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Interleukin-23-Mediated Inflammation in *Pseudomonas aeruginosa* Pulmonary Infection

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***Pseudomonas aeruginosa* is an opportunistic pathogen that is capable of causing acute and chronic pulmonary infection in the immunocompromised host. In the case of cystic fibrosis (CF), chronic *P. aeruginosa* infection causes increased mortality by promoting overly exuberant airway inflammation and cumulative lung damage. Identifying the key regulators of this inflammation may lead to the development of new therapies that improve *P. aeruginosa*-related mortality. We report here that interleukin-23 (IL-23), the cytokine most clearly tied to IL-17-mediated inflammation, also promotes IL-17-independent inflammation during *P. aeruginosa* pulmonary infection. During the early innate immune response, prior to IL-17 induction, IL-23 acts synergistically with IL-1β to promote early neutrophil (polymorphonuclear leukocyte [PMN]) recruitment. However, at later time points, IL-23 also promoted IL-17 production by lung $\gamma\delta$ T cells, which was greatly augmented in the presence of IL-1β. These studies show that IL-23 controls two independent phases of neutrophil recruitment in response to *P. aeruginosa* infection: early PMN emigration that is IL-17 independent and later PMN emigration regulated by IL-17.**

Pseudomonas aeruginosa pulmonary infection occurs in immunocompromised hosts and is associated with significant morbidity and mortality. The pathogen induces a robust neutrophil response that causes significant airway damage and does not always eliminate the pathogen. In cystic fibrosis (CF), where infection is chronic, the inflammation causes cumulative airway damage that is a major contributor to morbidity and mortality (41, 46, 62). Identifying the immune mediators that promote this inflammation is the first step in developing new therapies that can improve outcomes in chronic *P. aeruginosa* pulmonary infection.

We and others have previously shown that airway interleukin-23 (IL-23) and IL-17 levels correlate with infection status and inflammation in individuals with CF (12, 15, 38) and that lymphocytes from CF-derived draining lymph nodes produce higher levels of IL-17 than non-CF disease controls (3). We have also shown that both IL-23 and IL-17 are critical for neutrophil recruitment in a murine model of chronic *P. aeruginosa* pulmonary infection (14). While these data demonstrate that the IL-23/IL-17 proinflammatory axis is active during *P. aeruginosa* airway infection and in CF, the studies did not isolate the actions of IL-23 from those of IL-17.

Historically, IL-17 has been credited with promoting both neutrophil recruitment and granulopoiesis through its induction of neutrophil growth factors and chemokines, including IL-6, granulocyte colony-stimulating factor (G-CSF), keratinocyte chemoattractant (KC), and macrophage inflammatory protein 2 (MIP-2) (14, 24, 30, 38, 49, 54, 64). IL-23, a recently identified IL-12 family member (42, 60), has been primarily characterized in the context of this Th17 response (1, 25, 33). Of note, however, IL-23-overexpressing mice have a hyperinflammatory phenotype and notable elevations in serum IL-1β and tumor necrosis factor alpha (TNF-α) levels, without mention of elevated IL-17 (60). These data suggested to us that IL-23 may have significant functions beyond promoting IL-17 production.

A study by Ferretti and colleagues demonstrated that in response to intratracheal lipopolysaccharide (LPS) there are two phases of neutrophil recruitment: an early IL-17-independent response that peaks at 6 h and a later IL-17-dependent response that peaks at 36 h (17). Whether this model holds up in a live bacterial infection has not been determined. Due to the fact that IL-23p19 is induced within 60 to 90 min of *P. aeruginosa* infection, we questioned whether IL-23 might be responsible for this first wave of neutrophil recruitment and whether IL-23 might promote inflammation in concert with the other inflammatory mediators that were elevated at very early time points, such as IL-1β and TNF-α. Surprisingly, our studies demonstrate that IL-23 plays significant roles in early, innate neutrophil recruitment, in addition to later control of IL-17 production by $\gamma\delta$ T cells. In addition to a critical role for IL-23, IL-1β was required for both early polymorphonuclear leukocyte (PMN) recruitment and maximal IL-17 responses from $\gamma\delta$T cells. These data highlight the dual roles these innate cytokines play in orchestrating both phases of PMN emigration in response to *P. aeruginosa* infection. Furthermore, neutralization of IL-23 did not result in worsened infection, suggesting that IL-23 may be a proximal immunotherapeutic target for *P. aeruginosa*-induced lung inflammation.

MATERIALS AND METHODS

Mice. IL-23p19$^{-/-}$ (20), IL-1R1$^{-/-}$ (Jackson Laboratory, Bar Harbor, ME), IL-17RA$^{-/-}$ (64), T-cell receptor delta$^{-/-}$ (TCR-$\delta^{-/-}$) (subsequently referred to as $\gamma\delta$ TCR$^{-/-}$) (Jackson Laboratory), and control

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C57BL/6 (subsequently referred to as wild type [WT]) (Taconic, Hudson, NY) male mice were used at 6 to 8 weeks of age. The mice were maintained under pathogen-free conditions at the animal facility of the Children's Hospital of Pittsburgh under IACUC-approved protocols. All mice were acclimated to our animal facility for a minimum of 1 week prior to use.

***P. aeruginosa* infections.** Mice were challenged by noninvasive intratracheal instillation (i.t.) (31) with *P. aeruginosa* strain PAO1 (ATCC number BAA-47; American Type Culture Collection, Manassas, VA) at an inoculum of 1×10^6 to 2.5×10^6 CFU. PAO1 was grown in 100 ml Luria Bertani broth for 12 h at 37°C. After 12 h, 1 ml of the culture was added to fresh Luria Bertani broth and grown for an additional 12 h at 37°C. The pellet was centrifuged at 6,000 × *g* for 10 min, and the supernatant was discarded. The bacterial pellet was washed twice with phosphate-buffered saline (PBS) and diluted to deliver the target inoculum in a total of 50 μl. The inocula were retrospectively confirmed through plating of serial dilutions and counting of CFU on Luria Bertani plates. Control mice were administered 50 μl of sterile PBS.

Cytokine exposure studies. Carrier-free murine IL-23 (R&D Systems, Minneapolis, MN) was administered at 1 μg per mouse, and IL-1β (R&D Systems) was administered at 25 ng per mouse via i.t. Sterile PBS (50 μl) was administered to controls. All were administered in a total of 50 μl of sterile PBS. Dosages for IL-23 and IL-1β were determined based on the prior published literature (18, 28, 61) and on our data that demonstrate that these doses recapitulate levels seen in infection.

Sample acquisition and processing. All mice were anesthetized with isoflurane for i.t., and lungs were subsequently harvested at 1, 3, or 6 h postexposure. Bronchoalveolar lavage (BAL) fluid (1 ml) was obtained and processed for total cell counts and differential and cytokine measurements. Samples were centrifuged, and the cell pellets were resuspended in fresh PBS to assess total cell counts (Coulter Counter; Becton-Dickinson, Franklin Lakes, NJ) and differential (cytospin; ThermoFisher, Waltham, MA). The supernatant of the initial 1 ml of BAL fluid was stored at −80°C for later processing for enzyme-linked immunosorbent assay (ELISA) (R&D Systems) or Luminex (Millipore, Billerica, MA). Left lungs were collected in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and RNA was purified according to the manufacturer's instructions. Right lungs and spleens were collected and homogenized in 1 ml of nonbacteriostatic PBS, and serial dilutions were plated on Luria Bertani plates to determine bacterial CFU.

In additional studies, BAL fluid and lung homogenate were also acquired for analysis of myeloperoxidase (MPO). Tissues and BAL fluid were processed according to the protocol, and MPO levels were measured by ELISA (Hycult Biotech, Plymouth Meeting, PA).

Histological examination of lung sections. All mice were anesthetized with isoflurane for i.t., and lungs were subsequently harvested at 3 h postexposure. The lungs were inflated, fixed in 10% formalin, and embedded in paraffin; 5-μm sections were cut and stained with hematoxylin-eosin. Images were captured using an Olympus BX41 microscope and Spot software (Diagnostic Instruments, Sterling Heights, MI) and scored for neutrophil counting by a blinded reader. The reader selected and read three fields (magnification ×60) for each slide and chose the most inflamed areas on the slide for each captured field. There were three mice per group, resulting in nine fields analyzed for each condition.

Measurement of cytokines. IL-17A, IL-17F, and IL-22 were measured by ELISA (R&D Systems) according to the manufacturer's instructions. All other cytokines were measured by multiplex analysis using Luminex (Millipore), according to the manufacturer's instructions.

Alveolar macrophage and dendritic cell studies. Alveolar macrophages (AMs) were harvested from WT and IL-23p19$^{-/-}$ mice by lavaging with a total of 10 ml of sterile PBS per mouse. The AMs were plated at 1×10^5 cells per well and assessed for purity by flow cytometry using F4/80 (BD Biosciences) as the primary surface marker (purity > 95%). Bone marrow dendritic cells (DCs) were cultured in RPMI, 10% fetal bovine serum (FBS), IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF) for 6 days; enriched by CD11C column (Miltenyi Biotec, Auburn, CA); assessed for purity by flow cytometry with CD11C as the primary marker (purity > 95%); and plated at 1×10^5 cells per well. PAO1 was administered at an inoculum of 1×10^6 CFU/well. The supernatant was removed after 3 h, centrifuged to remove cellular and bacterial debris, and frozen at −80°C for later ELISA and Luminex analysis.

Assessment of $\gamma\delta$ T cell response. Three different studies were conducted. In the first two studies, the response of $\gamma\delta$ T cells to IL-23 was measured by assessing $\gamma\delta$ T cell-dependent cytokine production using two different $\gamma\delta$ T cell depletion strategies. In the first study, the cytokine production by single-cell suspension of lungs of WT mice and that by $\gamma\delta$ TCR$^{-/-}$ mice were directly compared. Because the $\gamma\delta$ TCR$^{-/-}$ mice might have had developmentally mediated compensatory changes in T cell function (29), we conducted the second study comparing cytokine production by cultures of WT lungs that had been sorted to remove CD3$^+$ $\gamma\delta$ TCR$^+$ cells or left intact (stained and run through the sorter without removing any cell populations).

In both of these experiments, whole lungs were removed from the mice and processed into a single-cell suspension by mechanical dissociation. Red blood cell lysis was performed, and the cells were plated at 1×10^6 cells per well in RPMI with 10% FBS (first study) or processed for flow sorting and then plated (second study). For the second study, lung cells were stained for CD3 (BD biosciences) and $\gamma\delta$ TCR (BD Biosciences) and either passed through the analyzer (FACS Aria) without sorting or sorted to remove $\gamma\delta$ TCR$^+$ cells. The cultures were exposed to recombinant murine IL-23 (R&D Systems), IL-1β (R&D Systems), or both (10-ng/ml final concentration) *in vitro* for 3 h. The culture supernatants were assessed for IL-17A, IL-17F, and IL-22 as the endpoint.

In the third set of studies, $\gamma\delta$ T cell production of cytokines in response to *P. aeruginosa* was assessed. WT mice were infected with PAO1 for 6 h, and the lungs were sorted into CD3$^+$ $\gamma\delta$ TCR$^+$ or CD3$^+$ $\gamma\delta$ TCR$^-$ fractions, which were assessed for IL-17A, IL-17F, and IL-22 gene expression by real-time PCR. The samples in these studies were pooled due to the low prevalence of $\gamma\delta$ TCR$^+$ cells in the lung.

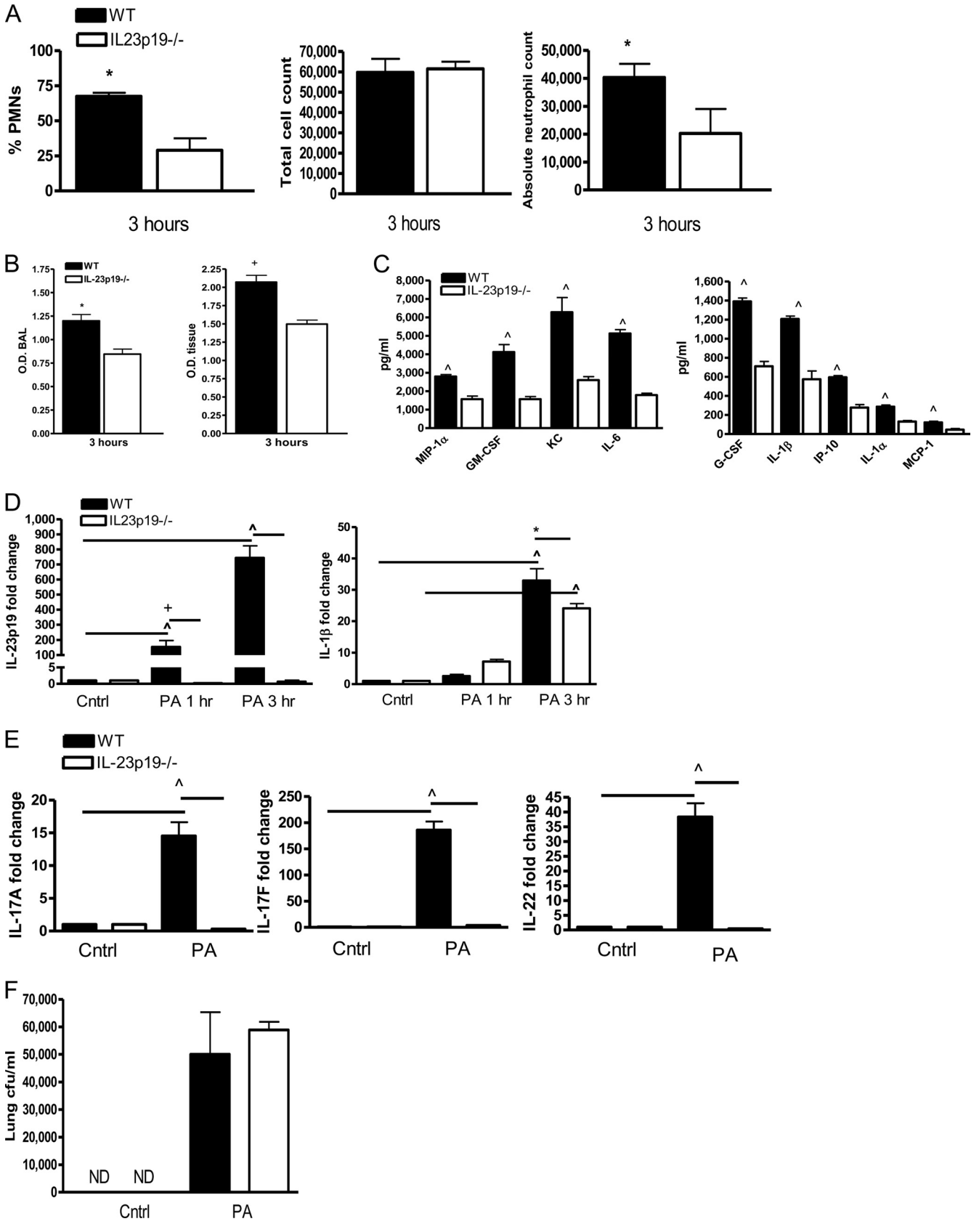
Real-time PCR. Isolated RNA was converted to cDNA (iscript; Bio-Rad, Hercules, CA) following the manufacturer's protocol. RNA analysis was performed by real-time (RT)-PCR using Assay on Demand TaqMan probes and primers (Applied Biosystems, Foster City, CA) with the ABI Prism 7700 detection system (Applied Biosystems, Foster City, CA).

Statistics. All data are presented as the mean and standard error of the mean (SEM), except where samples were pooled (see Fig. 7). Significance was tested using an unpaired *t* test, a Mann-Whitney U test, or one-way analysis of variance (ANOVA) with a Bonferonni *post hoc* test. All statistics were analyzed using GraphPad Prism 4 software (Graph Pad Software, La Jolla, CA). All experiments were conducted a minimum of two times.

RESULTS

IL-23 promotes neutrophil recruitment and inflammation in an IL-17-independent manner. We initially hypothesized that IL-23 causes inflammation only by promoting IL-17 production. However, to determine if IL-23 regulates early neutrophil responses, we infected WT, IL-23p19$^{-/-}$, and IL-17RA$^{-/-}$ mice with 1×10^6 CFU of PAO1; sacrificed the mice at 1, 3, or 6 h; and determined neutrophil counts and cytokine induction/elaboration as outcome measures. We chose these time points because we had previously determined that IL-17 could not be detected in BAL fluid or lung tissue at 1 and 3 h after intratracheal inoculation of *P. aeruginosa* but could be detected in both by 6 h. At the 1- and 3-h time points, we compared pulmonary inflammation in the BAL fluid and tissue of WT and IL-23p19$^{-/-}$ mice. For the 6-h time point, when IL-17 is consistently detected during *P. aeruginosa* infection, we compared inflammation levels in WT and IL-17RA$^{-/-}$ mice to determine the contributions of IL-17RA signaling in the inflammatory response.

Neither WT nor IL-23p19$^{-/-}$ mice had measurable neutrophil


A
WT
IL23p19-/-
% PMNs
Total cell count
Absolute neutrophil count
3 hours
3 hours
3 hours
B
WT
IL-23p19-/-
O.D. BAL
O.D. tissue
3 hours
3 hours
C
WT
IL-23p19-/-
pg/ml
MIP-1α
GM-CSF
KC
IL-6
pg/ml
G-CSF
IL-1β
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PA 1 hr
PA 3 hr
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WT
IL-23p19-/-
IL-17A fold change
IL-17F fold change
IL-22 fold change
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PA
Cntrl
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F
Lung cfu/ml
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recruitment or cytokine production in the BAL fluid at 1 h (data not shown). At 3 h, WT mice had a substantially greater inflammatory response than the IL-23p19$^{-/-}$ mice (Fig. 1). WT mice consistently recruited a higher percentage of neutrophils to the airway than the IL-23p19$^{-/-}$ mice, as measured in the BAL fluid ($P < 0.05$) (Fig. 1A). The absolute neutrophil count was also significantly higher in the BAL fluid over the three studies conducted ($P = 0.0005$; Mann-Whitney U test) and was significant in two of three studies by *t* test ($P < 0.05$) (Fig. 1A). MPO levels in BAL fluid and tissue, as well as tissue infiltrate with neutrophils, were also significantly higher in the WT groups than in the IL-23p19$^{-/-}$ group ($P < 0.05$) (Fig. 1B). Histology also demonstrated increased neutrophil infiltration in the WT compared to the IL-23p19$^{-/-}$ mice (see Fig. S1 in the supplemental material). The levels of the cytokines, chemokines, and growth factors that are central to neutrophil recruitment during infection were also significantly higher in WT than in IL-23p19$^{-/-}$ BAL fluid (Fig. 1C). All analytes (G-CSF, GM-CSF, KC, MIP-1α, MCP-1, IP-10, IL-6, IL-1β, and IL-1α) are associated with increased neutrophil recruitment, neutrophil activation, or neutrophil production, and all of the levels recovered in WT BAL fluid were at least double the levels seen in IL-23p19$^{-/-}$ BAL fluid.

To further determine the importance of IL-23 in promoting this inflammatory response, we measured the induction of IL-23p19 by RT-PCR and found that IL-23p19 expression was highly induced by 1 h after infection and further induced by 3 h in the WT mice (Fig. 1D). This rise in IL-23p19 expression preceded the induction and elaboration of IL-1β. IL-1β induction did not reach significance until 3 h ($P < 0.001$) and was partially dependent on IL-23 ($P < 0.05$) (Fig. 1D). We did not observe detectable levels of IL-17A, IL-17F, or IL-22 protein at 1 or 3 h of infection with *P. aeruginosa* (data not shown). We did note significant induction of IL-17A, IL-17F, and IL-22 mRNA transcripts in the WT mice as early as 3 h, and this occurred in an IL-23-dependent manner (Fig. 1E).

Despite the significant abrogation of inflammation in the IL-23p19$^{-/-}$ group, there was no difference in bacterial burden in the lung between WT and IL-23p19$^{-/-}$ mice (Fig. 1F), nor was there dissemination of infection in either group (data not shown). Taken together, these data demonstrate that IL-23 production promotes inflammation and IL-1β expression during the early innate immune response but is dispensable for bacterial clearance. Based on the lack of measurable IL-17, these effects also appear to be IL-17 independent.

IL-17RA knockout mice are capable of producing IL-17A and IL-17F but lack the receptor component necessary to respond to both of these IL-17 family members. In the study shown in Fig. 2, we demonstrate that while WT mice produce IL-17 family members during *P. aeruginosa* infection (Fig. 2B), there is no difference in neutrophil recruitment (Fig. 2A), cytokine and chemokine production (Fig. 2B), or bacterial load (Fig. 2C) and bacterial dissemination (data not shown) compared to infected IL-17RA$^{-/-}$ mice. These data demonstrate that while IL-17 is produced at 6 h of *P. aeruginosa* pulmonary infection, it is not responsible for the early (0 to 6 h) neutrophil recruitment and inflammation that occur. Taken together with the studies in IL-23p19$^{-/-}$ mice, these data demonstrate that the IL-23-mediated neutrophil recruitment in early *P. aeruginosa* airway infection is IL-17RA independent.

Antigen-presenting cells and the IL-23-mediated inflammatory response. Because antigen-presenting cells (APCs) are a likely source of IL-23 production, we postulated that antigen-presenting cells, either macrophages or dendritic cells, were responsible for these IL-23-mediated effects. To determine whether either of these two cell populations might be responsible for this early inflammation, we infected alveolar macrophage and dendritic cell cultures with PAO1 for 3 h and measured cytokine, chemokine, and growth factor levels in the cell culture supernatants, specifically assessing the magnitude of the response and IL-23 dependence. The pattern of cytokine production seen in the macrophage cultures (Fig. 3A) demonstrates a pattern similar to that seen in the BAL fluid of *P. aeruginosa*-infected mice (Fig. 1C), with detection of significant levels of neutrophil-associated cytokines that were produced in an IL-23-dependent manner. MIP-1α, KC, IL-1β, IL-1α, G-CSF, and IL-6 are all produced in an IL-23-dependent manner, are all associated with neutrophil recruitment and production, and are all elevated in an IL-23-dependent manner in BAL fluid during *P. aeruginosa* infection. In contrast to the AMs, the pattern of cytokine production seen in the DC cultures (Fig. 3B) involves fewer of the same cytokines detected in the BAL fluid (Fig. 1C), with only MIP-1α, KC, MCP-1, and IL-6 produced in significant amounts. Furthermore, the production of cytokines is less robust than that by alveolar macrophages, with the exception of IL-6. Also, cytokine production by DCs demonstrates only modest IL-23 dependence. These data demonstrate that alveolar macrophages produce cytokines in an IL-23-dependent manner when exposed to *P. aeruginosa in vitro* and suggest that these cells may play a significant role in promoting *P. aeruginosa*-induced, IL-23-mediated inflammation *in vivo*.

IL-23 and IL-1β synergistically mediate inflammation *de novo*. Our data from IL-23p19$^{-/-}$ mice suggested that IL-23 was a critical mediator of the innate inflammatory response. To further understand the role of IL-23 in mediating inflammation, we administered recombinant murine IL-23 intratracheally. These data suggest that IL-23 administered alone is insufficient to cause inflammation *de novo* (Fig. 4). Because IL-1β plays such a prominent role in neutrophil recruitment and CF inflammation and because a link between IL-23 and IL-1β production was suggested

FIG 1 Neutrophil recruitment and cytokine, chemokine, and growth factor levels are expressed in an IL-23-dependent manner in response to *P. aeruginosa* (PA) infection. WT and IL-23p19$^{-/-}$ mice were infected with *P. aeruginosa* or exposed to a control (PBS). (A) Inflammatory-cell counts in BAL fluid were measured and reported at 3 h. Data for 1 h is not shown, as there was no significant neutrophil recruitment to the airway. (B) Myeloperoxidase measured from BAL fluid and lung tissue is reported at 3 h. (C) Cytokines, chemokines, and growth factors in the BAL fluid were measured and reported at 3 h. IL-17A, IL-17F, and IL-22 proteins were undetectable (data not shown). There was no significant production of inflammatory cytokines at 1 h (data not shown). (D) IL-23 and IL-1β induction was measured by RT-PCR performed on whole-lung homogenate at 1 and 3 h after infection. Cntrl, control. (E) IL-17A, IL-17F, and IL-22 induction was measured by RT-PCR performed on whole-lung homogenates at 3 h (negative at 1 h [data not shown]). (F) Bacterial load in the lung homogenate (presented as lung CFU/ml); dissemination of infection to the spleen was negative (data not shown). ND, not detectable. For panels A and C to F, $n = 4$ mice per group, and the study was repeated two times. For panel B, $n = 4$ mice per group, and the tudy was repeated once. *, $P < 0.05$; +, $P < 0.01$; ^, $P < 0.001$. The error bars indicate SEM.

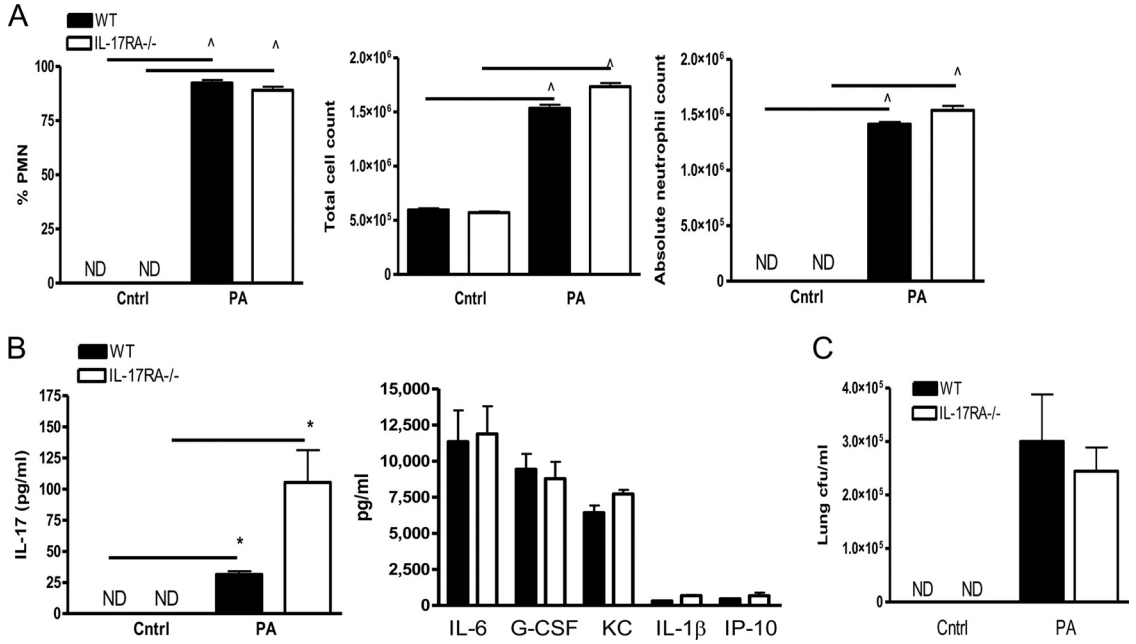

FIG 2 IL-17RA$^{-/-}$ mice do not exhibit decreased inflammation during *P. aeruginosa* pulmonary infection. WT and IL-17RA$^{-/-}$ mice were infected with *P. aeruginosa* or exposed to control (Cntrl) (sterile PBS) for 6 h. (A) Inflammatory-cell counts in BAL fluid were measured. (B) Cytokines, chemokines, and growth factors in the BAL fluid were measured. IL-17 production was documented in both WT and IL-17RA$^{-/-}$ mice. (C) Bacterial load in the lung homogenate presented as lung CFU/ml; dissemination of infection to the spleen was negative (data not shown). $n = 3$ mice per group. The study was repeated once. ^, $P < 0.001$; *, $P < 0.05$. The error bars indicate SEM.

in the literature, we focused subsequent studies on the actions of IL-23 and IL-1β together.

While intratracheal administration of exogenous IL-23 alone did not result in any neutrophil recruitment to the BAL fluid, IL-1β administration resulted in neutrophil recruitment (roughly 30% of the cells in the BAL fluid), and the combination of IL-23 and IL-1β resulted in synergistic neutrophil recruitment to the airway (accounting for over 75% of the cells in BAL fluid) (Fig. 4A). The intratracheal administration of IL-23 alone did not result in production of any of the cytokines measured (Fig. 4B). Administration of IL-1β alone resulted in production of significant amounts of KC, G-CSF, and IL-6; IL-1β, IP-10 and GM-CSF production was significant compared to control only ($P < 0.05$; *t* test). Coadministration of IL-23 and IL-1β resulted in synergistic production of KC, IL-1β, MCP-1, G-CSF, IP-10, IL-1α, IL-6, GM-CSF, IL-17A, IL-17F and IL-22 (Fig. 4B). These cytokines, chemokines, and growth factors are strongly associated with neutrophil recruitment, are synergistically produced, and are the same as those produced in response to *P. aeruginosa* infection (Fig. 1C).

We also assessed induction of IL-23R, IL-23p19, IL-1β, IL-17A, IL-17F, and IL-22 after intratracheal administration of IL-23, IL-1β, and IL-23 plus IL-1β, using quantitative real-time PCR on RNA isolated from whole lungs. Induction is reported as fold change over control, and the data were analyzed using ANOVA. At 3 h postexposure, IL-23p19, IL-1β, IL-17A, IL-17F, and IL-22 were significantly induced in response to coadministration of IL-23 and IL-1β, while IL-23R was not (Fig. 4C). These data dem-

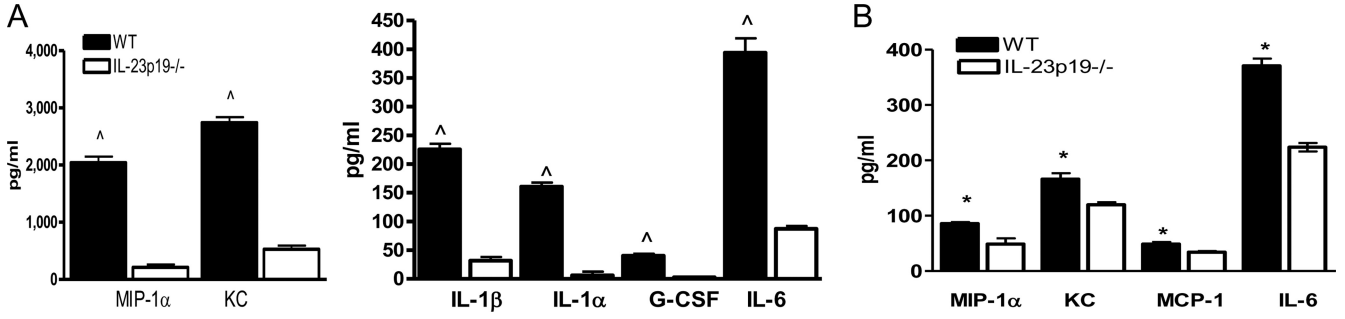

FIG 3 AMs demonstrate robust cytokine and chemokine production with IL-23-dependent elaboration; DCs demonstrate less robust cytokine production and less evident IL-23 dependence. *In vitro* cultures of AMs and DCs were plated under identical conditions and exposed to *P. aeruginosa* for 3 h. Cytokines were measured in the supernatant. (A) Cytokines, chemokines, and growth factors in supernatants of WT and IL-23p19-deficient AMs. (B) Cytokines, chemokines, and growth factors in supernatants of WT and IL-23p19-deficient myeloid DCs. The results reflect averages of triplicate wells. $n = 3$ wells per group. The study was repeated once. *, $P < 0.05$; ^, $P < 0.001$. The error bars indicate SEM.

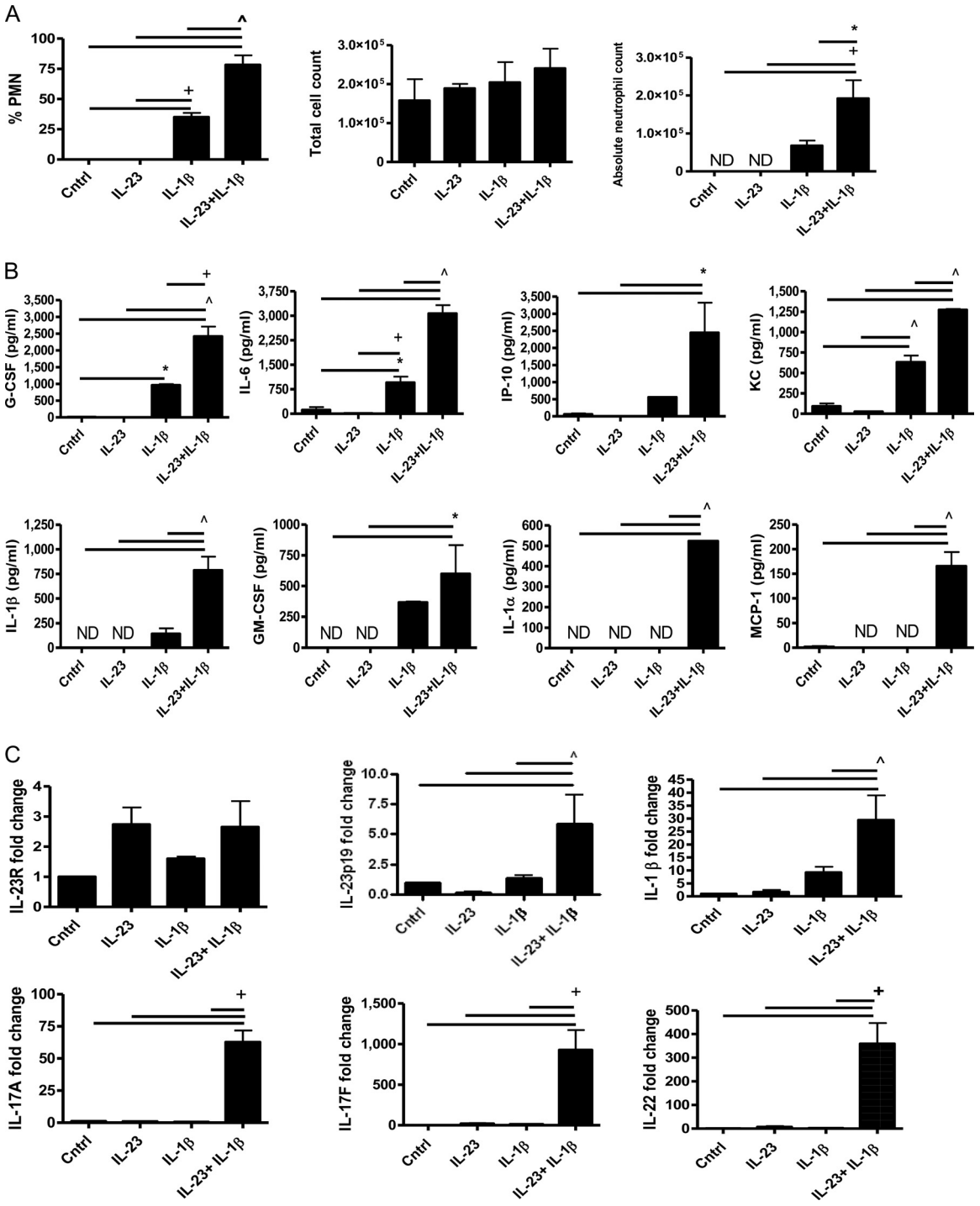

FIG 4 The combination of IL-23 and IL-1β administered to murine airways synergistically elicits inflammation *de novo*. IL-23, IL-1β, and the combination of IL-23 plus IL-1β were administered to mice via noninvasive i.t., and mice were harvested at 3 h. (A) Inflammatory-cell counts in BAL fluid were measured. (B) Cytokines, chemokines, and growth factors in the BAL fluid were measured. (C) IL-23R, IL-23, IL-1β, IL-17A, IL-17F, and IL-22 induction was measured by RT-PCR performed on whole-lung homogenates. The results are reported as fold change compared to control. $n = 3$ mice per group. The study was repeated twice. $*$, $P < 0.05$; $+$, $P < 0.01$; $\wedge$, $P < 0.001$. The error bars indicate SEM.

onstrate that IL-23 and IL-1β act synergistically and are sufficient to cause significant inflammation *de novo*. These data also demonstrate that IL-23 plus IL-1β synergistically promote the induction of both IL-23 and IL-1β.

We next determined if IL-17 was induced at the protein level after IL-23 administration with or without IL-1β. As shown in Fig. 5, we demonstrate that WT mice produce significant levels of IL-17 in response to IL-23 plus IL-1β at 3 h (Fig. 5B).

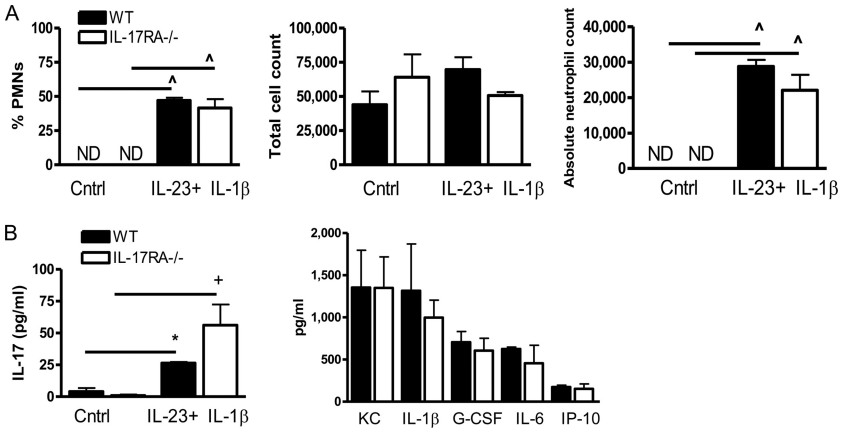

FIG 5 IL-17RA$^{-/-}$ mice do not exhibit decreased inflammation in response to IL-23 plus IL-1β. WT and IL-17RA knockout mice were intratracheally challenged with IL-23 plus IL-1β or sterile PBS (Cntrl) and harvested after 3 h. (A) Inflammatory-cell counts in BAL fluid were measured. (B) IL-17 production was documented in both WT and IL-17RA$^{-/-}$ mice. Cytokines, chemokines, and growth factors in the BAL fluid were measured. $n = 3$ mice per group. The study was repeated once. $\wedge$, $P < 0.001$; +, $P < 0.01$. The error bars indicate SEM.

However, there is no difference in neutrophil recruitment (Fig. 5A) or cytokine and chemokine production (Fig. 5B) compared to IL-17RA$^{-/-}$ mice treated with IL-23 plus IL-1β. These data demonstrate that IL-17 does not contribute to the early inflammation that we observe at 3 h in response to these cytokines.

IL-23 and IL-1 are critical mediators of the innate inflammatory response to *P. aeruginosa* pulmonary infection. Recognizing that IL-23 may mediate inflammation primarily through production of IL-1β, we undertook the following studies to determine the relative contributions of IL-23 and IL-1β to *P. aeruginosa*-induced inflammation. Our data show that IL-23-deficient mice have significantly reduced inflammation compared to WT mice without increased bacterial burden or dissemination (Fig. 1). We expected that the IL-1β-deficient mice would exhibit less inflammation than the IL-23p19$^{-/-}$ mice because IL-1β is a potent cytokine that is clearly implicated in neutrophil recruitment and is an effector independent of IL-23. Because IL-1 regulation is very complex and IL-1β neutralization can be incomplete, we undertook these studies in IL-1R1$^{-/-}$ mice, recognizing that this would neutralize all IL-1 activity. In this study, we infected WT, IL-1R1$^{-/-}$, and IL-23p19$^{-/-}$ mice with *P. aeruginosa* and harvested the mice at 3 h. Neutrophil recruitment was significantly abrogated in both IL-1R1$^{-/-}$ and IL-23p19$^{-/-}$ mice compared to the WT response, with significantly lower neutrophil recruitment in IL-1R1$^{-/-}$ mice compared to the IL-23p19$^{-/-}$ mice (Fig. 6A).

The stepwise decrease in the percentage of neutrophils measured in the BAL fluid of WT, IL-23p19$^{-/-}$, and IL-1R1$^{-/-}$ mice was not mirrored in the cytokine elaboration (Fig. 6B). There was significantly more GM-CSF, IL-1β, KC, IL-6, G-CSF, IP-10, IL-1α, and MCP-1 in the WT mice than in both knockouts (Fig. 6B). However, GM-CSF and KC were the only cytokines that were more abundant in the IL-23p19$^{-/-}$ BAL fluid than in the IL-1R1$^{-/-}$ BAL fluid, and while MIP-1α levels were higher in the WT BAL fluid than in the IL-23p19$^{-/-}$ BAL fluid, they were not significantly higher than in the IL-1R1$^{-/-}$ BAL fluid (Fig. 6B). Focusing specifically on IL-1β, we note that there was less IL-1β recovered in the IL-1R1$^{-/-}$ BAL fluid than in the WT BAL fluid, but more than that in the IL-23p19$^{-/-}$ BAL fluid (Fig. 6B). Taken together, these findings suggest that while IL-1β autoinduction does contribute to IL-1β production during *P. aeruginosa* pulmonary infection, IL-23 also contributes significantly to IL-1β production, thus promoting IL-1β-mediated inflammation. While there was no dissemination of bacteria in any of the groups (data not shown), there was a significantly higher bacterial burden in the IL-1R1$^{-/-}$ lungs than in WT and IL-23p19$^{-/-}$ mice (Fig. 6C).

These studies demonstrate that while IL-23 and IL-1 both promote neutrophil recruitment and proinflammatory cytokine production, there is a striking difference in their effects on the bacterial burden. This difference may be dependent on neutrophil recruitment, but the root cause of the difference cannot be concluded from these data.

$\gamma\delta$ T cells are a source of IL-17A, IL-17F, and IL-22 during the innate immune response. While IL-17 production appears not to contribute to inflammation at these early time points, our previous work demonstrated that IL-23 and IL-17 production results in the subsequent production of other neutrophil-associated cytokines that promote neutrophil inflammation at later time points (14). Because the IL-17 production observed in these studies occurs on the order of hours rather than weeks and persists to promote the neutrophil recruitment seen at later time points, we felt it was important to identify the source of this IL-17 production.

Because the mice used in these studies are naïve and have been maintained under specific-pathogen-free conditions, we thought that it was unlikely that the cytokines were being elaborated by Th17 cells. Furthermore, based on our prior work (unpublished) and the work of others (47, 54), we suspected that resident $\gamma\delta$ T cells were the primary or exclusive early source of IL-17A, IL-17F, and IL-22. To determine whether $\gamma\delta$ T cells were the source, we obtained lung cells from WT mice that were infected with *P. aeruginosa* or exposed to PBS for 6 h, sorted them into CD3$^+$ $\gamma\delta^+$ and CD3$^+$ $\gamma\delta^-$ populations, and processed them for RNA (due to small cell numbers). We measured IL-17A, IL-17F, and IL-22 induction by real-time PCR and report the results as fold change over control. IL-17A, IL-17F, and IL-22 are induced in the CD3$^+$ $\gamma\delta^+$, but not the CD3$^+$ $\gamma\delta^-$, population (Fig. 7). Interestingly, the pattern of induction demonstrates a greater magnitude of change

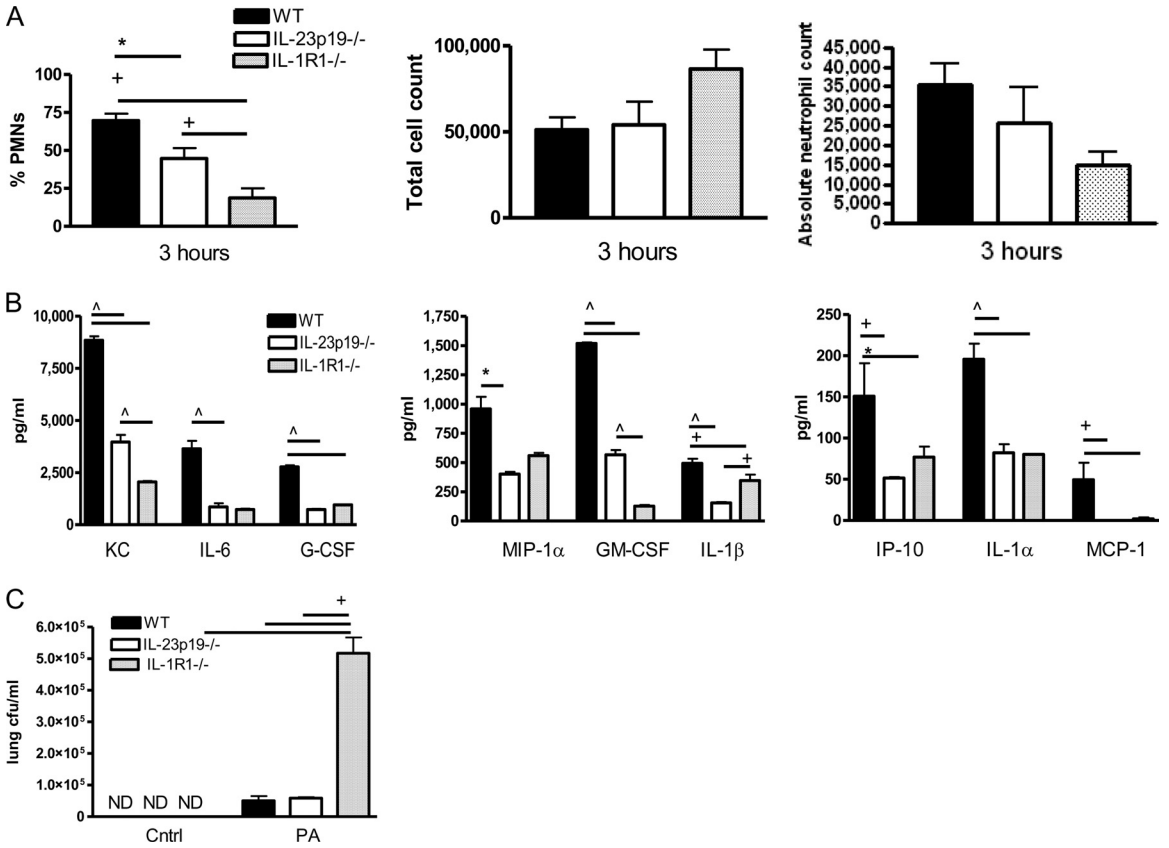

FIG 6 IL-1R1$^{-/-}$ mice show decreased neutrophil recruitment and worse pulmonary infection than WT and IL-23p19$^{-/-}$ mice. WT, IL-23p19$^{-/-}$, and IL-1R1$^{-/-}$ mice were infected with *P. aeruginosa* or exposed to a control (sterile PBS). All groups were sacrificed 3 h after exposure. (A) Inflammatory-cell counts in BAL fluid were measured for the infected groups. (B) Cytokines, chemokines, and growth factors in the BAL fluid of infected groups. (C) Bacterial load in the lung homogenate presented as lung CFU/ml (mouse); dissemination of infection to the spleen was negative (data not shown). Comparisons among infected groups are shown, as are comparisons to uninfected controls that had no detectable bacterial growth. $n = 3$ mice per group. The study was repeated once. *, $P < 0.05$; +, $P < 0.01$; ^, $P < 0.001$. The error bars indicate SEM.

for IL-17F and IL-22 than for IL-17A, which mirrors what is seen in whole lungs (Fig. 1E). Taken together, the studies shown in Fig. 1 and 7 strongly suggest that γδ T cells are the primary, if not the exclusive, source of IL-17A, IL-17F, and IL-22 during early *P. aeruginosa* pulmonary infection.

To determine the roles of IL-23, IL-1β, and the combination of IL-23 plus IL-1β in IL-17A, IL-17F, and IL-22 production, we isolated γδ T cell function by comparing cytokine production in WT- and γδ TCR$^{-/-}$-derived lung cultures (Fig. 8A) or between WT and WT cultures depleted of γδ T cells by fluorescence-activated cell sorter (FACS) (Fig. 8B). In the first set of studies, we plated single-cell suspensions of lungs obtained from WT and γδ TCR$^{-/-}$ mice and exposed them to IL-23, IL-1β, or IL-23 plus IL-1β. In these *in vitro* studies, recombinant IL-23 alone was sufficient to stimulate WT cultures to produce IL-17A, and IL-22 (IL-17F did not reach significance) (Fig. 8A). Exposure to IL-23 plus IL-1β resulted in synergistic production of IL-17A, IL-17F, and IL-22 (Fig. 8A). The γδ TCR$^{-/-}$ cultures made significantly less IL-17A, IL-17F, and IL-22 than WT-derived cultures in response to IL-23 alone and IL-23 plus IL-1β (Fig. 8A). These data suggest that γδ T cells are the predominant source of the Th17 cytokines produced during the innate inflammatory response and that IL-23 is sufficient to elicit this response.

Because our study (Fig. 7) demonstrated that γδ T cells are the

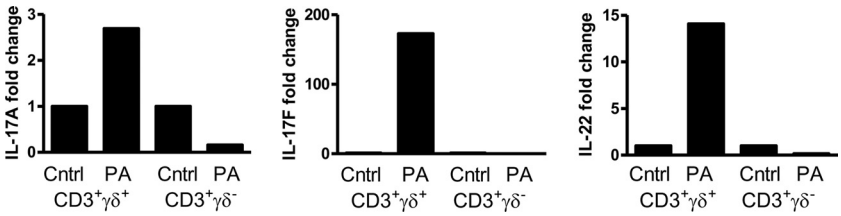

FIG 7 γδ TCR^{+} T cells demonstrate IL-17A, IL-17F, and IL-22 induction. WT mice were infected with *P. aeruginosa* or exposed to control (Cntrl) (sterile PBS) for 6 h, and lungs were harvested. Lung cells were sorted for γδ TCR^{+} versus γδ TCR^{-} T cells and processed for RT-PCR for IL-17A, IL-17F, and IL-22. The results are presented as fold change compared to the control. Lung cells from 2 or 3 mice were pooled and sorted for each group. The study was repeated twice, and a representative study is shown.

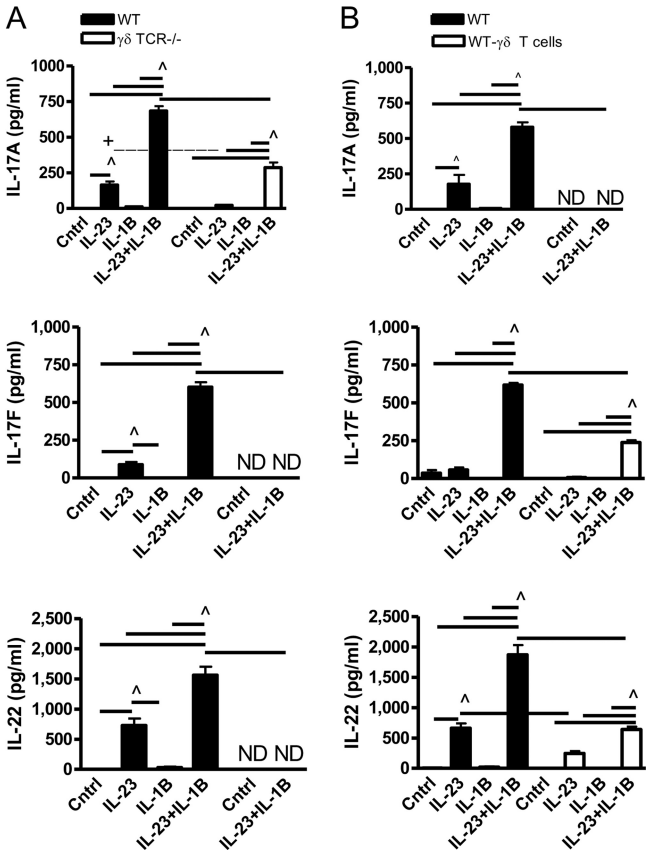

FIG 8 γδ TCR$^+$ T cells produce IL-17A, IL-17F, and IL-22 in response to IL-23 and IL-23 plus IL-1β. (A) Single-cell lung suspensions from γδ TCR$^{-/-}$ mice and WT mice were exposed to medium, IL-23, IL-1β, or IL-23 plus IL-1β. IL-17A, IL-17F, and IL-22 were measured in the supernatant. (B) A single-cell suspension of lung cells from WT mice was processed via FACS with and without removal of the γδ TCR$^+$ T cells (labeled WT-γδ or WT, respectively). IL-17A, IL-17F, and IL-22 were measured in the supernatant. For both sets of studies, the data presented represent triplicate wells. The studies were repeated once. +, $P < 0.01$; ∧, $P < 0.001$. The error bars indicate SEM.

only CD3$^+$ T cells induced for Th17 cytokines during *P. aeruginosa* infection, we questioned whether previously reported developmental abnormalities or compensatory mechanisms that could result in early IL-17A, IL-17F, and IL-22 production by a non-γδ T cell population(s) was occurring in the studies represented in Fig. 8A. To determine if this might be the case, we carried out similar exposure studies comparing cytokine production by WT cells and WT cells depleted of γδ T cells by FACS. In these studies, we observed significant IL-17A, IL-17F and IL-22 production by WT cells and no measurable IL-17A, IL-17F, or IL-22 in cultures with the γδ T cells removed (Fig. 8B). These data support the findings of our *in vivo* infection study shown in Fig. 7, demonstrating that γδ T cells are the T cell population responsible for IL-17A, IL-17F, and IL-22 production. Taken together, the data show that IL-23 plays a novel dual role in regulating pulmonary inflammation in response to *P. aeruginosa*. The first is in regulation of IL-1β expression and early PMN emigration into the lung at early time points, and this effect is IL-17RA independent. Subsequently, IL-23 and IL-1β synergize to induce IL-17 by γδ T cells. This γδ T cell production of IL-17 can sustain neutrophil emigration by sustaining expression of CXCR2 ligands and regulation of G-CSF-mediated granulopoiesis (53).

DISCUSSION

P. aeruginosa airway infection results in robust neutrophil inflammation that is associated with increased morbidity and mortality in CF (41, 46, 62). Development of therapeutic strategies that decrease this neutrophil inflammation without compromising host defense would lead to decreased airway damage and improved outcomes in CF. Our prior work, in a chronic model of *P. aeruginosa* pulmonary infection, demonstrated that the IL-23/IL-17 proinflammatory axis promotes robust neutrophil recruitment but does not improve protection against *P. aeruginosa* infection (14), suggesting that IL-23 or IL-17 may be an appropriate immunotherapeutic target. Because our prior studies could not differentiate between IL-23- and IL-17-mediated effects, we conducted these studies, in an acute-infection model, to determine the relative contributions of IL-23 and IL-17 to neutrophil recruitment and inflammation during *P. aeruginosa* pulmonary infection.

These studies demonstrate for the first time that IL-23 can mediate IL-17-independent inflammation during pulmonary infection. Infection of IL-23p19$^{-/-}$ mice demonstrated significant abrogation of neutrophil recruitment and proinflammatory cytokine production in the absence of IL-17 production (Fig. 1). Furthermore, IL-23p19 induction occurs as early as 1 h after infection and significantly increases at 3 h in WT mice, preceding the robust neutrophil recruitment that occurs at 3 and 6 h. By 6 h after infection, studies in IL-17RA knockout and WT mice (Fig. 2) demonstrate that while *P. aeruginosa* infection stimulates significant IL-17A production, there was no measurable decrease in neutrophil recruitment or cytokine production or change in the bacterial load in the IL-17RA$^{-/-}$ mice. These data demonstrate that even when IL-17 is produced during early *P. aeruginosa* pulmonary infection, it does not contribute to this early neutrophil recruitment. Furthermore, this IL-17 production has not yet promoted the production of neutrophil-associated cytokines, chemokines, and growth factors. Taken together, these data strongly suggest that the early neutrophil recruitment noted during *P. aeruginosa* pulmonary infection is IL-17 independent and IL-23 mediated.

APCs are well described as robust producers of IL-23 in a variety of models (4, 34, 60, 65) and are on the front lines of the innate immune response, poised to respond during early airway infections. For these reasons, we undertook studies to determine if AMs or myeloid DCs (mDCs), the most common APCs in the airways, were capable of mediating IL-23-dependent inflammation in response to *P. aeruginosa* infection. Our studies demonstrate that *P. aeruginosa* exposure of AMs results in IL-23-dependent production of cytokines and chemokines (Fig. 3A) and mirrors that seen in BAL fluid from infected mice (Fig. 1). mDCs produce a more limited range of these cytokines, demonstrate less IL-23 dependence, and are less robust in their production (Fig. 3B). While AM depletion studies *in vivo* would definitively determine if AMs are the key regulator of the IL-23-mediated inflammatory response to *P. aeruginosa* infection, the current methodological approaches to AM depletion are inadequate to answer this question. Commonly used approaches, such as clodronate depletion or the use of CD11b$^-$ diphtheria toxin receptor (DTR) mice, do deplete AMs; however, these approaches also deplete DC populations (19, 32, 50, 55). Based on our data, we believe that AMs

are capable of driving the IL-23-dependent innate inflammatory response, and more likely than mDCs to do so.

Because elimination of IL-23 led to decreased inflammation during pulmonary *P. aeruginosa* infection, as noted in our IL-23p19$^{-/-}$ mice (Fig. 1), we conducted corollary experiments to determine if administration of recombinant IL-23 was sufficient to promote inflammation *de novo* (Fig. 4). In these studies, the intratracheal administration of recombinant IL-23 did not result in neutrophil recruitment or production of the cytokines, chemokines, and growth factors shown in Fig. 1. Furthermore, it did not lead to the induction of the IL-23 receptor, IL-23, IL-1β, or the Th17 cytokines (Fig. 4). We hypothesized that the lack of response was due to either the route of administration or a requirement for costimulation.

We chose to coadminister IL-1β in the IL-23 exposure studies for the reasons noted above. In addition to providing costimulation that might elicit a more robust response, IL-1β may promote IL-23 access to the interstitial compartment (9, 22), where most tissue-associated lymphocytes are located, thus overcoming the problem of "access." For these reasons, we focused our sterile inflammation model on the combination of IL-23 and IL-1β.

Our data from the studies shown in Fig. 8A and B demonstrate that IL-23 is sufficient to promote cytokine production *de novo* and suggest that the route of administration and "compartmentalization" of IL-23 may explain the absence of IL-23-mediated inflammation in the *in vivo* studies (Fig. 4). However, the studies shown in Fig. 8 also demonstrate, even *in vitro*, that IL-1β acts synergistically with IL-23 to effect significant inflammation. While the synergy is evident for all of the analytes reported, the mechanism of this synergy is not clear. The fact that IL-1β is sufficient to drive neutrophil recruitment and non-Th17 cytokine production and that IL-1β is induced in an IL-23-dependent manner (Fig. 4) suggests that IL-23 promotes inflammation by driving IL-1β production; however, IL-23 appears to be necessary to drive IL-17A/IL-17F and IL-22 production (Fig. 4 and 8), which argues that increased IL-1β production cannot be the only mechanism for synergistic action of these two cytokines. Furthermore, MCP-1 and IL-1α are produced only when IL-23 and IL-1β are coadministered. These findings are informative, because they demonstrate that IL-23 and IL-1β act synergistically, in addition to amplifying each other's production. Our data suggest that neither IL-23 nor IL-1β is the "dominant" driver of inflammation, while each cytokine may have a dominant or exclusive role.

These findings are significant, because this is the first report of IL-23 driving non-IL-17-mediated inflammation during pulmonary infection. Furthermore, this is the first report of IL-23-amplified induction/production of IL-1β and demonstrates that IL-23 has the potential to potently amplify IL-1β-mediated processes, such as neutrophil inflammation and autoinflammation (21, 27, 39). This synergistic induction can create autoamplification loops that promote dysfunctional inflammation, as has been documented with IL-1β (13, 58, 59). Further, this autoamplification can significantly promote IL-17A, IL-17F, and IL-22 production and downstream inflammation. These findings also have direct disease relevance to CF, because we have shown that IL-23 levels are correlated with active infection (15, 38) and that IL-1β is highly expressed in CF airways, is expressed concurrently with IL-23 and IL-17 during infection (15, 38), and is associated with more severe CF disease (5, 6, 8, 36).

While these studies of sterile inflammation clarify some of the mechanisms of IL-23-mediated inflammation, we conducted additional studies to help isolate the functions of IL-23 and IL-1β in mediating *P. aeruginosa*-induced inflammation. Using IL-23p19$^{-/-}$ mice to model IL-23 neutralization, we compared the impacts of IL-23 neutralization and IL-1 neutralization. While IL-1β neutralization would have been more specific, we thought that it might lead to spurious results due to incomplete neutralization of IL-1β or compensatory responses of IL-1α, which has been previously demonstrated (2, 16, 40). The use of IL-1R1$^{-/-}$ mice results in neutralization of both IL-1β and IL-1α; however, it does effectively isolate IL-1 function from that of IL-23. Recognizing that these results may reflect activity of both IL-1α and IL-1β, we demonstrate that IL-1R1$^{-/-}$ mice have significantly fewer neutrophils in their BAL fluid than either WT or IL-23p19$^{-/-}$ mice after *P. aeruginosa* infection. Interestingly, this decrease in BAL neutrophils is accompanied by an increased bacterial load, demonstrating that while IL-23 is dispensable for control of infection, IL-1 is not. Our findings agree with other studies that also demonstrate the importance of IL-1 function during Gram-negative infection (44, 51, 56, 57). Explanations of this difference in IL-23- versus IL-1-mediated host defense include neutrophil recruitment to the airway, downstream effects of GM-CSF and KC, and other downstream effects of IL-1 function. Further studies that are beyond the scope of this project would be necessary to determine the precise mechanism of IL-1-mediated host defense.

Recently, Liu et al. (37) reported that in their model of acute *P. aeruginosa* pulmonary infection, early IL-17 production is responsible for control of infection, a finding that contrasts with our current and prior studies (14). We believe that this difference in findings and conclusions between our studies might be explained on the basis of experimental technique. In the Liu et al. studies, the bacterial load was measured in the BAL fluid, which can be subject to significant sampling error. In our studies, the bacterial load was measured in the lung tissue and spleen. We chose to assess the bacterial loads in these two compartments because we believe that they best represent active pulmonary infection and dissemination, and these data are directly comparable to other studies of pulmonary infection in murine models.

While our data demonstrate that IL-17 activity is not responsible for control of *P. aeruginosa* pulmonary infection and does not promote concurrent inflammation during early infection, the IL-17 produced during the innate immune response may serve to prolong the inflammatory response through stabilization of chemokine mRNA and G-CSF production (11, 26, 53) or by producing the conditions that foster the development of a Th17-polarized phenotype. IL-17, IL-23, and IL-1β promote production of IL-6, IL-21, and transforming growth factor beta (TGF-β) (35, 52), the key differentiation factors for Th17 cells (7, 43, 48, 63). For these reasons, we thought it was important to determine the source of this early IL-17 production and the conditions that prompted it. As demonstrated by the studies shown in Fig. 7 and 8, $\gamma\delta$ T cells appear to be the primary, if not the exclusive, source of IL-17A, IL-17F, and IL-22 production, and production of these cytokines appears to be dependent on IL-23, with more robust production elicited by the combination of IL-23 and IL-1β. While the IL-17 field has primarily focused on Th17 cells and the adaptive immune response, a nascent literature describing IL-17 production by $\gamma\delta$ T cells is developing (10, 23, 45, 47), and we believe that this work contributes to that growing body of literature. Based on these data and our knowledge of the IL-23/IL-17 proin-

flammatory axis, IL-23 and IL-1β production during the innate inflammatory response may have a far-reaching impact during inflammation, with $\gamma\delta$ T cells providing the link between the IL-23/IL-1β-driven innate and IL-17/Th17-mediated adaptive responses. This is obviously an area that deserves further exploration.

In summary, the data presented here demonstrate for the first time that IL-23 acts independently of IL-17 to promote inflammation in an acute model of *P. aeruginosa* pulmonary infection. Furthermore, our data demonstrate that IL-23 acts synergistically with IL-1 to promote neutrophil recruitment; amplifies IL-1 induction and production; and promotes IL-17A, IL-17F, and IL-22 production by $\gamma\delta$ T cells. These data also confirm our prior finding that IL-23 is not necessary to host defense against *P. aeruginosa* pulmonary infection, demonstrating no effect of IL-23 neutralization on the bacterial load or dissemination. Furthermore, acting with IL-1β, innate IL-23 production may promote polarization of the adaptive response toward a Th17 phenotype, in addition to promoting the production of Th17 cytokines by $\gamma\delta$ T cells and, later, Th17 cells. These studies demonstrate that IL-23 is a central mediator of the neutrophil inflammation seen in *P. aeruginosa* pulmonary infection. While further studies are necessary, this work suggests that IL-23 is a promising target for the development of immunotherapeutic drugs that prevent morbidity and mortality in chronic *P. aeruginosa* pulmonary infection.

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