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IL-17 Is a Critical Component of Vaccine-induced Protection against Lung Infection by LPS-heterologous Strains of *Pseudomonas aeruginosa*

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Abstract

In a murine model of acute fatal pneumonia, we previously showed that nasal immunization with a live-attenuated *aroA* deletant of *P. aeruginosa* strain PAO1 elicited LPS serogroup-specific protection, indicating that opsonic antibody to the LPS O antigen was the most important immune effector. Because *P. aeruginosa* strain PA14 possesses additional virulence factors, we hypothesized that a live-attenuated vaccine based on PA14 might elicit a broader array of immune effectors. Thus, an *aroA* deletant of PA14, denoted PA14Δ*aroA*, was constructed. PA14Δ*aroA*-immunized mice were protected against lethal pneumonia caused not only by the parental strain but also by cytotoxic variants of the O-antigen-heterologous *P. aeruginosa* strains PAO1 and PAO6a,d. Remarkably, serum from PA14Δ*aroA*-immunized mice had very low levels of opsonic activity against strain PAO1 and could not passively transfer protection, suggesting an antibody-independent mechanism was needed for the observed cross-serogroup protection. Compared with control mice, PA14Δ*aroA*-immunized mice had more rapid recruitment of neutrophils to the airways early after challenge. T cells isolated from *P. aeruginosa* Δ*aroA*-immunized mice proliferated and produced IL-17 in high quantities after co-culture with gentamicin-killed *P. aeruginosa*. Six hours following challenge, PA14Δ*aroA*-immunized mice had significantly higher levels of IL-17 in bronchoalveolar lavage fluid compared with unimmunized, *E. coli*-immunized, or PAO1Δ*aroA*-immunized mice. Antibody-mediated depletion of IL-17 prior to challenge or absence of the IL-17 receptor abrogated the PA14Δ*aroA* vaccine's protection against lethal pneumonia. These data show that IL-17 plays a critical role in antibody-independent vaccine-induced protection against LPS-heterologous strains of *P. aeruginosa* in the lung.

Keywords

Animals-Rodent; Infections-Bacterial; Molecules-Cytokines; Processes-Vaccination; Tissues-Lung

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Disclosures
The authors declare no conflict of interest.

Introduction

Pseudomonas aeruginosa is a significant pathogen for hospitalized and immunocompromised patients. It is the most commonly isolated Gram-negative rod in the setting of hospital-acquired pneumonia (1) and, in the setting of ventilator-associated pneumonia, has a strikingly high attributable mortality (2). Vaccine development for lipopolysaccharide (LPS)-smooth strains of *P. aeruginosa*, which are the type of strains that cause nosocomial infections and are the initial colonizing strains in patients with cystic fibrosis (CF) (3), has been hampered by the inability to achieve broad protection across different LPS O antigen types (called serotypes or serogroups). In both mice (4) and humans (5), O antigen-based vaccine candidates have largely been abandoned due to lack of LPS-heterologous protection.

We are developing live-attenuated *P. aeruginosa* strains as intranasal vaccines and have shown previously that an unmarked *aroA* deletion mutant of the *P. aeruginosa* laboratory strain PAO1, denoted PAO1Δ*aroA*, is highly attenuated and, after intranasal immunization of mice, engenders high levels of serum opsonic antibody directed against LPS-homologous strains (6). Protection studies showed that after active immunization the PAO1Δ*aroA* vaccine prevented lethal pneumonia after inoculation with the parental strain PAO1, its cytotoxic variant ExoU$^+$ PAO1, or some LPS-subtype related strains; but there was no protection after inoculation with LPS-heterologous strains (7). Passive immunization using antisera generated in nasally immunized rabbits protected only against lethal pneumonia due to ExoU$^+$ PAO1 and failed to protect against LPS-heterologous strains or even against the parental strain PAO1 despite serum opsonic antibody titers that were identical to or higher than those observed after active immunization where protection was achieved (6). These results suggested both that the immunodominant nature of the LPS O antigen of PAO1Δ*aroA* may be hindering a broad antibody response and also that immune effectors elicited by active vaccination in addition to antibody are likely to be important for full protection against pneumonia.

Because of the broad host range of *P. aeruginosa* strain PA14 (8), we hypothesized that a live-attenuated vaccine based on this strain might elicit a broader array of immune effectors and thus constructed and tested an *aroA* deletion mutant in PA14 called PA14Δ*aroA*. Remarkably, we found that active intranasal immunization with PA14Δ*aroA* could protect against lethal pneumonia caused by LPS-heterologous strains in the absence of opsonic antibody. Further experiments showed that the neutrophil-attracting, T cell-secreted cytokine IL-17 is critical for the protective efficacy of this vaccine. Our results suggest that when opsonic antibody levels are low, rapid recruitment of neutrophils by IL-17-secreting vaccine-induced Th17 cells can achieve broad-based protections against *P. aeruginosa* pneumonia.

Materials and Methods

Bacterial strains

The bacterial strains and plasmids used in these experiments, along with their relevant characteristics and sources, are listed in Table I. PA14Δ*aroA* was constructed as previously described (6).

Preparation of bacterial inocula for in vivo challenge studies

Frozen bacterial stocks were plated and grown overnight at 37°C on tryptic soy agar (TSA) or TSA containing the appropriate antibiotics (carbenicillin at 400 μg/mL, gentamicin at 150 μg/mL). For intranasal inoculation experiments, bacteria were suspended in PBS. For some survival experiments, bacteria were suspended in PBS containing 1% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT). Concentrations were adjusted spectrophotometrically

and confirmed after serially diluting in PBS containing 1% FCS and enumerating growth on TSA after overnight incubation at 37°C.

Immunization of mice

Six- to 8-week-old female C3H/HeN mice were purchased from Harlan Sprague-Dawley Farms (Chicago, IL) while C57BL/6 mice were purchased from Taconic (Hudson, NY). The C57BL/6 mice were age- and gender-matched with IL-17R knockout (KO) mice (also in C57BL/6 background) that were originally obtained from Amgen (Thousand Oaks, CA) and bred in-house. Mice were housed under virus-free conditions, and all animal experiments complied with institutional and federal guidelines regarding the use of animals in research. Immunizations were performed as described previously (6). Briefly, mice anesthetized with ketamine and xylazine were inoculated by placing 10 μL of the live *P. aeruginosa* Δ*aroA* vaccine strain or live *E. coli* HB101 as a control on each nostril. Escalating doses of 1×10^8 CFU, 5×10^8 CFU, and 1×10^9 CFU were administered at weekly intervals. For passive immunization experiments, mice were given pooled mouse antiserum (0.4 mL i.p.) 1 day prior to challenge. IL-17 depletion studies were done using IgG (1 mg i.p.) purified with protein G-linked agarose (Invitrogen, Carlsbad, CA) from an anti-mouse IL-17 rabbit antiserum kindly provided by A. Tzianabos (9). Control rabbit IgG was purchased from Sigma (St. Louis, MO).

Murine pneumonia model

All animal experiments complied with institutional and federal guidelines regarding the use of animals in research. We used our previously described model of acute fatal pneumonia following intranasal inoculation (10). In this model, 67-100% of the bacterial inoculum is consistently and rapidly translocated to the lungs from the nose of anesthetized 6- to 8-week-old mice (10) . For quantitation of CFU, neutrophils, and cytokines in bronchoalveolar lavage fluid (BALF), mice were sacrificed using CO_2 at the indicated time points and then BALF obtained by cannulation of the trachea followed by two instillations of 1 mL of cold PBS containing 0.5 mM EDTA. For CFU measurements, BALF was diluted in 1% proteose peptone (BD, Franklin Lakes, NJ) and then plated on TSA for enumeration of CFU after overnight growth at 37°C. The limit of detection was 1 CFU in 100 μL of the undiluted BALF, which corresponded to 10 CFU per mL. BALF was centrifuged and the supernatant frozen at −80 °C prior to measurement of IL-17 by ELISA (R&D Systems, Minneapolis, MN). Erythrocytes in the cell pellets were lysed using a Mouse Erythrocyte Lyse Kit (R&D Systems) according to the manufacturer's instructions. Total cell counts were determined with a hemacytometer and Trypan Blue counterstaining. Percent neutrophils was measured by flow cytometry using PE-labeled anti-Ly6G (1A8) after treatment with anti-CD16/CD32 (2.4G2, FcBlock, BD Biosciences, San Jose, CA). For survival analyses, mice were followed daily for at least 7 days to assess mortality. Moribund animals were sacrificed and considered nonsurvivors.

Opsonophagocytic assays

Standard methods were employed, as described (6), with infant rabbit serum (Accurate Chemical, Westbury NY) as the complement source and polymorphonuclear leukocytes (PMNs) isolated from human volunteers. By convention, the serum concentrations listed were the input rather than final concentration (the initial serum dilution is further diluted 4-fold in the assay tube). Under routine conditions, killing of ≥50% is considered biologically significant and therefore serves to classify a serum as positive for opsonic killing activity. Although killing <50% is sometimes statistically significant, this level of killing is not considered biologically significant.

Anti-P. aeruginosa *IgG titers*

P. aeruginosa-specific IgG binding curves were measured by ELISAs using plates coated with whole live bacteria as previously described (6).

T cell proliferation, stimulation, and cytokine measurements

Splenic T cells were isolated using CD3 cell selection columns from R&D Systems. Cell purity was routinely >90% $CD3^+$ cells as assessed by flow cytometry (not shown). Lung leukocytes were isolated after perfusion of the lungs in situ with PBS containing heparin (10 Units/mL) followed by incubation with EDTA, then digestion with collagenase and DNase, then sedimentation over a discontinuous Percoll gradient, all as previously reported (11). Bacteria used as antigens were killed by incubation of a 10^9 CFU/mL suspension with gentamicin (1 mg/mL in PBS) for 1 h at 37 °C, with killing verified by absence of growth of 100 μL samples plated on TSA. Staphylococcal enterotoxin A (SEA) was purchased from Sigma (St. Louis, MO) and used at final concentration of 10 ng/mL. For proliferation experiments, each well of a 96-well round-bottom plate was seeded with 1×10^5 T cells, 1×10^5 irradiated (1500 rad) splenocytes isolated from unimmunized C3H/HeN mice and purified using density centrifugation over Lympholyte-M (Accurate Chemical), and 1×10^6 gentamicin-killed bacteria, all suspended in RPMI containing 10% heat-inactivated FCS as well as standard concentrations of glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, and 1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA). On day 5 of stimulation, wells were pulsed with [^{3}H]thymidine (1 μCi/well) for 8 hours prior to scintillation counting.

For cytokine secretion experiments, 96-well plates were used containing 1×10^5 T cells and 1×10^5 irradiated splenocytes as feeder cells. For both proliferation and cytokine secretion experiments, anti-CD4 (GK1.5), anti-CD8 (53-6.7), and rat IgG isotype controls were purchased from BD Biosciences and used at 1 μg per well. For stimulation of bulk lung leukocytes isolated 18 h after challenge with live bacteria, no feeder cells were added. IL-17 and IFN-γ were measured in supernatants after 7 days of stimulation using ELISA (R&D Systems). Intracellular cytokine staining was performed using a kit from BD Biosciences according to the manufacturers instructions. Lung leukocytes isolated as described above 2 h after challenge with live bacteria were stimulated for 15 h ex vivo with PMA and ionomycin (Leukocyte Activation Cocktail, BD Biosciences) in the presence of Brefeldin A (GolgiPlug, BD Biosciences). After treatment with anti-CD16/CD32, cells were stained with FITC-labeled antibodies to mouse TCRβ (H57-597) or CD3ε (145-2C11), followed by fixation and permeabilization and then staining with PE-labeled anti-mouse IL-17 (TC11-18H10.1) and analysis on a MoFlo (Dako, Carpinteria, CA) flow cytometer. Antibodies and appropriate isotype controls were obtained from BD Biosciences.

Statistical analyses

Nonparametric data were evaluated by Mann Whitney U test (for 2-group comparisons) or Kruskal Wallis (for 3 or more groups) with Dunn's multiple comparison test for pairwise comparisons. Parametric data were analyzed by t test (for 2-group comparisons) or ANOVA with Dunnett's multiple comparison test using *E. coli*-immunized mice or cells as the control comparator group. All analyses were performed using Prism software (GraphPad Software, San Diego, CA). Survival data were analyzed by Fisher's exact test or by survival analysis with the Kaplan-Meier method, also using Prism.

Results

Protective efficacy of PA14ΔaroA vaccination

We initially evaluated the protective efficacy of the PA14Δ*aroA* vaccine against LPS-homologous and heterologous strains. In our prior work, we showed that the serogroup O2/O5 vaccine PAO1Δ*aroA* elicited high levels of opsonic antibody as well as protection from lethality after lung challenge with LPS-homologous strains but not LPS-heterologous strains. As shown in Table II, nasal immunization with PA14Δ*aroA* resulted in significantly better survival compared with *E. coli*-immunized mice after challenge with a high dose of the parental strain PA14. There was no protection after challenge of PAO1Δ*aroA*-immunized mice with a lower dose of strain PA14. Notably, PA14Δ*aroA*-immunized mice were protected from lethality after challenge with ExoU-producing strains of the LPS-heterologous strains PAO1 (serogroup O2/O5) and PAO6a,d (serogroup O6). There was no protection, however, after challenge with the non-cytotoxic strains Fisher IT-7 (serogroup O2/O5) or 6294 (serogroup O6). This is likely related to the higher LD_{50}s of the non-cytotoxic strains in this model and thus the very high challenge doses required to achieve 100% mortality in the *E. coli*-immunized control mice.

Opsonic killing activity in antisera raised to P. aeruginosa PA14ΔaroA

For most vaccines effective against *P. aeruginosa* infections, serum opsonic antibody directed against the LPS O antigen has been the most important immune effector (12). To assess the role of serum antibodies and antibodies to specific antigens such as the ExoU cytotoxin in the protection afforded by the PA14Δ*aroA* vaccine, we first tested the opsonic killing activity of pooled sera obtained from immunized mice 3 weeks after the third (final) nasal immunization. As depicted in Fig. 1A, there was minimal opsonic killing of strain PAO1 in serum from mice immunized with the PA14Δ*aroA* strain, whereas good killing was achieved using sera from mice immunized with the LPS-homologous PAO1Δ*aroA* strain. Opsonic killing activity in antisera raised to PA14Δ*aroA* was present against the parental PA14 strain, although the opsonic titer (the calculated serum dilution at which the killing activity dropped to 50% as determined by linear regression) was approximately 50 (not shown), which is much lower than the titer of approximately 1000 seen with antiserum to PAO1Δ*aroA* against its parental strain PAO1 (7). Because PA14Δ*aroA* possesses the genes for ExoU while PAO1Δ*aroA* does not, and thus the PA14-based vaccine could induce antibody to this antigen, we next evaluated whether antibody to ExoU could be mediating opsonic killing if for unexpected reasons this antigen was retained on the bacterial cell surface. As shown in Fig 1B, there was no significant opsonic killing of an ExoU-producing strain compared with the strain containing the empty vector.

Passive immunization and whole-cell ELISA experiments

Next, we determined whether passive immunization with pooled antisera from PA14Δ*aroA*-immunized mice mediated protection against lethal pneumonia due to the LPS-heterologous strain $ExoU^+$ PAO1. Mice given the PA14Δ*aroA* antiserum i.p. 1 day prior to challenge had significantly higher mortality compared with the 0% mortality of mice given identical amounts of antisera raised to the LPS-homologous PAO1Δ*aroA* strain (Fig. 1C). These results suggested, surprisingly, that an antiserum-independent mechanism is a key component of the heterologous protection engendered by active vaccination with the PA14Δ*aroA* vaccine. We also tested for O antigen-specific and ExoU-specific antibodies by comparing IgG titers of the PAO1Δ*aroA*- and PA14Δ*aroA*-immunized mouse antisera to whole cells of the wild-type parental strain PAO1 (containing the empty vector) compared with strain $ExoU^+$ PAO1 as well as ExoU-producing and non-producing LPS-rough *galU* mutants of strain PAO1 (*galU* mutants are O antigen deficient with a truncated outer LPS core (13)). As shown in Fig. 2, the PA14Δ*aroA* antisera had the expected lower reactivity than did the antisera raised to

PAO1Δ*aroA* against *P. aeruginosa* strains PAO1 (pUCP19) and ExoU$^+$ PAO1 based on its different LPS serogroup. The titers to the O antigen-deficient *galU* mutants were identical, suggesting that the PA14Δ*aroA* vaccine elicited a comparable level of antibody to non-O-side-chain antigens as did the PAO1*ΔaroA* vaccine.

T cell responses elicited by P. aeruginosa *PA14Δ*aroA *vaccine*

To determine whether immune T cells are induced by nasal immunization with the *P. aeruginosa* Δ*aroA* vaccines, we isolated splenic T cells 3 weeks after immunization (prior to challenge) and co-cultured them with irradiated splenocytes as antigen-presenting cells (APCs) and gentamicin-killed *P. aeruginosa* as antigen and measured T cell proliferation by [^{3}H] thymidine incorporation after 5 days. As shown in Fig. 3A, there was a high level of proliferation of both PAO1Δ*aroA* and PA14Δ*aroA*-immune T cells to either PAO1 or PA14, although the PA14Δ*aroA*-immune T cells proliferated less well when stimulated with PAO1 compared with the PAO1Δ*aroA*-immune T cells. Proliferation of PAO1Δ*aroA*-immune T cells was inhibited by an antibody to CD4 but not to CD8 (Fig. 3B). Proliferation of PAO1Δ*aroA* and PA14Δ*aroA*-immune T cells was high when stimulated with multiple *P. aeruginosa* strains regardless of LPS serogroup or ability to produce ExoU (Figs. 3C). Interestingly, the *P. aeruginosa* Δ*aroA*-immune T cells proliferated when cultured with *E. coli* but the *E. coli*-immune T cells were not significantly stimulated by *P. aeruginosa* strains.

Cytokine secretion by P. aeruginosa *Δ*aroA *vaccine-elicited T cells*

As neutrophils are known to be essential mediators of protective immunity in the lung against *P. aeruginosa* infection (14,15), we hypothesized that the recently described Th17 subset of CD4 helper T cells, which secrete the neutrophil-attracting cytokine IL-17, might play a role in rapid recruitment of neutrophils to the airways. We thus evaluated IL-17 secretion after 7-day co-culture of splenic T cells from naïve and immunized mice cultured with irradiated splenocytes and gentamicin-killed *P. aeruginosa* strains PAO1 and PA14 as antigens (Figs. 4A and 4B). For comparison, we also measured levels of IFN-γ, the predominant Th1 effector cytokine, and found them not to be significantly different among the vaccine groups. In contrast, the levels of IL-17 in the supernatants of the *P. aeruginosa* Δ*aroA*-immune T cells stimulated with *P. aeruginosa* were significantly higher than those of control T cells. Addition of the anti-CD4 monoclonal antibody (clone GK1.5, which blocks class II MHC antigen-specific binding to CD4 and thereby blocks CD4 T cell function (16)) during co-culture returned the IL-17 levels to those of the control T cells (Fig. 4B), indicating that CD4 T cells are the predominant source of IL-17 in this system. We also isolated intraparenchymal and airway lung leukocytes 18 h after challenge of immune and non-immune mice by digesting the lung tissue with collagenase and DNase followed by a discontinuous Percoll gradient. Numbers of T cells in the lungs prior to challenge were too low to allow accurate assessment of cytokine secretion from cells prior to challenge so we therefore only evaluated cytokine secretion from lung T cells after challenge. At the 18-h time point after challenge, the cellular infiltrate is approximately 35% lymphocytes (with the remainder neutrophils) as assessed by forward-and side-scatter FACS analysis; and the composition was not significantly different between the experimental groups (not shown). The isolated lung leukocytes were co-cultured with gentamicin-killed PAO1 for 7 days. The IL-17 levels in the supernatants of these in vivo-immunized, ex vivo-stimulated lung leukocytes are shown in Fig. 4C and were remarkably higher in the Δ*aroA*-immune groups, with the PAO1Δ*aroA*-immune and PA14Δ*aroA*-immune T cells having approximately 10-fold and 40-fold higher IL-17, respectively, than the control T cell groups.

To determine the cellular source of the IL-17 in vivo, we measured intracellular staining for IL-17 in lung parenchymal and airway lymphocytes isolated as described above but from mice sacrificed just 2 h after challenge with live bacteria. Again, numbers of lymphocytes in the

lungs of immunized mice prior to challenge were too low to allow intracellular cytokine analysis. Cells were stimulated for 15 h with PMA and ionomycin in the presence of brefeldin A. As shown in Fig. 4D, there was a small population of IL-17-producing TCRβ-positive cells in the lungs of Δ*aroA*-immune mice, with 3-fold more in the PA14Δ*aroA*-immunized group. Similar proportions of IL-17-positive cells were seen in CD3ε-positive cells, and no IL-17-positive cells were seen outside the lymphocyte gate of Δ*aroA*-immune mice (not shown). We were unable to assess CD4-staining in these experiments due to apparent decreased expression of CD4 during stimulation (not shown). Unstimulated cells showed no detectable IL-17 (not shown). Lung leukocytes (lymphocytes and non-lymphocytes) isolated 18 h after challenge with live bacteria showed no intracellular staining for IL-17 with or without PMA/ionomycin stimulation (not shown).

Neutrophil recruitment to the lungs of P. aeruginosa *Δ*aroA*-immunized mice*

Because IL-17 is known to stimulate neutrophil recruitment to the lung, we tested whether the numbers of neutrophils recruited to the airways are greater in the *P. aeruginosa* Δ*aroA*-immunized mice and whether the different *P. aeruginosa Δ*aroA vaccines elicited different neutrophil recruitment. As shown in Fig. 5A, there were significantly more neutrophils in BALF 6 h after challenge of *P. aeruginosa* Δ*aroA*-immunized mice compared with the *E. coli*-immunized and unimmunized controls. Notably, even at this early time point after challenge, there were significantly fewer CFU of *P. aeruginosa* in the BALF (Fig. 5B) and homogenized lung tissue (not shown) in the *P. aeruginosa* Δ*aroA*-immunized mice compared with the control groups. IL-17 levels in BALF (Fig. 5C) obtained 6 h after challenge with a lethal dose of ExoU$^+$ PAO1 were significantly higher in the PA14Δ*aroA*-immunized compared with the *E. coli*-immunized mice ($p < 0.01$, ANOVA with Dunnett's multiple comparison test). At the later time point (18 h), neutrophil numbers and IL-17 levels in BALF were highest in the *E. coli*-immunized mice. Bacterial CFU were very high in the control groups at this time after challenge, indicating that these neutrophils were not effective at clearing the challenge strain.

Effect of loss of IL-17 on protective efficacy of P. aeruginosa *Δ*aroA *vaccines against LPS-heterologous strain challenge*

We next determined the effects of neutralization of IL-17 prior to lung challenge with the LPS-heterologous strain PAO1 ExoU$^+$ in mice immunized intranasally with the *P. aeruginosa* PA14Δ*aroA* vaccine or with *E. coli* as control. As expected, there was no effect of neutralization of IL-17 on survival following challenge of the *E. coli*-immunized mice (left panel of Fig. 6A). However, there was significantly higher mortality ($p < 0.02$, log rank test) in the PA14Δ*aroA*-immunized mice that received anti-IL-17 IgG compared with the mice given control IgG (Fig. 6A, right panel), indicating that depletion of IL-17 abrogates vaccine-induced protection. In contrast, mice immunized with the PAO1Δ*aroA* vaccine and then similarly depleted of IL-17 had no change in median survival and were fully protected against LPS-homologous strain PAO1 ExoU$^+$ because these mice had high levels of serum opsonic antibody specific for the LPS of strain PAO1 (not shown). We also found that when we immunized mice genetically deficient in production of the IL-17 receptor (IL-17R KO) with PA14Δ*aroA* (or with *E. coli* as control) and then challenged them with ExoU$^+$ PAO1, the IL-17R KO mice had significantly decreased survival compared with wild-type mice (Fig. 6B, right panel). We observed lower percent survival in the PA14Δ*aroA*-immunized wild-type C57BL/6 mice challenged with 5×10^7 CFU/mouse (40% survival, Fig. 6B) compared with PA14Δ*aroA*-immunized wild-type C3H/HeN mice given the same challenge dose (88%, Table 2). This was likely due to the high susceptibility of mice in the C57BL/6 background to *P. aeruginosa* lung infection (17) and the relative resistance of C3H/HeN mice (18).

Consistent with the IL-17 neutralization experiments in PAO1Δ*aroA*-immunized C3H/HeN mice, IL-17R KO mice immunized with the PAO1Δ*aroA* vaccine and then challenged with the LPS-homologous strain ExoU$^+$ PAO1 were fully protected (not shown). These PAO1Δ*aroA*-immunized IL-17R KO mice had normal levels of opsonic antibody to strain PAO1 (not shown), which very likely accounted for the lack of an impact of deficient IL-17 signaling. We next evaluated the neutrophil recruitment to the airways 6 h after challenge of PA14Δ*aroA*-immunized wild-type C57BL/6 mice compared to PA14Δ*aroA*-immunized IL-17R KO mice. We again used *P. aeruginosa* strain ExoU$^+$ PAO1 (7×10^6 CFU/mouse) as the challenge strain. As shown in Fig. 6C, there were significantly fewer BALF neutrophils normalized to bacterial load in the IL-17R KO mice. Of note, unimmunized IL-17R KO and wild-type C57BL/6 mice had similar survival curves when challenged with 2×10^6 CFU of strain ExoU$^+$ PAO1 (Fig. 6D) or 7×10^5 CFU (100% survival in both groups, n=6 mice per group, not shown), indicating that IL-17R signaling is not important for the innate host defense against acute lethal pneumonia caused by *P. aeruginosa*.

Discussion

In the current study, we tested the hypothesis that a live-attenuated *P. aeruginosa* vaccine based on the broad-host-range *P. aeruginosa* strain PA14 would elicit a broader array of immune effectors than a vaccine derived from strain PAO1. We found that unlike the PAO1Δ*aroA* vaccine, active immunization with the PA14Δ*aroA* vaccine could protect against acute lethal pneumonia caused by LPS-heterologous *P. aeruginosa* strains. Surprisingly, we observed that this protection was not due to serum opsonic activity but rather was dependent on the T cell-secreted cytokine IL-17. The protective efficacy was coincident with a rapid reduction in bacterial CFU in BALF as well as higher neutrophil numbers and higher IL-17 in the BALF 6 h after lung challenge. These findings indicate that active immunization with *P. aeruginosa* vaccines that induce T cells secreting IL-17 could be a key component of full immunity to lung infection by this pathogen and, furthermore, might be an explanation for why a large clinical trial of passively administered hyperimmune IgG obtained from volunteers given a multivalent *P. aeruginosa* O-antigen conjugate vaccine did not reduce the incidence of infection in recipients of the antibody (5).

Recent evidence suggests that IL-17-secreting CD4$^+$ T helper cells comprise a distinct lineage called Th17 (19,20) and play an important role in innate antibacterial host defense in the lung (21-23) as well as in autoimmunity (24). IL-17 is a proinflammatory cytokine expressed primarily by activated memory CD4$^+$ T cells and is the prototypic member of a family of cytokines named IL-17A-F (where IL-17A is what is typically denoted IL-17, and IL-17E is IL-25) (25). IL-17-secreting NKT cells (specifically, CD1d-restricted, NK1.1-negative NKT cells with invariant TCR) have also recently been shown to recognize glycolipid ligands derived from *Sphingomonas wittichii* and *Borrelia burgdorferi* and to be involved in neutrophil recruitment to the airways after exposure to LPS (26). Th17 cell development has been linked to IL-23, an IL-12 cytokine family member that shares a common subunit with IL-12 (IL-12p40) but has a distinct subunit, p19 (27). TGF-β appears to be critical for commitment to Th17 development by increasing IL-23R expression, thereby conferring responsiveness to IL-23 (28). An inflammatory milieu (in particular the proinflammatory cytokine IL-6, with amplification by IL-1β and TNF-α) is also required Th17 polarization (29).

The mechanisms whereby the PA14Δ*aroA* vaccine induces more robust IL-17 responses than the PAO1Δ*aroA* vaccine after subsequent challenge with live bacteria are not clear. Presumably, the PA14-based vaccine induces higher levels of IL-23, IL-6, IL-1β, TNF-α, and/or TGF-β during the priming of the immune response in the lung or local lymph nodes. This could be related to differential inflammatory activity of the different LPS O antigens of the two vaccine strains and/or to one or several of the additional genes and gene clusters possessed

by strain PA14 that are absent from strain PAO1, including the pathogenicity islands PAPI-1 (108 kb) and PAPI-2 (11 kb) (30). Notably, a subtractive hybridization technique called representational difference analysis found not only that several genes, including *pilA* and *pilC*, in strain PA14 differed substantially from their counterparts in strain PAO1 but also that only strain PA14 possessed a homolog of the gene for the siderophore-associated ABC transporter protein YbtQ of *Yersinia pestis* that, when mutated, led to significant attenuation in *G. mellonella* and in a burned mouse model of sepsis (31). While some siderophores are known to suppress T cell proliferation and function (32) others such as the siderophore lactoferrin have been shown to induce T helper responses to the BCG vaccine (33), so it is conceivable that differences in siderophore production might skew a vaccine-induced T cell response.

The action of IL-17 in the lung centers on neutrophil recruitment via induction of CXC chemokine secretion (KC and MIP-2 in rodents, GRO and IL-8 in humans) along with granulopoietic factors (such as G-CSF) that lead to increased bone marrow production and/or prolonged survival of neutrophils (21). In studies of innate immunity to *Klebsiella pneumoniae*, Kolls and co-workers found that IL-17R-deficient mice were highly susceptible in an acute pneumonia model, with increased mortality and bacterial dissemination associated with delayed neutrophil recruitment to the lung as well as lower levels of G-CSF and MIP-2 in the knockout mice (23,34). In acute *P. aeruginosa* pneumonia, on the other hand, we found that non-immune IL-17R-deficient mice had similar susceptibility compared with wild-type mice, although the IL-17R-deficient mice were much more susceptible in the setting of vaccination. In a different lung infection model, the Kolls group has shown that IL-23-deficient mice had less lung inflammation after challenge with a mucoid *P. aeruginosa* strain encased in agar beads (35).

Overexpression of IL-17 in the lung via a recombinant adenoviral vector led to local induction of TNF-α, MIP-2, and G-CSF and augmented neutrophil recruitment and bacterial clearance after challenge with *K. pneumoniae* (22). In this model, signaling through TLR4 was shown to be critical for IL-23 generation (36). Interestingly, CF patients were found to have high levels of IL-17 in bronchoalveolar lavage fluid (BALF), particularly during pulmonary exacerbations (37). Whether IL-17 plays a role in the excessive neutrophilic infiltration and IL-8 secretion characteristic of CF lung disease (38) remains to be determined.

While the time course of IL-17 production in innate immunity models is relatively slow (12-16 h) (23), the production of IL-17 in settings wherein the adaptive response has been activated is far more rapid (as early as 3 h) (39). There is a growing body of evidence that there is a critical role of IL-17 in adaptive immunity to extracellular bacterial respiratory pathogens, particularly *Streptococcus pneumoniae* and *Bordetella pertussis*. Malley and co-workers have shown that IL-17 mediates antibody-independent, vaccine-induced protection against nasopharyngeal pneumococcal colonization in mice immunized with the species-common cell wall polysaccharide called the C polysaccharide (40). Higgins et al. have demonstrated that T cell secretion of IL-17 is critical for protective efficacy of the whole-cell *B. pertussis* vaccine and is also dependent on intact TLR4 signaling (41). Antibody-mediated neutralization of IL-17 in the pertussis studies resulted in an approximately 10-fold increase in CFU 10 days after infection, but the impact on survival was not assessed. Those workers also demonstrated that exposure of peritoneal macrophages or macrophage cell lines to IL-17 enhanced bactericidal activity (41). Evaluation of IL-17R-deficient mice has not been reported in either of these models.

A number of investigators have demonstrated T cell-mediated control of *P. aeruginosa* infections in rodent models. A high-molecular-weight polysaccharide antigen prepared from the LPS O antigen elicited *P. aeruginosa*-specific T cells capable of killing the organism in

vitro and protecting neutropenic mice against infection in a mechanism whereby anti-LPS IgG on the T cell surface mediated antibody-dependent cellular cytotoxicity (42). Other workers have observed $CD4^+$ T cell-mediated control of *P. aeruginosa* pneumonia after vaccination with paraformaldehyde-killed *P. aeruginosa* given to rats orally or injected into Peyer's patches (43,44). Intravenous immunization of mice with *P. aeruginosa*-pulsed dendritic cells has also been shown to protect against subsequent *P. aeruginosa* pneumonia (45). The *P. aeruginosa*-protective T cell epitopes have not been defined, although studies in humans in the early 1980s reported that the alkaline phosphatase of *P. aeruginosa*, a secreted protein, was the principle antigen recognized by clones of T cells derived by stimulation of peripheral blood mononuclear cells from healthy volunteers using crude bacterial culture fluids (46). More recently, immunization with a replication-deficient adenoviral vector expressing a 14-amino acid epitope of *P. aeruginosa* outer membrane protein OprF showed that $CD4^+$ and $CD8^+$ T cell responses to the OprF peptide could be elicited as well as protection against lethal pneumonia in mice (47). Investigations into whether Th1 (IFN-γ dominated) versus Th2 (IL-4 and IL-10 dominated) $CD4^+$ T cell responses are more important for protective immunity against *P. aeruginosa* lung infections have yielded conflicting results, with some reports suggesting that Th1-prone strains of mice are more susceptible and other reports concluding just the opposite (14,17,18,48). None of those studies evaluated Th17 cells or the cytokine IL-17 as they preceded identification of the Th17 lineage.

The importance of a clear understanding of the role of IL-17 in vaccine-induced protection against bacterial pneumonia relates not only to the design of more effective vaccines but also to the fact that inhibitors of the IL-17 pathway are under active development as anti-inflammatory agents for treatment of a host of autoimmune disorders ranging from rheumatoid arthritis to multiple sclerosis (49,50). Our results suggest that disabling the IL-17 response could nullify the protective effects of some vaccines – a situation that will need to be studied extensively and weighed carefully in the risk-benefit analysis of IL-17-directed treatment of autoimmune diseases.

The present study suggests that IL-17 is a critical component of vaccine-induced immunity to LPS-heterologous strains of *P. aeruginosa* and thus paves the way towards a broadly protective *P. aeruginosa* vaccine using either mixtures of multiple live-attenuated *aroA* deletants or potentially by expressing heterologous LPS O antigens from a single live-attenuated strain such as PA14Δ*aroA*. The importance of the neutrophil in control of *P. aeruginosa* infections is also underscored by our results, as is the critical interplay between components of innate and adaptive immunity (neutrophils with T cells and antibody) during vaccine-induced protection from infection.

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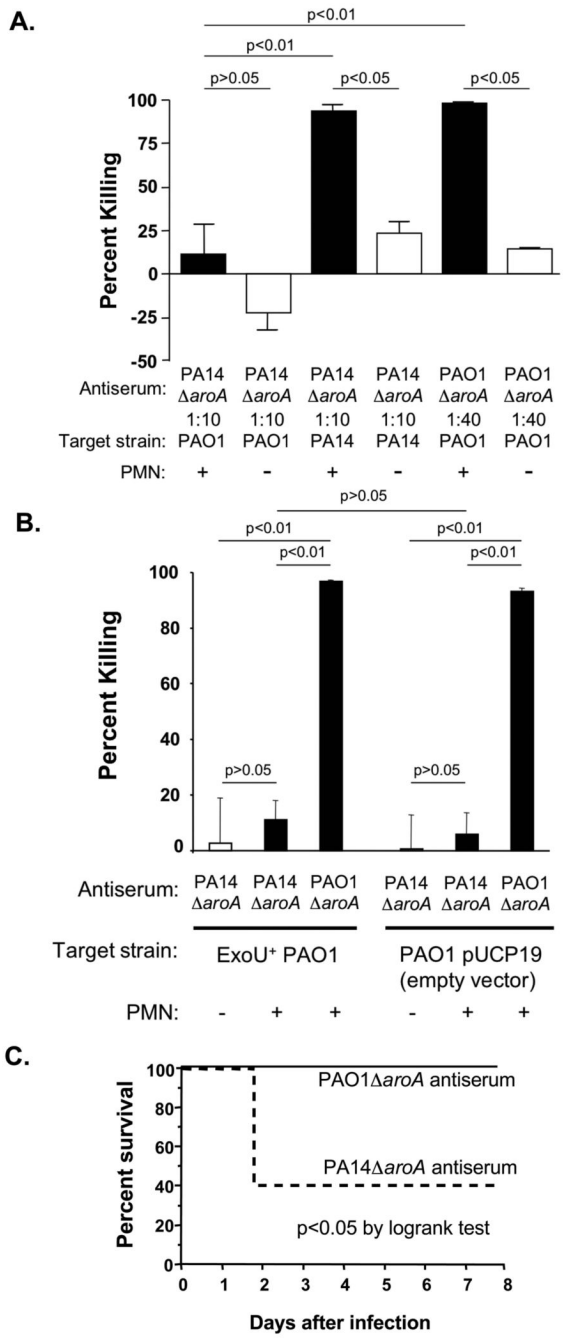

Fig. 1.

Absence of PAO1-targeted (**A**) and ExoU-specific (**B**) opsonic killing activity of sera from PA14Δ*aroA*-immunized mice 3 weeks after the final immunization. Bars are means and error bars the SEM. P values calculated by ANOVA with Tukey's multiple comparison test. **C.** Survival after passive immunization with PA14Δ*aroA* mouse antiserum (0.4 mL i.p., n=5 mice/group) given 1 day prior to lung challenge with *P. aeruginosa* strain ExoU$^+$ PAO1 (1.4×10^7 CFU per mouse, a dose that resulted in 100% lethality of mice passively immunized with *E. coli* antiserum [not shown]).

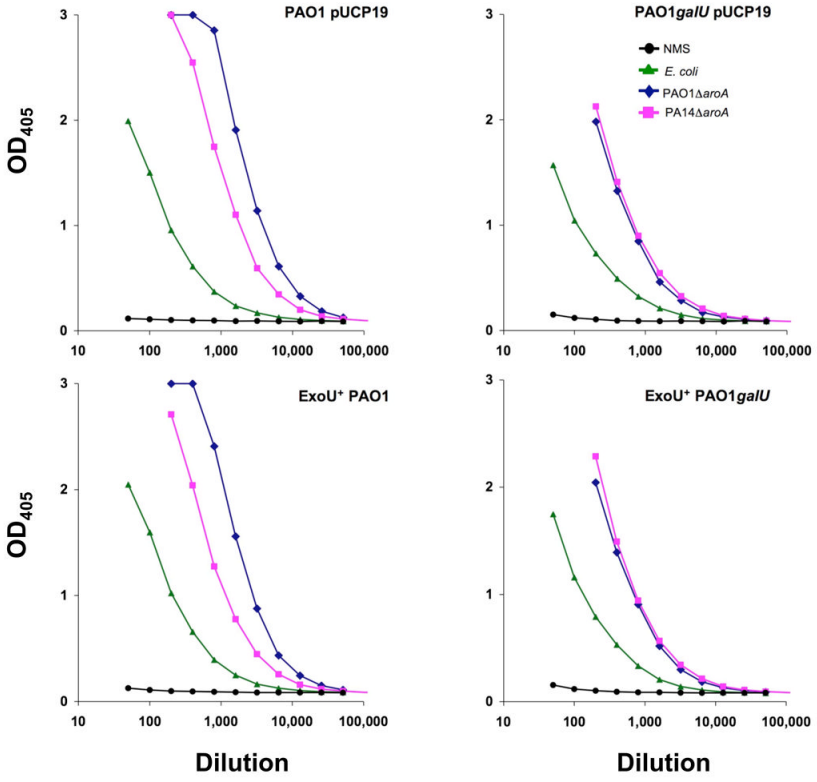

Fig. 2.

Comparison of IgG binding curves to *P. aeruginosa* strains PAO1 (with empty vector pUCP19), ExoU^{+} PAO1, and their LPS-rough isogenic *galU* mutant cells by ELISA using antisera from C3H/HeN mice immunized with live *E. coli*, PAO1Δ*aroA*, or PA14Δ*aroA* bacteria. NMS: normal mouse serum. Each point is the mean of duplicates using pooled sera from 3-5 mice.

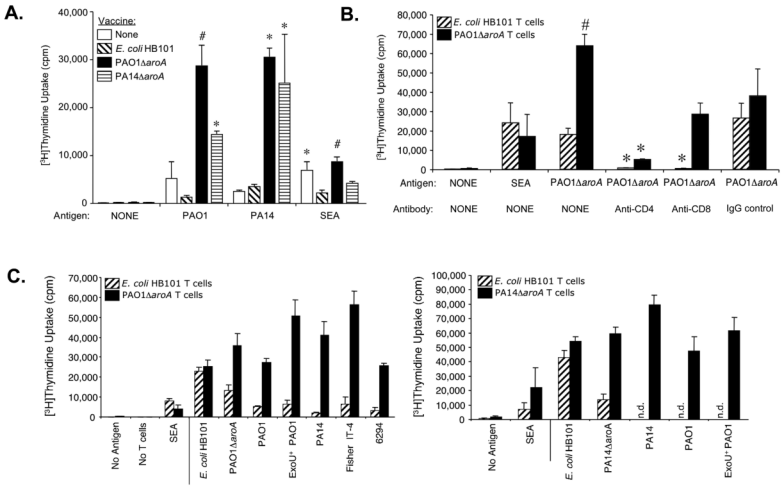

Fig. 3.

Nasal immunization with *P. aeruginosa* Δ*aroA* vaccines primes splenic T cells. **A.** Proliferation of splenic T cells from unimmunized mice compared with mice immunized with *E. coli*, PAO1Δ*aroA*, or PA14Δ*aroA*. **B.** Proliferation of PAO1Δ*aroA*-immune T cells was significantly decreased by anti-CD4 monoclonal antibody. **C.** Proliferation of T cells from PAO1Δ*aroA* (left panel) or PA14Δ*aroA* (right panel) immunized mice was similar regardless of LPS serogroup or cytotoxic genotype of the stimulating gentamicin-killed, whole bacterial cell antigen (MOI: 10 bacteria per T cell). Bars represent means and error bars SEM. Cells were pooled from 3-5 mice per group. In **A**, *$p<0.05$ and $^{\#}p<0.01$ in comparison to *E. coli* control group by ANOVA with Dunnett's multiple comparison test. In **B**, *$p<0.05$ compared with IgG control of corresponding vaccine group and $^{\#}p<0.01$ by t test compared to *E. coli*-immune T cells. In **C**, proliferation of Δ*aroA*-immune T cells was significantly greater ($p<0.01$, t test) compared to *E. coli*-immune T cells in the presence of *P. aeruginosa*. SEA: staphylococcal enterotoxin A, positive control; n.d., not determined.

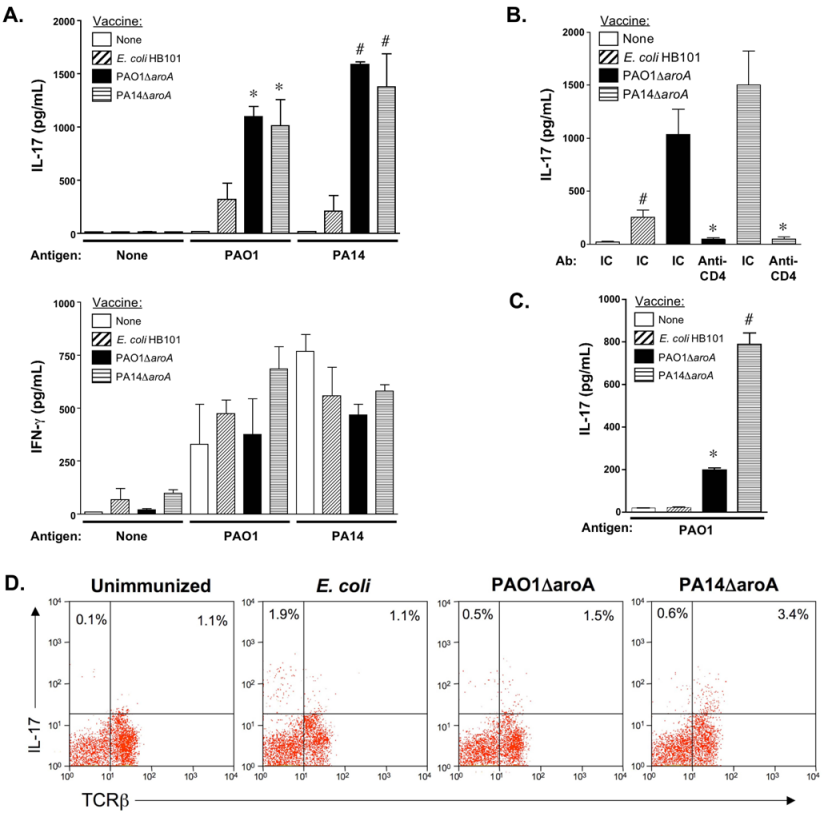

Fig. 4.

Nasal immunization with *P. aeruginosa* Δ*aroA* vaccine strains elicits Th17 cells in the spleen and lung. **A.** IL-17 (upper panel) and IFN-γ (lower panel) production by splenic T cells isolated 3 weeks after nasal immunization and then stimulated for 7 days in the presence of gentamicin-killed whole bacterial cells (MOI: 10 bacteria per T cell) of *P. aeruginosa* strains PAO1 or PA14 along with irradiated splenocytes. Cells were pooled from 3-5 mice per group. **B**. IL-17 secretion decreases to baseline when splenic T cells are co-cultured as in **(A.)** with strain PAO1 along with anti-CD4 monoclonal antibody (clone GK1.5, 1 μg/well). IC, isotype control IgG2b monoclonal antibody (1 μg/well). Bars indicate means of triplicate wells, error bars the SEM. $^{\#}p<0.05$ compared with *E. coli* control group by ANOVA with Dunnett's multiple comparison test. *p<0.01 compared with corresponding vaccine group treated with IC by ANOVA with Tukey's multiple comparison test. **C.** IL-17 production by lung leukocytes isolated from vaccinated and unvaccinated mice (3 mice per group)18 h after challenge with strain ExoU^{+} PAO1 and then co-cultured for 7 days with gentamicin-killed PAO1 whole bacterial cells (MOI: 10 bacteria per T cell). Bars indicate means of triplicate wells, error bars the SEM. $^{\#}p<0.01$ and *p<0.05 compared with *E. coli* control group by ANOVA with Dunnett's multiple comparison test. **D.** Intracellular staining for IL-17 in lymphocytes isolated from collagenase-digested lungs of vaccinated and unvaccinated mice 2 h after challenge with *P. aeruginosa* strain ExoU^{+} PAO1. Cells were pooled from 3 mice per group.

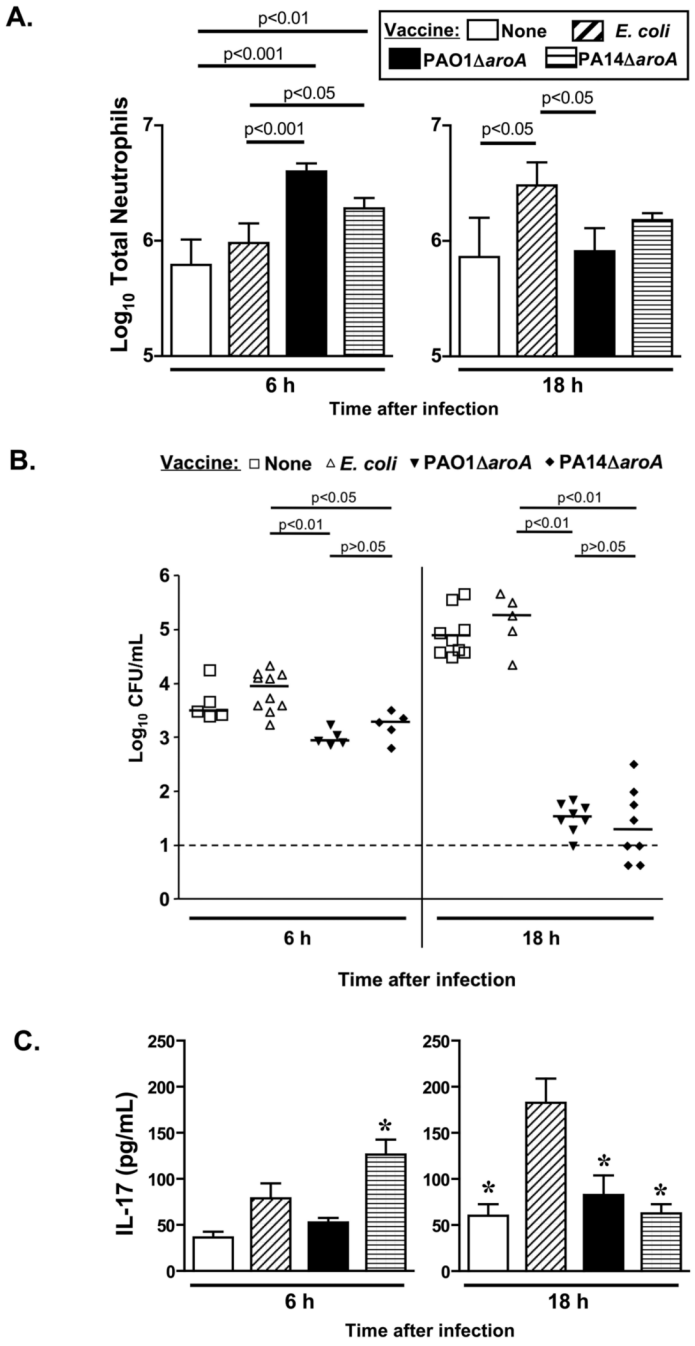

Fig. 5.

Increased neutrophil recruitment to airways early after bacterial challenge is coincident with lower bacterial CFU and higher IL-17 levels in BALF. Total BALF neutrophils (**A**) and bacterial CFU (**B**) isolated after infection of immune and non-immune mice (n=5-10 mice per group) with *P. aeruginosa* strain ExoU$^+$ PAO1 (5×10^6 CFU). Bars represent medians and error bars the interquartile range. P values are by Kruskal-Wallis with Dunn's multiple comparison test. **C.** IL-17 concentration in BALF following infection of immune and non-immune mice (n=5-15 mice per group) with strain ExoU$^+$ PAO1 (5×10^6 CFU). Bars depict means and error bars the SEM. *p<0.05 compared with *E. coli* control group by ANOVA with Dunnett's multiple comparison test.

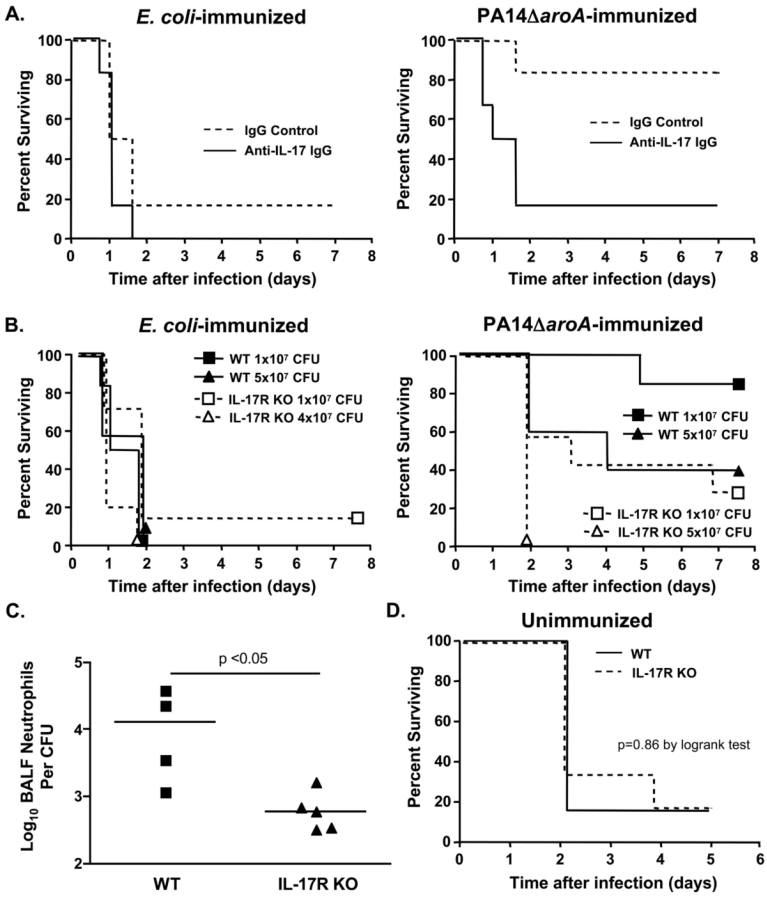

Fig. 6.

Neutralization of IL-17 or absence of its receptor diminishes vaccine-induced protection from *P. aeruginosa* pneumonia. **A.** Survival of immunized wild-type C3H/HeN mice after administration of IL-17-specific rabbit IgG (solid line) or control IgG (dashed line) 1 day prior to challenge with ExoU$^+$ PAO1 (n=6 mice/group, 5×10^7 CFU). **B.** Survival of immunized wild-type C57BL/6 (WT, solid line) or IL-17R KO (dotted line) mice after challenge with ExoU$^+$ PAO1 (n=5-7 mice per group; $p<0.05$ by logrank test for comparison of PA14Δ*aroA* WT vs. PA14Δ*aroA* IL-17R KO mice at either challenge dose). **C.** Neutrophil recruitment in PA14Δ*aroA*-immunized mice is deficient in the absence of IL-17R signaling. BALF total neutrophil numbers were measured 6 h after challenge with ExoU$^+$ PAO1 (7×10^6 CFU) and normalized to bacterial CFU. Each point represents one mouse. P value by Mann Whitney U test. **D.** Innate immunity to *P. aeruginosa* pneumonia is intact in IL-17R KO mice. Survival of unimmunized wild-type C57BL/6 (solid line) and IL-17R KO (dashed line) mice after challenge of with ExoU$^+$ PAO1 (n=6 mice per group, 2×10^6 CFU). All mice (6 per group) survived after a challenge dose of 7×10^5 CFU (not shown).

TABLE I

Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
P. aeruginosa		
PAO1	Wild-type strain, LPS-smooth, non-cytotoxic, serogroup O2/O5	M. Vasil[a]
PAO1Δ*aroA*	*aroA* deletion mutant of PAO1, LPS-smooth, serogroup O2/O5	(6)
PA14	Wild-type strain, LPS-smooth, serogroup O10	F. Ausubel[b]
PA14Δ*aroA*	*aroA* deletion mutant of PA14	This study
ExoU$^+$ PAO1	PAO1(pUCP19*exoUspcU)*. Cytotoxic variant of PAO1 carrying the plasmid pUCP19*exoUspcU*; Cb^r	(10)
PAO1 *galU* pUCP19	*galU* mutant of PAO1 in which the *galU* gene is disrupted by an *aacC1* cassette; also carries the plasmid pUCP19; Gm^r and Cb^r	(51)
ExoU$^+$ PAO1 *galU*	PAO1 *galU*(pUCP19*exoUspcU)*; Gm^r and Cb^r	(13)
ExoU$^+$ PAO6a,d	PAO6a,d(pUCP19*exoUspcU)*; Cb^r	(10)
Fisher IT-7	Wild-type strain, LPS-smooth, noncytotoxic, serogroup O2/O5	(4)
6294	Clinical isolate (corneal infection), LPS-smooth, noncytotoxic, serogroup O6	BPEI[c], (52,53)
6206	Clinical isolate (corneal infection), LPS-smooth, cytotoxic, serogroup O11	BPEI[c], (52,53)
PA103	Clinical isolate, LPS-smooth, cytotoxic, serogroup O11	D. Frank[d]
E. coli HB101	*supE*44 *hsdS*20($r^-_B m^-_B$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	(54)
Plasmid		
pUCP19	Broad host range shuttle vector; Ap^r/Cb^r	(55,56)
pUCP19*exoUspcU*	pUCP19-based plasmid carrying the *exoU* gene and its chaperone gene, *spcU*	(10)

Abbreviations: Ap, ampicillin; Cb, carbenicillin; r, resistant

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[b] F. Ausubel, Massachusetts General Hospital, Boston, MA

[c] Bascom-Palmer Eye Institute, Miami, FL

[d] D. Frank, Medical College of Wisconsin, Milwaukee, WI

TABLE II

Protective efficacy against acute fatal *P. aeruginosa* pneumonia following active nasal immunization of C3H/HeN mice with *E. coli* HB101, PAO1Δ*aroA*, or PA14Δ*aroA*

Challenge Strain	Sero-group	LD_{50} [b] (CFU)	Inoculum (CFU)	Vaccine Group: *E. coli* HB101	Vaccine Group: PAO1Δ*aroA*	Vaccine Group: PA14Δ*aroA*
ExoU$^+$ PAO1	O2/O5	7×10^5	2×10^7	1/7[a]		7/8*
PA14	O10	3×10^6	1×10^8	0/4	0/4	
			8×10^8	2/9		9/9**
ExoU$^+$ PAO6a,d	O6	1×10^6	2×10^7	1/5		10/10**
			7×10^6	3/11	1/4	
Fisher IT-7	O2/O5	7×10^6	6×10^8	1/4		1/4
6294	O6	5×10^7	2×10^8	1/6		1/5
6206	O11	4×10^6	4×10^6	1/8		4/8#
PA103	O11	2×10^5	1×10^6	3/6		3/6

[a] No. surviving/No. challenged.

[b] LD_{50}s are for unimmunized mice and are from refs. (7,10,13).

* $p < 0.05$

** $p < 0.01$ by Fisher's exact test.

p=0.28 by Fisher's exact test but p=0.02 by logrank test.