Contrasting roles of IL-22 and IL-17 in murine genital tract infection by *Neisseria gonorrhoeae*

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Murine genital tract infection with *Neisseria gonorrhoeae* has previously been found to induce IL-17 which is important in both recruitment of neutrophils and prompt clearance of the infection. As IL-22 is another Th17-related cytokine that has been implicated in the immune responses in several infection models, we investigated its role in vaginal gonococcal infection of mice. Production of IL-22 was observed in response to stimulation with *N. gonorrhoeae* in both mouse splenic mononuclear cells and vaginal tissue explants cultured *ex vivo*. Tissue from mice genetically deficient in IL-22 showed diminished production of IL-6 and the CXC chemokine KC in response to *N. gonorrhoeae*, whereas IL-17 and the chemokines LIX and MIP-2α were produced to the same extent as in wild-type tissue. IL-22-deficient mice were unexpectedly resistant to genital tract infection with *N. gonorrhoeae in vivo*, but showed no change in the influx of neutrophils to the site of infection. These results reveal divergent roles for IL-17 and IL-22 in response to gonococcal infection.

Keywords: interleukin-22, immune response, *Neisseria gonorrhoeae*, genital infection, mouse

INTRODUCTION

Neisseria gonorrhoeae is an exclusively human pathogen that has probably been associated with humans for many millennia, and consequently has become extremely well adapted to survival within its host. Although it can infect any exposed mucosal surface, it is mainly a sexually transmitted infection of the genital tract. Typically the infection elicits an acute inflammatory response marked by an influx of neutrophils, although clinically inapparent cervical infection can persist for prolonged periods in women, so that timely treatment may not be sought. In men urethral infection usually becomes symptomatic within a few days, prompting presentation for treatment. If left untreated, cervical infection can ascend into the upper reproductive tract, causing pelvic inflammatory disease and leading to tubal scarring, infertility, and risk for ectopic pregnancy (Hook and Handsfield, 1999). The infection has usually been treated effectively with antibiotics, but there are now concerns that strains of *N. gonorrhoeae* are emerging with resistance to multiple classes of antibiotics (Allen et al., 2011). Repeated infections are common, and no state of protective immunity appears to develop as a consequence of infection. Thus the immune response to *N. gonorrhoeae* has been somewhat puzzling.

Recently we have reported that, in a mouse model of female genital tract infection, *N. gonorrhoeae* induces the production of IL-17 which has an important role in directing host response including the neutrophil influx (Feinen et al., 2010). Moreover, abrogation of signaling through the principal IL-17 receptor, IL-17RA, in IL-17RA-knockout (ko) mice results in a diminished neutrophil

influx and prolongation of the infection (Feinen et al., 2010). However, it is unlikely that clearance of the infection depends on phagocytosis by neutrophils; other factors induced by IL-17 may be responsible for this (reviewed in Liu et al., 2011; Packiam et al., 2011).

Interleukin-22, a member of the IL-10 family of cytokines, is also secreted by Th17 cells (Liang et al., 2006), as well as by a subset of natural killer cells (designated NK22; Cupedo et al., 2009) and even by some Th1 cells (Duhen et al., 2009). Other studies have suggested that a distinct Th22 lineage of cells exists (Eyerich et al., 2009; Fujita et al., 2009). Many of the same cytokines that induce differentiation and proliferation of IL-17-producing cells also lead to the secretion of IL-22 by Th17 cells, NK22 cells, and putative Th22 cells, including IL-6, IL-23, IL-1β, TGFβ, and TNFα (reviewed in Wolk et al., 2010). IL-17 and IL-22 are therefore frequently produced together in response to infections.

Interleukin-22 interacts with a heterodimeric receptor, IL-10R2/IL-22R1 (Wolk et al., 2004), which is expressed on a variety of non-lymphoid cells, especially epithelial cells, and ligation of this receptor leads to both protective and detrimental effects. In synergy with IL-17, IL-22 induces proinflammatory cytokines in human bronchial epithelial cells (Aujla et al., 2008) and colonic myofibroblasts (Andoh et al., 2005). Independently or in synergy with IL-17, IL-22 acts in defense against intestinal infection of mice with *Citrobacter rodentium* (Zheng et al., 2008). In this model, IL-22 was produced earlier in the infection than IL-17, and was essential for clearance of the bacteria and survival of the

animals, while IL-17 was dispensable. Blockade of IL-22 downregulates the expression of CXC chemokines which are involved in the recruitment of neutrophils in response to infection with *Klebsiella pneumoniae* (Aujla et al., 2008). IL-22 appears to have a particularly important role in inducing epithelial cells to secrete antibacterial proteins, including β-defensins, S100 proteins, RegIIIγ, and lipocalin-2 (Ouyang and Valdez, 2008).

We previously found that IL-22 was among the Th17 cytokines produced by explants of mouse vaginal tissue cultured *in vitro* with *N. gonorrhoeae* (Feinen et al., 2010). We hypothesized that production of IL-22 is an important component of the response to *N. gonorrhoeae* and is essential for the recruitment of innate defense mechanisms against it including neutrophils and soluble antibacterial factors. We have therefore investigated further the production of IL-22 by murine lymphocytes in response to stimulation with *N. gonorrhoeae*, and have examined its role in gonococcal genital tract infection using IL-22-ko mice, anticipating that the course of infection would be prolonged in the absence of IL-22, as we had found for interference with IL-17RA-dependent signaling.

MATERIALS AND METHODS

MICE AND VAGINAL INFECTION MODEL

Wild-type (WT) C57BL/6 and BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). Breeding pairs of IL-22-ko mice on a C57BL/6 background were provided by Genentech (South San Francisco, CA, USA) and bred in the Laboratory Animal Facility of the University at Buffalo. Mice were maintained in HEPA-filtered cages with autoclaved food, water, and bedding. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee, and in compliance with all applicable laws and guidelines.

For the vaginal gonococcal infection model (Jerse, 1999), aged-matched groups of 10 female C57BL/6 WT and IL-22-ko mice, between 8 and 10 weeks of age and in anestrus or diestrus, were injected with estradiol on days −2, 0, and 2, and commenced on an antibiotic regimen as described previously (Feinen et al., 2010). On day 0, mice were infected by intravaginal instillation of 20 μl of phosphate-buffered saline containing 2×10^6 colony-forming units (CFU) of piliated Opa^+ *N. gonorrhoeae* strain FA1090 that had been freshly harvested from GC agar plates supplemented with hemoglobin and IsoVitalex, as described (Feinen et al., 2010). Infection was monitored daily by vaginal swabbing and serial dilution of the recovered mucus on chocolate agar plates containing selective antibiotics, which were incubated overnight at 37°C in 5% CO_2/air (Feinen et al., 2010). Replicate cultures were also performed on brain–heart infusion agar for the commensal microbiota, and any mice that developed an overgrowth of commensals were promptly removed from experiment, leaving no fewer than seven mice per group. Duplicate swabs were smeared onto glass slides, stained with Hema 3 (Fisher Scientific), and examined microscopically to count the numbers of neutrophils relative to 100 epithelial cells.

SPLEEN CELL CULTURE

Mononuclear cells were isolated from BALB/c mouse spleens by centrifugation on Histopaque 1083 (Sigma, St. Louis, MO, USA)

and cultured in 24-well plates at a density of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in 5% CO_2/air. Cultures were stimulated by adding *N. gonorrhoeae* FA1090 cells (2×10^7 CFU/ml), gonococcal outer membrane vesicles (OMV; 5 μg/ml), obtained by shearing *N. gonorrhoeae* FA1090 cells in lithium acetate buffered as described (Feinen et al., 2010), or ConA (2 μg/ml). Supernatants were harvested after 3 days, and IL-22 was assayed by ELISA using a kit obtained from eBioscience (San Diego, CA, USA).

VAGINAL EXPLANTS

Vaginas were excised aseptically from WT or IL-22-ko C57BL/6 mice, and fragments of approximately 10 mg were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES, penicillin, streptomycin, and fungizone, as described previously (Feinen et al., 2010). Cultures were stimulated with *N. gonorrhoeae* at a concentration of 2×10^7 CFU/ml, prepared as described above. Supernatants were removed after 3 days and assayed for IL-6, IL-17, IL-22, LIX, KC, and MIP-2α by means of ELISA using reagents and kits obtained from eBioscience or R&D Systems (Minneapolis, MN, USA).

STATISTICS

Statistical analysis was performed using InStat 3 or Prism 5 (GraphPad Software, San Diego, CA, USA). Student's *t* or two-way ANOVA with Bonferroni *post hoc* test were used to determine the significance of difference of means. Kaplan–Meier survival analysis with the log-rank test was used to evaluate the duration of infection in animals. $P < 0.05$ in two-tailed tests was considered significant.

RESULTS

To determine whether *N. gonorrhoeae* can induce production of IL-22 from murine immune cells, splenic mononuclear cells were cultured with or without *N. gonorrhoeae* or OMV, which we had previously shown to replicate stimulation with live *N. gonorrhoeae* (Feinen et al., 2010), or with ConA as a positive control. As observed previously for IL-17, IL-22 was secreted into the supernatants of cells cultured with live gonococci or OMV (**Figure 1**). This result indicates that mononuclear cell populations from mice are capable of producing IL-22 in response to *N. gonorrhoeae*, without requiring additional factors from infected tissue.

We have previously shown that mouse vaginal explants produced IL-22 in response to *N. gonorrhoeae* in an *ex vivo* culture model, and that generation of IL-22 was independent of signaling through IL-17RA (Feinen et al., 2010). To expand on this finding, mouse vaginal explants from either WT or IL-22-ko mice were cultured alone or with *N. gonorrhoeae*, and the secretion of inflammatory cytokines and CXC chemokines into the supernatants was determined by ELISA. The results confirmed that IL-22 was produced by WT vaginal tissue in response to *N. gonorrhoeae*, but no IL-22 was detected in the supernatants of vaginal explants obtained from IL-22-ko mice (**Figure 2**). The IL-22-ko explants also showed unaltered IL-17 production relative to explants from WT mice, although generation of IL-6 was significantly diminished (**Figure 2**). In addition, production of the chemokine KC (CXCL1) in response to *N. gonorrhoeae* was diminished in IL-22-ko tissue,
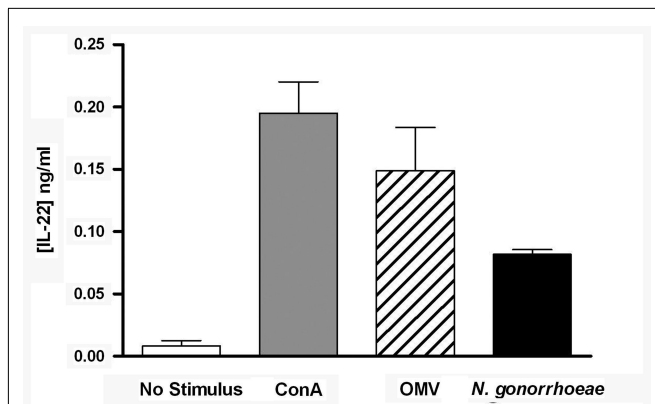
FIGURE 1 | Production of IL-22 from mouse splenic mononuclear cells, incubated in medium only (control), ConA (2 μg/ml), *N. gonorrhoeae* (2 × 10⁷ CFU/ml), or gonococcal OMV (5 μg/ml) for 3 days. Supernatants were assayed for IL-22 by ELISA. Data are shown as mean ± SD, *N* = 3, from one of three experiments yielding similar results.
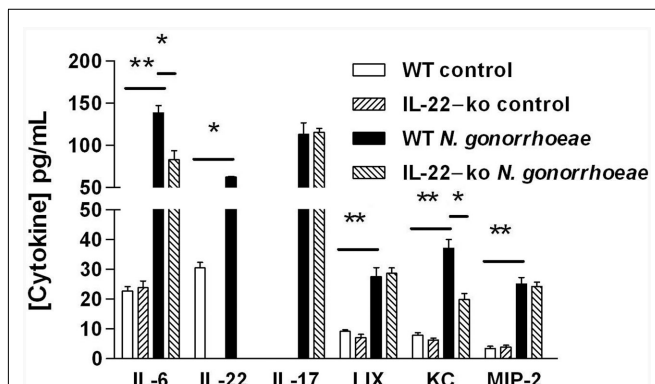

FIGURE 2 | Production of cytokines and chemokines by mouse vaginal explants cultured with *N. gonorrhoeae*. Vaginal tissue from WT or IL-22-ko mice was incubated *in vitro* in medium only (control) or with *N. gonorrhoeae* at a concentration of 2 × 10⁷ CFU/ml. Supernatants were assayed after 3 days for cytokines and chemokines by ELISA. Results are shown as mean ± SD of triplicate cultures. Cytokine or chemokine production by WT tissue in the presence of *N. gonorrhoeae* was significantly higher than in corresponding controls, or than by IL-22-ko tissue in the presence of *N. gonorrhoeae*, as indicated by the bars: ** *P* < 0.01; * *P* < 0.05 (Student's *t*).

whereas production of MIP-2α (CXCL2) and LIX (CXCL5) were unaltered (**Figure 2**). These results suggested that IL-22 could be involved in neutrophil recruitment in response to *N. gonorrhoeae*.

To determine whether production of IL-22 is important in the clearance of gonococcal infection and neutrophil recruitment into the genital tract, we compared the course of infection and neutrophil response in WT and IL-22-ko mice. Female mice were pretreated with estradiol and antibiotics to suppress the indigenous vaginal microbiota, and challenged intravaginally with *N. gonorrhoeae*. Vaginal swabs were collected daily for gonococcal culture, and smears were made and stained for microscopic evaluation of
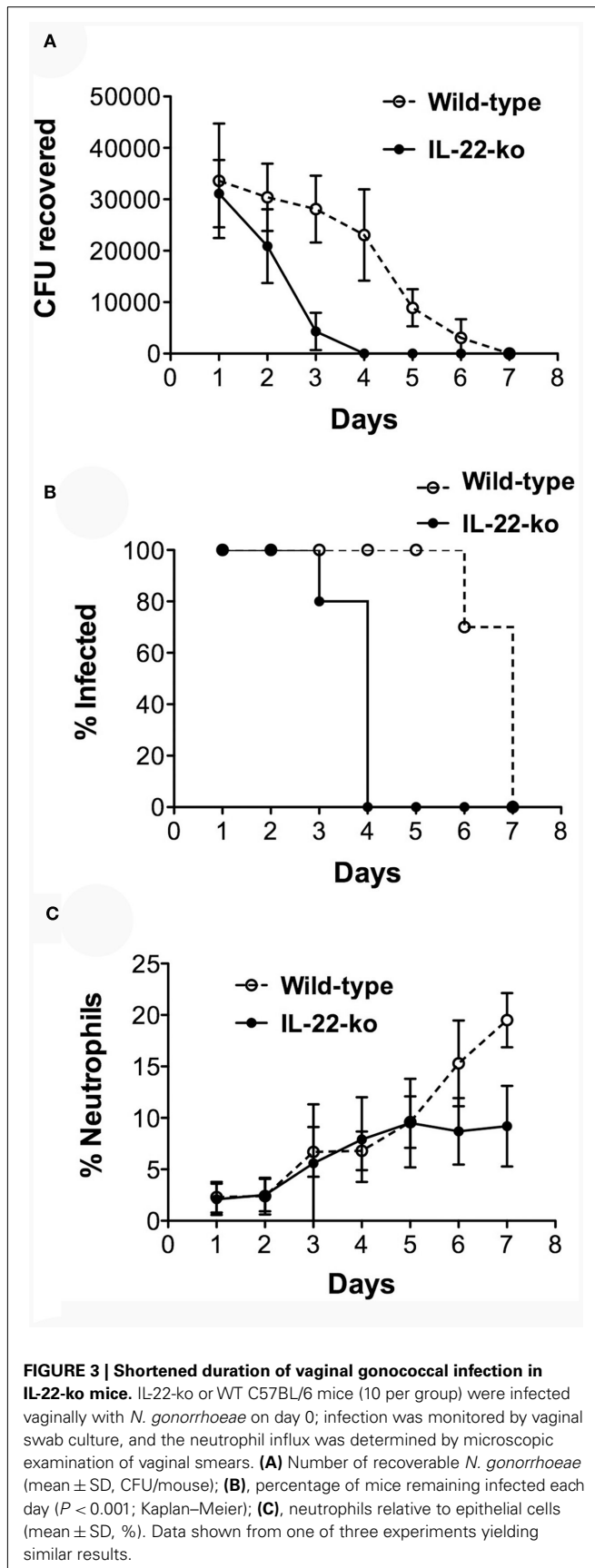
infiltrating neutrophils relative to epithelial cells. The experiment was performed three times to confirm the results.

In complete contrast to our previous findings in IL-17RA-ko mice (Feinen et al., 2010), the infection was cleared much faster in IL-22-ko mice compared to WT mice (**Figure 3**). Whereas in WT mice the numbers of recoverable gonococci began to decrease from about day 4 and the infection was cleared in all animals by day 8, similar to previous findings (Feinen et al., 2010), in IL-22-ko mice the infection began to decrease after day 1, and was completely cleared in all animals by day 4 (**Figures 3A,B**). The neutrophil influx followed the same course in both groups until day 5, after which it leveled off in the IL-22-ko mice and continued to rise in the WT mice, probably in ongoing response to the infection in these animals (**Figure 3C**).

DISCUSSION

We found that the production of IL-22 is a component of the murine response to *N. gonorrhoeae*, both *in vitro* and *in vivo*. Normal mouse spleen cells or explants of vaginal tissue stimulated with gonococci released IL-22 into the supernatants. Secretion of IL-22 was also detected when iliac lymph node or vaginal cells isolated from mice infected with *N. gonorrhoeae* were cultured *in vitro* (Liu et al., 2012). Given that IL-22 is known to be produced by Th17 cells (Liang et al., 2006), and that *N. gonorrhoeae* induces IL-17 and a Th17 response (Feinen et al., 2010) this is not surprising. However, early production of IL-22 in response to *N. gonorrhoeae* infection suggests that it may originate from cells other than those of the Th17 lineage. IL-22 has been demonstrated in the response to other murine models of infection, such as *K. pneumoniae* and *C. rodentium* (Aujla et al., 2008; Zheng et al., 2008); indeed in these models, IL-22 appears to have a more prominent role in protection than IL-17. Whereas IL-17 induces the recruitment of neutrophils to sites of infection, through the production of cytokines such as GCSF which mobilizes neutrophils from bone marrow reserves and of CXC chemokines which attract neutrophils (Ouyang et al., 2008), IL-22 targets epithelial cells to upregulate the production of several antimicrobial peptides including β-defensins, S100 proteins, lipocalin-2, and RegIIIγ (Ouyang and Valdez, 2008). Blockade or deletion of IL-22 inhibits the production of these factors and enhances the survival of *K. pneumoniae* and *C. rodentium* (Aujla et al., 2008; Zheng et al., 2008).

We therefore expected that vaginal gonococcal infection of IL-22-ko mice would result in increased recovery of *N. gonorrhoeae* and delayed clearance of infection, as we found in IL-17RA-ko mice. Instead, we found the complete opposite: numbers of *N. gonorrhoeae* recoverable from the genital tract of infected IL-22-ko mice declined rapidly from day 1 after inoculation, and the infection was completely cleared by day 4. The rapid fall in recoverable gonococci suggests that the inoculum failed to take hold in these animals, whereas in normal WT mice the infection persists with evidence for growth and phase-variation of Opa protein expression (Jerse, 1999; Simms and Jerse, 2006). Production of other cytokines in response to gonococcal stimulation of vaginal explants, IL-6 and the chemokine KC (CXCL1), was also diminished in tissues from IL-22-ko mice, but notably the production of IL-17 and chemokines MIP-2α (CXCL2) and LIX (CXCL5) were not affected. The impact on CXC chemokines was the opposite of

FIGURE 3 | Shortened duration of vaginal gonococcal infection in IL-22-ko mice. IL-22-ko or WT C57BL/6 mice (10 per group) were infected vaginally with *N. gonorrhoeae* on day 0; infection was monitored by vaginal swab culture, and the neutrophil influx was determined by microscopic examination of vaginal smears. **(A)** Number of recoverable *N. gonorrhoeae* (mean ± SD, CFU/mouse); **(B)**, percentage of mice remaining infected each day ($P < 0.001$; Kaplan–Meier); **(C)**, neutrophils relative to epithelial cells (mean ± SD, %). Data shown from one of three experiments yielding similar results.

the effect of IL-17RA deficiency, in which production of MIP-2α and LIX were abrogated while that of KC was unaffected (Feinen et al., 2010). In addition, there was no observable change in neutrophil influx in response to gonococcal infection of IL-22-ko mice at least until after the infection was cleared, suggesting there is some redundancy in the chemokines involved in recruiting neutrophils into the genital tract.

The biological activities of IL-22 are complex (Sonnenberg et al., 2011). There is strong evidence for its importance in mediating innate mucosal defense by inducing the production of antimicrobial proteins by mucosal epithelial cells (Ouyang and Valdez, 2008), and IL-22 cooperates with IL-17 in protection against bacterial infections (Aujla et al., 2008; Zheng et al., 2008). However, IL-22 interacts with IL-17 in a complex manner. In a model of sterile airway inflammation induced by bleomycin, IL-22 could be either proinflammatory or protective, depending upon the presence or absence of IL-17 respectively (Sonnenberg et al., 2010). In this model IL-17A also inhibited the production of IL-22 by Th17 cells. In skin IL-22 induces production of antimicrobial peptides and affects the differentiation and proliferation of skin cells, indicating a role in wound healing and tissue rearrangement (Boniface et al., 2005). In addition, IL-22 is implicated in epithelial repair, and has been shown to contribute significantly to the inflammatory skin condition, psoriasis (Wolk et al., 2009). IL-22 is also involved in systemic innate immune defense against some bacteria (Siegemund et al., 2009). However, immune defense against *Listeria monocytogenes* is independent of IL-22 (Zenewicz et al., 2007), and IL-22 has only a minimal role in defense against oropharyngeal *Candida albicans* (Conti et al., 2009). In polymicrobial sepsis neutralization of IL-22 enhances the influx of phagocytes and diminishes bacterial loads (Weber et al., 2007). Clearly, IL-22 has divergent roles in immune defense against various infections.

We therefore propose an alternative explanation for our findings, and hypothesize that IL-22 is necessary to create the conditions for gonococcal infection of the murine genital tract, such that in its absence, *N. gonorrhoeae* is unable to establish infection. This is in accordance with our observations. We have further hypothesized that *N. gonorrhoeae* proactively induces in its host Th17-driven innate responses that it is capable of resisting and concomitantly suppresses Th1/Th2 adaptive immune responses that might be capable of eliminating it (Liu et al., 2011). We have evidence to support this hypothesis in the murine model (Feinen et al., 2010; Liu and Russell, 2011; Liu et al., 2012). It is also possible that IL-17-governed inflammatory responses induced by *N. gonorrhoeae* might facilitate its infection of the mouse vagina by suppressing competing organisms that are susceptible to innate defense factors, to which gonococci are relatively resistant, as has been shown for *Salmonella enterica* Typhimurium infection of the mouse intestine (Raffatellu et al., 2009).

It is not known what is responsible for the eventual clearance of *N. gonorrhoeae* from the murine genital tract, and it should be noted that the mouse is not its natural host. Most probably, as *N. gonorrhoeae* is an exclusively human pathogen, numerous factors limit its ability to persist in the murine genital tract. These include species-specificity in recognition of receptors for gonococcal colonization factors and of transferrin as an iron source, as well as selective resistance to human complement (reviewed in Jerse et al.,

2011). Although in our experiments clearance follows the neutrophil influx (Feinen et al., 2010), this is not proof of causation, and other studies indicate that neutrophils cannot be responsible for clearance (Packiam et al., 2010, 2011). Thus the apparent association of prolonged infection with abrogation of the neutrophil influx in IL-17RA-ko mice may be misleading, because the infection is still cleared after a delay of several days despite the absence of a neutrophil influx (Feinen et al., 2010). It may be that in the absence of IL-17 (or deficiency of IL-17RA), other defense factors were diminished despite the generation of IL-22, whereas in the absence of IL-22 (but presence of IL-17) gonococcal infection of the mouse genital tract could not occur. In that way, perhaps IL-22 deficiency is more informative than IL-17RA deficiency in demonstrating how *N. gonorrhoeae* exploits host Th17 responses to create an environment favorable for its survival.

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