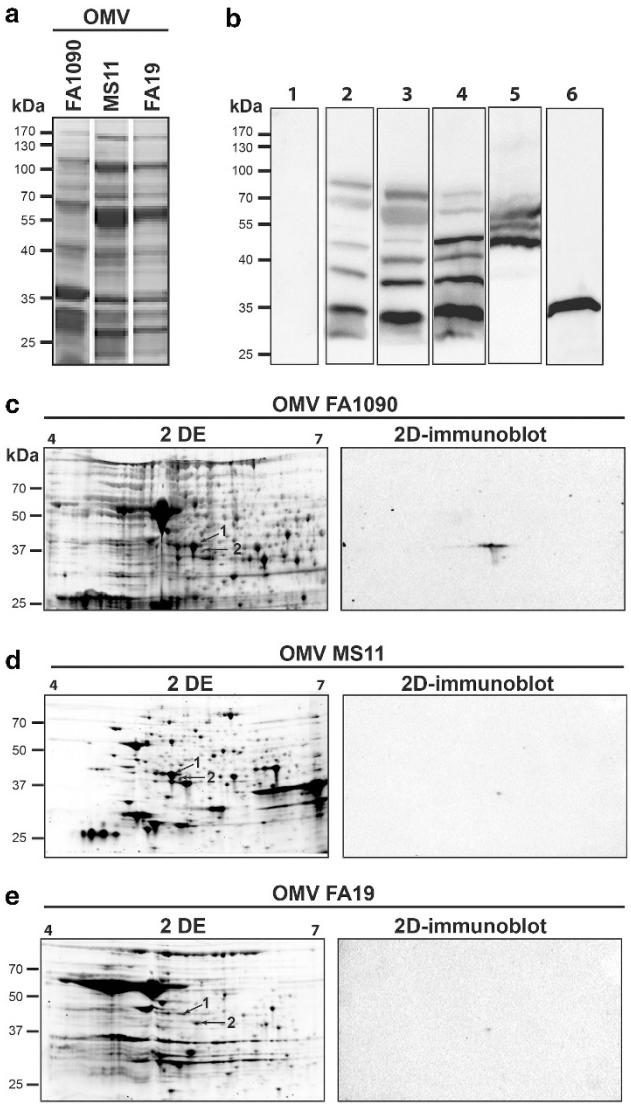

Figure 6 Immunoproteomics of gonococcal outer membrane vesicles (OMVs). (**a**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of OMV preparations from *Neisseria gonorrhoeae* strains FA1090, MS11, and FA19, stained with Coomassie blue. (**b**) Western blot analysis of mouse sera tested on gonococcal OMV preparations separated by SDS-PAGE. Lane 1, control serum from a mouse immunized with FA1090 OMV plus blank microspheres (ms), tested against FA1090 OMV; lanes 2–4, serum 1 from a mouse immunized with FA1090 OMV plus interleukin-12 encapsulated in microspheres (IL-12/ms), tested against OMV from FA1090 (lane 2), MS11 (lane 3), or FA19 (lane 4); lane 5, serum 2 from a mouse immunized with FA1090 OMV plus IL-12/ms, tested against OMV from FA1090; lane 6, antibody H5 (anti-porin PIB3) tested against FA1090 OMV. (**c**–**e**) Proteome maps of gonococcal OMV derived from (**c**) FA1090, (**d**) MS11, and (**e**) FA19 revealed by two-dimensional (2D) electrophoresis (2-DE) and Flamingo fluorescent staining (left panels) and their corresponding immunoblots (right panels) obtained by probing with mouse serum 2. Immunoreactive spots subjected to tandem mass spectrometry (MS/MS) analysis are labeled as spots 1 and 2 (arrows). Molecular mass marker (kDa) indicated on the left.

comparative high-throughput proteomic analysis of OMV from these gonococcal strains.[31] Western blot analyses of serum from one mouse (no. 1) immunized with FA1090 OMV plus IL-12/ms against FA1090, MS11, or FA19 OMV separated by SDS-PAGE revealed IgG antibodies reactive with bands migrating at ~35–80 kDa, with reactivity against bands present in OMV from all three strains strains (**Figure 6b**, lanes 2–4). One of these bands at ~35 kDa may correspond to porin, as H5 antibody reacted with a band of similar mobility (**Figure 6b**, lane 6). Another serum (no. 2) displayed strong reactivity against three bands migrating at ~45–65 kDa (**Figure 6b**, lane 5), possibly similar to antigen bands detected by antibodies in mice that had been intranasally immunized with OMV.[32] In an effort to identify the ~45–65 kDa antigens, we used immunoproteomic approaches consisting of two-dimensional (2D) SDS-PAGE separation of OMV and parallel 2D SDS-PAGE followed by immunoblotting (2D immunoblot) and mass spectrometry. The three 2D protein maps of OMV revealed by Flamingo staining showed numerous protein species and significant differences in the OMV proteome between FA1090, MS11, and FA19 strains (**Figure 6c,d,e**). In contrast, the blotted protein maps showed two spots (spot 1 and spot 2) of masses corresponding to 45 kDa and 43 kDa, and pI 5.2 and 5.5, respectively (FA1090 OMV; **Figure 6c**), or one spot (spot 1) with an approximate mass 45 kDa and pI 5.2 (MS11 and FA19 OMV; **Figure 6d,e**). Mass spectrometry analysis of the tryptic peptides obtained from spot 1 and spot 2 (**Figure 6c,d,e**) revealed as top hits translation elongation factor-Tu (EF-Tu) and a putative periplasmic polyamine-binding protein, PotF3, respectively (**Supplementary Table S3**). EF-Tu appeared as the most confident antigen as it was immunoreactive in all three 2D immunoblots and was identified with the highest confidence (score ranging from 485.0 to 947.1) and coverage (64.2 to 90.6) in all OMV preparations (**Supplementary Table S3**).

Immune resistance to *N. gonorrhoeae* depends on IFNγ and antibody

To determine whether the protective effect of immunization with OMV adjuvanted with IL-12/ms is dependent on IFNγ or antibody responses, or on immunity governed by $CD4^+$ or $CD8^+$ T cells, we performed immunization experiments using mutant C57BL/6 mice deficient in IFNγ (IFNγ-KO) B cells (μMT), $CD4^+$ T cells (CD4-KO), or $CD8^+$ T cells (CD8-KO). Groups of 8 C57BL/6 wild-type (control) and immumnodeficient mice were immunized with FA1090 OMV plus IL-12/ms or blank ms, and challenged with *N. gonorrhoeae* FA1090 (5×10^6 CFUs) 1 month later. The course of vaginal gonococcal infection was not altered in unimmunized immunodeficient mice relative to wild-type controls. All wild-type and immunodeficient mice started to reduce the recoverable gonococcal load from days 7 to 11 and had cleared the infection by days 12–14 (median 9–13 days), similar to BALB/c mice used in experiments described in the previous sections (**Figure 7a,b,f,g** and **Supplementary Table S4**).

In contrast to wild-type mice, clearance of gonococcal infection was not accelerated in IFNγ-KO or μMT mice immunized with OMV plus IL-12/ms compared with immunization with OMV plus blank ms (**Figure 7a, b**, **Supple-**

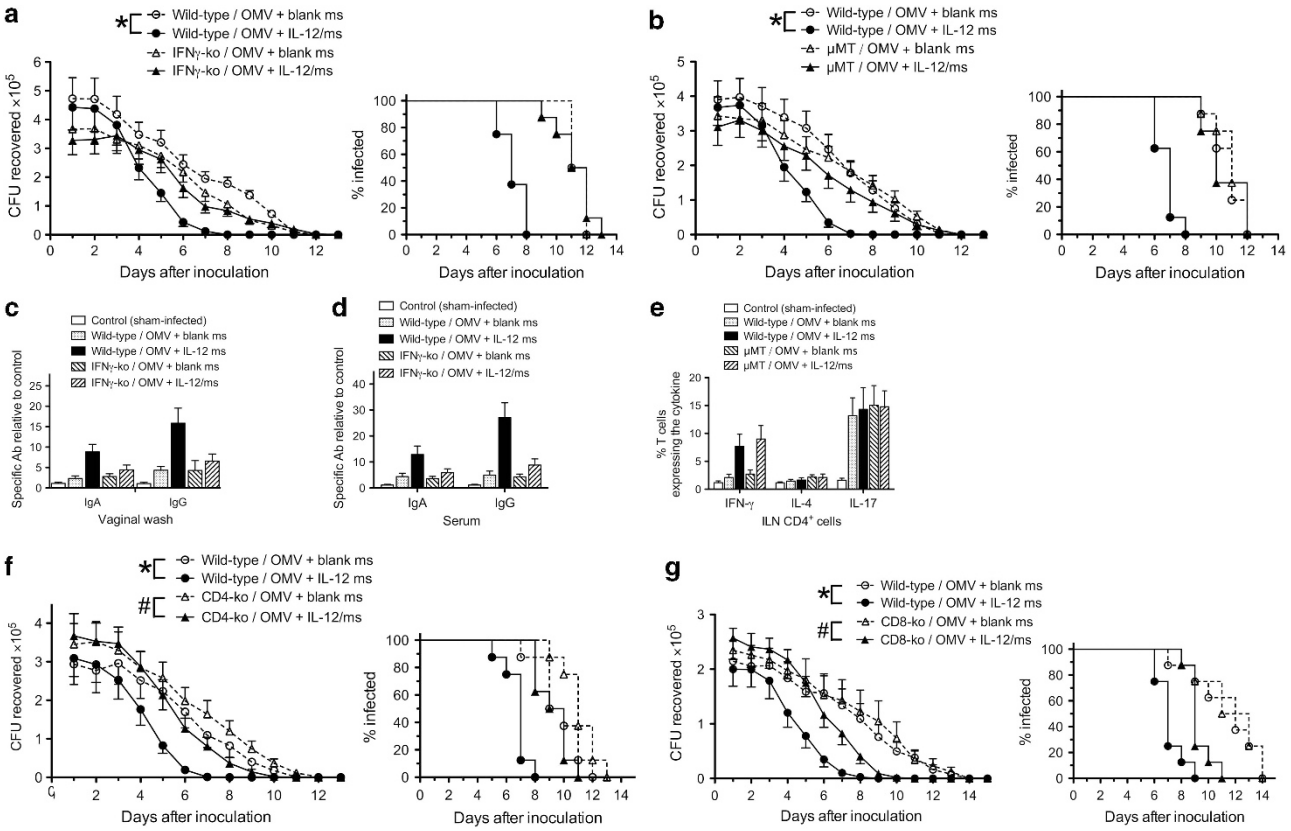

Figure 7 Resistance to challenge induced by immunization with gonococcal outer membrane vesicle (OMV) plus interleukin-12 encapsulated in microspheres (IL-12/ms) depended on interferon-γ (IFNγ) and B cells. (**a**) Course of infection (FA1090) in IFNγ-KO (knockout) vs. wild-type mice immunized with FA1090 OMV; left panel, recovery (colony-forming units (CFUs)) of *Neisseria gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^{*}P < 0.01$ (analysis of variance (ANOVA)); right panel, % of animals remaining infected at each time point, $P < 0.0001$ for wild-type mice, IL-12/ms vs. blank ms (Kaplan–Meier analysis, log-rank test). (**b**) Course of infection (FA1090) in μMT vs. wild-type mice immunized with FA1090 OMV; left panel, recovery (CFU) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^{*}P < 0.01$ (ANOVA); right panel, % of animals remaining infected at each time point, $P < 0.0001$ for wild-type mice, IL-12/ms vs. blank ms (Kaplan–Meier analysis, log-rank test). (**c**) Vaginal wash and (**d**) serum antibody responses in IFNγ-KO vs. wild-type (mean ± s.e.m., $N = 5$ samples) assayed at termination (day 13). Immunoglobulin A (IgA) and IgG responses in vaginal wash and serum were significant ($P < 0.05$, Student's *t*-test, OMV plus blank ms vs. OMV plus IL-12/ms) for wild-type mice, but not for IFNγ-KO mice. (**e**) T-cell cytokine responses in μMT vs. wild-type mice (mean ± s.e.m., $N = 3$ samples) assayed at termination (day 13). IFNγ response to immunization with OMV plus blank ms vs. OMV plus IL-12/ms was significant ($P < 0.01$) for both wild-type and μMT mice (ANOVA). (**f**) Course of infection (FA1090) in CD4-KO vs. wild-type mice immunized with FA1090 OMV; left panel, recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^{\#}P < 0.05$, $^{*}P < 0.01$ (ANOVA) for comparisons shown; right panel, % of animals remaining infected at each time point, $P < 0.001$ for wild-type mice IL-12/ms vs. blank ms, $P < 0.01$ for CD4-KO mice IL-12/ms vs. blank ms (Kaplan–Meier analysis, log-rank test). (**g**) Course of infection (FA1090) in CD8-KO vs. wild-type mice immunized with FA1090 OMV; left panel, recovery (CFUs) of *N. gonorrhoeae* (mean ± s.e.m., $N = 8$ mice), $^{\#}P < 0.05$, $^{*}P < 0.01$ (ANOVA) for comparisons shown; right panel, % of animals remaining infected at each time point, $P < 0.001$ for wild-type mice IL-12/ms vs. blank ms, $P < 0.02$ for CD8-KO mice IL-12/ms vs. blank ms (Kaplan–Meier analysis, log-rank test).

mentary Table S4, and **Supplementary Figure S8a,b**). Thus, deficiency of either IFNγ or B cells abrogated the adjuvant effect of IL-12/ms in generating immune resistance to genital gonococcal infection. The production of gonococcus-specific vaginal and serum IgA and IgG antibodies induced by OMV plus IL-12/ms in wild-type mice was abrogated in IFNγ-KO mice (**Figure 7c,d**), and as expected there was no generation of IFNγ by the ILN cells of immunized IFNγ-KO mice (not shown). Similarly, in μMT mice there was no detectable antibody response to immunization (not shown). In contrast, the numbers of $IFN\gamma^{+}/CD4^{+}$ T cells in ILNs of μMT mice immunized with gonococcal OMV plus IL-12/ms were not affected, and there was no IL-4 response, whereas IL-17 responses remained unaltered (**Figure 5e**). These findings indicate that resistance induced by immunization with gonococcal OMV plus IL-12/ms depended on both IFNγ and B cells, the latter presumably to produce gonococcus-specific antibodies.

The protective effect of immunization with gonococcal OMV plus IL-12/ms was incompletely diminished in CD4-KO, and partially also diminished in CD8-KO mice, in comparison with wild-type controls (**Figure 7f,g**, **Supplementary Table S4**, and **Supplementary Figure S8c,d**). These findings suggest that the requirement for $CD4^{+}$ T cells to generate immune resistance could be partially compensated by other cells, including $CD8^{+}$ or natural killer cells, that can also produce IFNγ. However, $CD8^{+}$ cells appeared to be less critical for protective immunity.

DISCUSSION

We have demonstrated for the first time that a vaccine-induced state of immune resistance to genital gonococcal infection can

be reliably generated by an intact mammalian immune system. This state of immunity appears to depend on antibody production by B cells, and on the generation of IFNγ mainly by $CD4^+$ T cells. I.vag. vaccination of mice with gonococcal OMV plus IL-12/ms as an adjuvant induced serum and vaginal IgG and IgA antibodies against gonococcal antigens, and IFNγ-secreting $CD4^+$ and $CD8^+$ T cells in the draining ILN. Both Th1 cellular and antibody responses persisted for several months after immunization, and were capable of eliciting resistance to challenge with *N. gonorrhoeae* for at least 6 months, with the recall of immune memory. I.vag. immunization with gonococcal OMV alone, either without adjuvant or with control (blank) ms, induced only weak antibody responses with no detectable IFNγ production, and no significant resistance to challenge infection. Control immunization with OMV prepared from NTHI plus IL-12/ms did not generate immune resistance or antibodies crossreactive with *N. gonorrhoeae*, although an IFNγ response was induced. Thus, although IFNγ appears to be necessary for resistance to *N. gonorrhoeae*, without specific antibodies it is not sufficient.

It should be noted that all mice eventually clear genital gonococcal infection, usually within 2–3 weeks regardless of immunization or other treatment. We previously reported that IL-17-dependent responses contribute to this as infection is prolonged in IL-17RA-deficient mice,[19] and, furthermore, i.vag. treatment with microencapsulated IL-17 shortens the course of infection, importantly however without enhancing resistance to reinfection.[26] Nevertheless, the infection is eventually eliminated in untreated or in IL-17RA-deficient mice, probably because *N. gonorrhoeae*, as an exclusively human pathogen, is not adapted to survive for longer periods in mice. For example, *N. gonorrhoeae* cannot utilize murine transferrin as a source of iron,[33] nor effectively bind murine factor H or C4B-binding protein to inhibit complement activation,[34] nor exploit murine epithelial cell receptors as effectively as it exploits the human equivalents.[35,36] Nevertheless, we have repeatedly found that IL-12/ms, given i.vag. either as a therapeutic treatment of an existing infection[26] or as an adjuvant with gonococcal OMV vaccine (this study), enhances Th1-driven protective immunity revealed by a significantly shortened course of genital gonococcal infection. Notably, a similar dose of free, soluble IL-12 is ineffective,[26] indicating that sustained release of IL-12 from the slowly hydrolyzing microspheres over several days is necessary to induce the protective immune responses. Possibly, however, the microspheres also facilitate uptake by the genital tissues, as reported for another microparticulate formulation.[37]

What is perhaps most remarkable about the findings presented here, given the well-known and extensive antigenic variation shown by *N. gonorrhoeae*, is that resistance extended to heterologous strains as well as against the homologous strain from which the OMV vaccine was prepared. The full extent of this cross-protection is unknown at present, but our results show that immunization with OMV derived from strain FA1090 enhances resistance equally well against strains MS11 and FA19, and vice versa, and that resistance extends to clinical isolates of *N. gonorrhoeae* in addition to these "laboratory strains". Among the major gonococcal surface antigens, we know that FA1090, MS11, and FA19 differ in their porin (PorB) molecules. FA1090 and MS11 possess different subtypes of PorB.1B, and FA19 has PorB.1A.[28] Although not as well characterized, the Opa proteins encoded in their genomes differ[29,38] and their LOS are different.[39] Opa proteins and LOS glycan chains are also phase-variable, resulting in the expression of different antigenic epitopes.[40] Recent quantitative proteomic studies revealed additional differences and commonalities in the composition of the extracellular proteome (cell envelopes and naturally secreted OMV) existing between these gonococcal strains.[31]

Consistent with cross-protective immunity, ELISA analysis of antibodies induced by immunization revealed quantitatively similar levels of antibodies detectable against the different strains, with respect to both IgG and IgA in serum and vaginal washes. The antibodies appeared to be specific for *N. gonorrhoeae* as they were not detected against *E. coli* or NTHI, and they were not generated by immunization with OMV prepared from NTHI. Western blot analysis of serum IgG antibodies, however, revealed evidence of antigens shared between different strains of *N. gonorrhoeae*. Bands migrating at 45–65 kDa resembled those observed in an earlier study on intranasal immunization with gonococcal OMV that found modest partial protection against genital gonococcal infection.[32] These bands migrated at higher molecular mass than major gonococcal outer membrane proteins such as porin and Opa that are in the range 30–40 kDa. Although in these studies we did not observe putative antibodies against gonococcal Rmp ($\sim$25 kDa), it will be important to determine whether anti-Rmp antibodies are induced, because of their counter-protective effect in blocking complement activation by antibodies against other antigens.[41] Further studies on many more samples of serum and vaginal wash from immunized mice need to be undertaken to determine the range of gonococcal antigens detected by the induced antibodies.

Immunoproteomics technology has provided vaccine candidate antigens for many infectious diseases,[42,43] and our initial efforts have identified two novel gonococcal vaccine candidates, EF-Tu in FA1090, MS11, and FA19 OMV, and PotF3 also in FA1090. Corroborating these findings, both proteins were also identified in quantitative proteomic profiling of cell envelopes and OMV derived from four common gonococcal isolates.[31] EF-Tu is of particular interest as it has been identified in both spots and in all analyzed OMV. EF-Tu is commonly perceived as a cytosolic GTP-binding protein and an essential factor in protein synthesis, but only 20–30% of its total cellular pool is involved in protein synthesis at any given time.[44] EF-Tu is encoded by at least two genes and has been identified previously in gonococcal periplasm as well as on the surface of bacterial cell envelopes and OMVs.[45–47] In *N. gonorrhoeae* two separate open reading frames of identical sequences encode EF-Tu (NGO1842 and NGO1858; **Supplementary Table S3**). In support of our findings, EF-Tu has been shown to exhibit many functions associated with pathogenesis and to elicit antibody

responses during infection by several bacteria, and by a meningococcal group B OMV vaccine.[48–51] The role of PotF3 in gonococcal pathogenesis and immunity is unknown at this point; however, PotF2 appears to function as a polyamine-binding protein, and spermine inhibits gonococcal biofilm formation.[52] Further immunoproteomic analysis of additional samples of serum and vaginal wash from immunized mice is likely to reveal many more, hitherto unsuspected gonococcal antigens that might contribute to protective immunity against *N. gonorrhoeae*.

Immune resistance induced by i.vag. immunization with gonococcal OMV plus IL-12/ms depended on IFNγ, the key cytokine in Th1-driven responses, as IFNγ-deficient mice did not generate protective immunity and antibody responses were substantially diminished. Similarly, immune resistance was abrogated in μMT mice that lack B cells and hence the ability to generate antibodies, although they retained IFNγ production. Consistent with Th1-driven immunity, serum and vaginal IgG antibodies were mainly of the IgG2a subclass that in mice is particularly effective in mediating complement activation by the classical pathway, and in opsonization for phagocytosis by neutrophils and macrophages.[53] However, it is unclear at this point which mechanisms of anti-gonococcal defense are most important for the observed protection in the murine genital tract. Murine vaginal secretions contain both IgG, probably derived largely from the circulation, and locally produced secretory IgA.[54] Both isotypes of anti-gonococcal antibodies were induced in response to i.vag. immunization with OMV plus IL-12/ms, but their relative contribution to defense against genital gonococcal infection is unknown at this point. The role of IFNγ may have been to skew the IgG isotype response toward IgG2a,[55] but it is also possible that it enhanced phagocytosis by upregulating the expression of high-affinity FcγR-I on neutrophils and macrophages, and by activating macrophages. Other mechanisms of IFNγ-driven T cell-mediated defense cannot be ruled out, although these might be considered less likely against a predominantly extracellular bacterial infection. In addition, it is possible that IL-12 may have had direct effects on B cells that express the IL-12 receptor[56] and can respond by differentiating and generating antibodies.[57–60] This may also help to explain why CD4 deficiency did not totally abrogate the adjuvant effect of IL-12/ms on immunization with OMV for inducing protective responses. In addition, other cells including $CD8^+$ T cells, as well as possibly natural killer cells, can serve as an alternative source of IFNγ; deficiency of CD8 was not strongly detrimental to the adjuvant effect of IL-12/ms.

Two major questions are the extent to which these findings relate to immunity to gonorrhea in humans, and whether this approach to vaccination can be developed for human use. The answers are not straightforward, in part because the absence of a demonstrable state of protective immunity to *N. gonorrhoeae* in humans means that the determinants or even correlates of protection are unknown.[9] Previous studies in humans with uncomplicated genital gonococcal infection have revealed a paucity of antibody responses in serum and genital secretions.[20,21] In addition, cytokine responses were weak, although inflammatory cytokines such as IL-1 and IL-6 were elevated in some women who were coinfected with *Chlamydia trachomatis* or *Trichomonas vaginalis* in addition to *N. gonorrhoeae*.[20] However, these studies performed nearly 20 years ago were limited by the technology for measuring cytokines available at the time, and by the existing knowledge of cytokines. For example, although IL-17 had been discovered in 1995,[61] its significance for antimicrobial defense did not become widely appreciated until after 2005 when the Th17 subset of lymphocytes was defined.[62,63] There is therefore a need to update these studies on human immune responses in gonorrhea, using current multiplex technology for detecting multiple cytokines in limited amounts of samples, and including more recently discovered cytokines.

Although the mouse model does not fully mimic the human infection or the consequent disease,[64] it nevertheless remains the only currently available model for the prospective investigation of immune responses to *N. gonorrhoeae*. There are certain parallels between what is known about the immune response of mice and humans to *N. gonorrhoeae*. In the absence of intervention in either species, the immune response to uncomplicated lower tract infection in females is minimal: there is little or no specific antibody response, and no evidence for the generation of effective, recallable immune memory involving Th1 or Th2 cells. However, two reports indicate that humans infected with gonorrhea have elevated IL-17,[22,23] as do female mice infected with *N. gonorrhoeae*.[19] Both mice and humans can be reinfected with *N. gonorrhoeae* with apparently no effective protection, whether measured in terms of probability of acquisition, duration or intensity of infection, or disease, arising from prior exposure. Therefore, although it would be mistaken to assume that findings in the murine model are directly translatable to humans, our findings raise questions and specific hypotheses for evaluation in humans.

The prevailing paradigm for lack of effective immunity to gonorrhea holds that the extensive antigenic variation shown by *N. gonorrhoeae*, involving most of its major surface antigens, coupled with multiple mechanisms for resisting complement, enable it to evade whatever adaptive immune responses the host may develop. Our studies in mice, presaged by conclusions derived from the studies of Hedges *et al.*[20,21] in humans, reveal that, in addition, *N. gonorrhoeae* suppresses adaptive immune responses by inducing high levels of regulatory cytokines TGFβ and IL-10, and type 1 regulatory T cells.[17,24,25] Counter-manipulating this suppression by means of neutralizing antibodies to TGFβ and IL-10,[17,25] or by the local administration of microencapsulated IL-12,[26] allows Th1-governed responses to emerge, with the development of specific antibodies, establishment of memory, and accelerated clearance of infection.

This study builds on these findings in the context of vaccination by demonstrating that mice can be immunized against *N. gonorrhoeae* by the i.vag. administration of a nonliving vaccine (OMV) with a Th1-driving adjuvant, IL-12/ms. Whether this approach is applicable to humans remains to be determined. Most likely, i.vag. immunization will not be

acceptable, and is inapplicable for males; therefore, another route of immunization will be needed. In addition, other Th1-driving adjuvants may be required. However, the findings imply that a vaccine against *N. gonorrhoeae* may indeed be feasible despite earlier setbacks and subsequent pessimism,[64] and they serve to indicate the type of immune responses that will need to be induced to generate protective immunity.

METHODS

Mice. All mice, including wild-type BALB/c and C57BL/6 mice, B6.129S7-*Ifng*^{tm1Ts}/J (IFNγ-deficient), B6.129S2-*Ighm*^{tm1Cgn}/J (B-cell deficient; also known as μMT), B6.129S2-*Cd4*^{tm1Mak}/J (CD4-deficient), and B6.129S2-*Cd8a*^{tm1Mak}/J (CD8-deficient) mice on a C57BL/6 background, were purchased from Jackson Laboratories (Bar Harbor, ME). BALB/c mice were used for the experiments unless otherwise specified. Mice were maintained in a BSL2 facility in the Laboratory Animal Facility at the University at Buffalo that is fully accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care). All animal use protocols were approved by the institutional animal care and use committee of the University at Buffalo.

Bacteria. *N. gonorrhoeae* strain FA1090 was provided by Dr Janne Cannon (University of North Carolina at Chapel Hill, Chapel Hill, NC); strain MS11 was provided by Dr Daniel Stein (University of Maryland, College Park, MD); strain FA19 and clinical isolates were obtained from the collection of clinical strains maintained at the University of North Carolina at Chapel Hill. The original clinical strains have been stored at −80 °C since initial isolation in 1992 and have been minimally subcultured.[65] For use in the murine infection model, *N. gonorrhoeae* strains 9087 and 0336 were transformed with the streptomycin-resistant *rpsL* gene from strain FA1090 to generate strains GC68 and GC69, respectively. *E. coli* K12 was provided by Dr Terry Connell (University at Buffalo, Buffalo, NY). NTHI strain 6P24H1 was provided by Dr Timothy Murphy (University at Buffalo). *N. gonorrhoeae* was cultured on GC agar supplemented with hemoglobin and Isovitalex (BD Diagnostic Systems, Franklin Lakes, NJ) and the resultant growth was checked for colony morphology consistent with Opa protein and pilus expression. NTHI was cultured on GC agar supplemented with hemoglobin only. *E. coli* was cultured on BHI agar. Bacteria were harvested from plates and the cell density was determined as detailed previously.[24]

IL-12 microspheres. Murine IL-12 (Wyeth, Philadelphia, PA) was encapsulated into poly-lactic acid microspheres using the Phase Inversion Nanoencapsulation technology as previously described except that bovine serum albumin was replaced by sucrose (0.1%, w/w).[66] Blank microspheres were prepared in the same way but without IL-12.

Gonococcal OMVs. After 18–22 h culture on supplemented GC agar, *N. gonorrhoeae* was harvested from plates into ice-cold lithium acetate buffer (pH 5.8) and passed through a 25-gauge needle 10–12 times to sheer the outer membranes from the bacteria. The suspensions were spun in microfuge tubes at 13,000 RPM for 1 min. The supernatants were collected and ultracentrifuged at 107,000 × *g* for 2 h. The pellet was washed with 50 mM Tris-HCl (pH 8.0) and resuspended in phosphate-buffered saline (PBS). Protein was assayed with the Micro BCA protein kit (Thermo Scientific, Rockford, IL) or RC DC Protein Assay kit (Bio-Rad, Hercules, CA).

Immunization schedule and mouse vaginal infection model. Groups of 8 female mice between 7 and 9 weeks of age were immunized i.vag. with gonococcal OMV (40 μg protein) of various strains as described, plus IL-12/ms (1 μg IL-12) or blank ms in a total volume of 40 μl PBS; control groups were sham-immunized with IL-12/ms or with blank ms alone. Mice were immunized 1 to 3 times with a 7–14 day interval, as indicated. After a further 2 weeks to 6 months, immunized mice were infected with 5×10^6 CFUs live *N. gonorrhoeae* as previously described,[15,26] with the modification that 0.5 mg Premarin (Pfizer, Philadelphia, PA) was used as estradiol administered subcutaneously on days −2, 0, and 2. Vaginal swabs collected daily were quantitatively cultured on GC agar supplemented with hemoglobin, Isovitalex, and selective antibiotics (vancomycin, streptomycin, nisin, colistin, and trimethoprim) to determine the bacterial colonization loads.[15,26] The limit of detection was 100 CFU recovered per mouse. Gonococcal recovery was counted by an individual who was "blinded" to the experimental treatments, and all experiments were repeated 2 or 3 times for verification.

Assay of serum and mucosal antibodies. Samples of vaginal wash and serum were collected from the mice at the indicated time points.[17,26] Gonococcus-specific IgA, IgG, IgM, and IgG subclass antibodies IgG1, IgG2a, IgG2b, and IgG3 in vaginal washes and sera were measured by ELISA on plates coated with whole gonococci, using undiluted vaginal wash and 10-fold diluted serum as starting dilutions.[17,26] Total IgA, IgG, and IgM concentrations in secretions were assayed by ELISA on plates coated with anti-IgA, -IgG, or -IgM antibodies (Southern Biotech, Birmingham, AL). H5 mouse monoclonal antibody (specific for *N. gonorrhoeae* porin serovar PIB3) or affinity-purified mouse IgA, IgG, and IgM (Southern Biotech) were used to establish standard curves. Bound antibodies were detected by alkaline phosphatase-conjugated goat anti-mouse IgA, IgG, IgM, IgG1, IgG2a, IgG2b, or IgG3 antibody (Southern Biotech) and *p*-nitrophenylphosphate substrate (Southern Biotech). Plates were read in a VersaMax microplate reader with SoftMax software (Molecular Devices, Sunnyvale, CA) or an EL_X800 Universal microplate reader with KC Junior software (Bio-Tek Instruments, Winooski, VT). Antibody data were expressed as relative (fold increase) to the antibody levels detected in control samples (from sham-immunized mice) assayed simultaneously.

Flow cytometry. Isolated cells were washed with staining buffer twice, then incubated with the indicated antibodies for 30 min on ice, washed, and analyzed on a FACSCalibur cytometer. For intracellular staining, cells were first fixed with Cytofix/Cytoperm (eBioscience, San Diego, CA). Antibodies to mouse CD4, CD8, IFNγ, IL-4, and IL-17A conjugated with fluorescein isothiocyanate, phycoerythrin, or allophycocyanin were purchased from eBioscience.

Lymphocyte isolation and culture. Mononuclear cells were isolated from aseptically harvested ILN using Histopaque 1083 (Sigma-Aldrich, St Louis, MO) density gradient centrifugation and pooled from two or three mice to provide sufficient numbers of cells for culture. $CD4^+$ T cells were purified through negative selection using a Dynal CD4 cell isolation kit (Invitrogen, Carlsbad, CA). Cells were cultured in 24-well culture plates at a density of 2×10^6 cells per ml in the presence of equal numbers of mitomycin C-inactivated spleen cells to serve as antigen-presenting cells, either with no stimulus or with 2×10^7 *N. gonorrhoeae* cells.

Proliferation assays. Cells were labeled with CFSE (Sigma-Aldrich).[67] CFSE-labeled cells were then washed twice in PBS, recounted, and stimulated as described above. Cultured cells were harvested and then stained with allophycocyanin-conjugated anti-mouse CD4 antibody. The data were acquired by gating on the $CD4^+$ cell populations in a FACSCalibur cytometer. The attenuation of CFSE fluorescence was used to measure cell proliferation.

Cytokine ELISA. IFNγ, IL-4, and IL-17A levels were measured in triplicate using ELISA kits purchased from eBioscience.

Real-time reverse transcriptase-PCR. Total cellular RNA of whole vaginas harvested from the mice was isolated with RNeasy Mini Kits (Qiagen, Valencia, CA), and was transcribed to complementary DNA

using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time reverse transcriptase-PCR was performed on an iCycler iQ detection system (Bio-Rad) using Sybergreen (Bio-Rad) for real-time monitoring of the PCR. The primers used were as follows: IFNγ, 5′-TACTGCCACGGCACAGTCATTGAA-3′, 5′-GCAGCGACTCC TTTTCCGCTTCCT-3′; IL-4, 5′-GAAGCCCTACAGACGAGCT CA-3′, 5′-ACAGGAGAAGGGACGCCAT-3′; IL-17A, 5′-TCAGG-GTCGAGAAGATGCTG-3′, 5′-TTTTCATTGTGGAGGGCAGA-3′; β-actin, 5′-CCTAAGGCCAACCGTGAAAAG-3′, 5′-GAGGCATA-CAGGGACAGCACA-3′. Relative quantification of target genes was analyzed based on the threshold cycle (Ct) determined by Bio-Rad iQ5 optical system software.

Western blot. *N. gonorrhoeae* OMV preparations were boiled for 5 min in SDS loading buffer containing 2-mercaptoethanol. Protein quantification was done with the RC DC Protein Assay kit. From each sample, 10 μg of protein was separated on 10% polyacrylamide SDS electrophoresis gels. Protein bands were transferred onto nitrocellulose membranes using the electrophoresis transfer system (Bio-Rad). The nitrocellulose membranes were blocked with PBS containing 3% skim milk overnight at 4 °C before incubation for 2 h with serum samples diluted 1:200, or vaginal wash samples diluted 1:20 in PBS containing 3% skim milk. Specific antibodies bound to *N. gonorrhoeae* OMV preparations were detected with horseradish peroxidase-conjugated goat anti-mouse-IgG (Santa Cruz Biotechnology, Paso Robles, CA) at a dilution of 1:4,000. The Pierce (Rockford, IL) detection kit was used for chemiluminescent detection and images were collected with a ChemiDoc MP imaging system (Bio-Rad).

Immunoproteomics. Protein concentration in OMV was measured using DC Protein Assay Kit (Bio-Rad). Samples of OMV (300 μg and 50 μg of protein, for 2D SDS-PAGE tandem mass spectrometry (MS/MS) analysis and immunoblotting, respectively) were precipitated overnight in 90% acetone, washed twice with 100% ice-cold acetone, and air-dried. Protein pellets were reconstituted in 200 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% ASB-14, 1% DTT, 2 mM TBP, 2% 3–10 IPG buffer, trace of Orange G) and used to rehydrate pH 4–7 ReadyStrip IPG strips (Bio-Rad) overnight at 25 °C. Isoelectric focusing was carried out using the PROTEAN i12 IEF System (Bio-Rad) for a total of 26,000 Vh with the following settings: 50 μA current limit, 8000 V rapid ramp for 26,000 Vh, 750 V hold. The 2D SDS-PAGE was performed using Criterion TGX Any kD gels (Bio-Rad). The proteins were stained overnight in Flamingo fluorescent stain (Bio-Rad) and the spots were visualized using the ChemiDoc Imaging System (Bio-Rad). For immunoblotting, separated proteins were transferred onto polyvinylidene difluoride membranes using the TurboBlott transfer system (Bio-Rad). The membranes were blocked for 2 h in 5% milk in PBS Tween, and probed by overnight incubation with sera from immunized mice, followed by incubation with anti-mouse horseradish peroxidase-conjugated antibodies (Bio-Rad). Spots were visualized using Clarity Western ECL Substrate and ChemiDoc MP Imaging System (Bio-Rad). Proteins on membranes were stained with Novex Reversible Membrane Protein Stain (Invitrogen) to overlay positions of selected "anchor" spots with the Flamingo-stained 2D gels. Matching spots were excised and the proteins were trypsin digested as previously described.[68] Samples containing extracted peptides were desalted using ZipTip C_{18} (Millipore, Billerica, MA) and eluted with 70% acetonitrile/0.1% trifluoroacetic acid, and dried in a speed vac. Desalted peptides were brought up in 2% acetonitrile in 0.1% formic acid (20 μl) and analyzed (2 μl) by liquid chromatography electrospray ionization MS/MS with a Thermo Scientific Easy-nLC II (Thermo Scientific, Waltham, MA) nano-HPLC system coupled to a hybrid Orbitrap Elite ETD (Thermo Scientific) mass spectrometer using an instrument configuration as previously described.[69] In-line desalting was accomplished using a reversed-phase trap column (100 μm × 20 mm) packed with Magic C_{18}AQ (5 μm 200 Å resin; Michrom Bioresources, Auburn, CA) followed by peptide separations on a reversed-phase column (75 μm × 250 mm) packed with Magic C_{18}AQ (5-μm 100 Å resin; Michrom Bioresources) directly mounted on the electrospray ion source. A 30 min gradient from 7% to 35% acetonitrile in 0.1% formic acid at a flow rate of 400 nl min^{-1} was used for chromatographic separations. The heated capillary temperature was set to 300 °C and a spray voltage of 2750 V was applied to the electrospray tip. The Orbitrap Elite instrument was operated in the data-dependent mode, switching automatically between MS survey scans in the Orbitrap (AGC target value 1,000,000, resolution 240,000, and injection time 250 ms) with MS/MS spectra acquisition in the linear ion trap (AGC target value of 10,000, and injection time 100 ms). The 20 most intense ions from the Fourier-transform full scan were selected for fragmentation in the linear trap by collision-induced dissociation with normalized collision energy of 35%. Selected ions were dynamically excluded for 15 s with a list size of 500 and exclusion mass by mass width ± 0.5. Data analysis was performed using Proteome Discoverer 1.4 (Thermo Scientific). All identified peptides were searched against a *N. gonorrhoeae* database (FA1090, FA19, and MS11) combined with cRAP.fasta, a database of common contaminants (http://www.thegpm.org/crap/); this creates a list of proteins commonly found in proteomics experiments that are present by accident or unavoidable contamination. Trypsin was set as the enzyme with maximum missed cleavages set to 2. The precursor ion tolerance was set to 10 p.p.m. and the fragment ion tolerance was set to 0.8 Da. Variable modifications included oxidation on methionine (+ 15.995 Da) and carbamidomethyl on cysteine (+ 57.021 Da). Data were searched using Sequest HT, and all search results were run through Percolator for scoring (Proteome Discoverer 1.4; Thermo Scientific).

Statistical analysis. Data are expressed as the mean ± s.e.m. Data on the effect of immunization on recovery of *N. gonorrhoeae* after inoculation were analyzed using two-way analysis of variance for repeated measures with Fisher's protected least significant difference *post hoc* tests. In addition, Kaplan–Meier analysis with log-rank tests was used to compare clearance of infection (defined as the first of 3 successive days of zero recovery) between treatment groups. For immune response data, unpaired two-tailed *t*-tests were used to compare the mean values between two groups, or analysis of variance with Bonferroni *post hoc* tests was used for multiple comparisons. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using Microsoft Excel or Prism 5 (GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS

Y.L. and M.W.R. conceived and designed the animal experiments, interpreted and analyzed the data, and wrote the manuscript with input from M.M.H., A.E.S. and A.E.J.; Y.L. and L.A.H. performed animal experiments; L.A.H. and W.L. performed *in vitro* analyses; M.M.H. collected, characterized, and transformed clinical neisserial isolates; R.A.Z. and A.E.S. designed and performed proteomics analyses; A.E.J. conceived, developed, and refined the animal model and advised on its use and interpretation; N.K.E. conceived and developed the cytokine microencapsulation process. All authors read and approved the final manuscript.

DISCLOSURE

N.K.E. has ownership interest in TherapyX that is developing sustained-release nanoparticulate adjuvants for inflammatory disease therapy. Y.L. and L.A.H. are salaried employees of TherapyX. M.W.R. serves as a paid consultant for TherapyX. The other authors declared no conflict of interest

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