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HOST RESPONSE AND INFLAMMATION

Role of Common γ-Chain Cytokines in Lung Interleukin-22 Regulation after Acute Exposure to *Aspergillus fumigatus*

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ABSTRACT Humans are constantly exposed to the opportunistic mold *Aspergillus fumigatus*, and disease caused by this pathogen is often determined by the magnitude of local and systemic immune responses. We have previously shown a protective role for interleukin-22 (IL-22) after acute *A. fumigatus* exposure. Here, employing IL-22^{Cre} R26R^{eYFP} reporter mice, we identified iNKT cells, γδ T cells, and type 3 innate lymphoid cells (ILC3s) as lung cell sources of IL-22 in response to acute *A. fumigatus* exposure. As these cells often utilize common γ-chain cytokines for their development or maintenance, we determined the role of IL-7, IL-21, and IL-15 in lung IL-22 induction and *A. fumigatus* lung clearance. We observed that IL-7, IL-21, and IL-15 were essential for, partially required for, or negatively regulated the production of IL-22, respectively. Deficiency in IL-7 and IL-21, but not IL-15R, resulted in impaired fungal clearance. Surprisingly, however, the absence of IL-7, IL-21, or IL-15R signaling had no effect on neutrophil recruitment. The levels of IL-1α, an essential anti-*A. fumigatus* proinflammatory cytokine, were increased in the absence of IL-7 and IL-15R but decreased in the absence of IL-21. IL-7 was responsible for maintaining lung iNKT cells and γδ T cells, whereas IL-21 was responsible for maintaining lung iNKT cells and ILC3s. In contrast, IL-15R deficiency had no effect on the absolute numbers of any IL-22 cell source, rather resulting in enhanced per cell production of IL-22 by iNKT cells and γδ T cells. Collectively, these results provide insight into how the IL-22 response in the lung is shaped after acute *A. fumigatus* exposure.

KEYWORDS *Aspergillus*, IL-22, innate-like lymphocyte, lung

Exposure to *Aspergillus fumigatus* may lead to variety of different infections and comorbidities, including development of an aspergilloma, chronic necrotizing aspergillosis, fungal asthma, and an invasive fungal infection (IFI) termed invasive aspergillosis (IA). Incidence rates for IFIs are ~7% in both solid organ transplants (1) (19% due to IA) and stem cell transplants (2) (43% due to IA). In addition to these individuals with suppressed immune systems, there are several genetic immunodeficiencies in which IA or infection with *A. fumigatus* is extremely high. The classic example is chronic granulomatous disease (CGD), in which NADPH oxidase deficiency is uniquely associated with the development of IA (3). Individuals with hyper-IgE syndrome have mutations in STAT3, are unable to produce Th17 cells (4), and are severely susceptible to an *A. fumigatus* lung infection, although most often when cavitary lung lesions are present (5). Additional studies have reported that single-nucleotide polymorphisms (SNPs) in Dectin-1 (6), Toll-like receptor 1 (TLR1)/TLR6 (7), DC-SIGN (6), plasminogen (8), and tumor necrosis factor receptor 1 (TNFR1) (9) are also associated with susceptibility to IA. Although IA is a known infectious complication of the above-mentioned conditions, there is a growing concern for the development of nosocomial IA in the intensive

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care unit (10), with the underlying disease in these nonneutropenic included patients being high-dose steroids for chronic obstructive pulmonary disease (COPD), cirrhosis/liver failure, and solid cancers (11–14). Finally, simple colonization with or sensitization to *A. fumigatus* may have dramatic effects on lung function in asthmatics (15–18) and individuals with cystic fibrosis (CF) (19–21) and COPD (22). Therefore, a clearer understanding of protective immune responses in the lung after acute or chronic *A. fumigatus* exposure may identify therapeutic targets that could improve outcomes in multiple lung diseases (lung transplant, CF, COPD, asthma etc.).

Interleukin-22 (IL-22) is widely acknowledged to promote epithelial antimicrobial responses (23). We have previously reported that mice deficient in Dectin-1 acutely exposed to *A. fumigatus* had multiple defects in host defense (24). We extended the antifungal contribution of Dectin-1 to the induction of IL-22, as Dectin-1 deficiency resulted in a near total loss of lung IL-22 production after acute *A. fumigatus* exposure (25). Importantly, genetic deficiency in or neutralization of IL-22 resulted in impaired clearance of *A. fumigatus* as early as 24 h postchallenge, illustrating a critical role for IL-22 in pathogen elimination during acute infection (25). Although multiple cell sources of IL-22, including $CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta$ T cells, NK cells, iNKT cells, LTi cells, and type 3 innate lymphoid cells (ILC3s), have been identified (reviewed in references 26 and 27), it is not clear which of these are functioning in an innate capacity after acute lung fungal exposure. However, we have previously reported that Dectin-1-dependent IL-22 was possibly produced by a non-$CD4^+$ T cell source in a model of fungal asthma associated with chronic *A. fumigatus* exposure (28).

The common γ-chain family of cytokines, which includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, are most well recognized as essential factors for T cell development and B cell lymphopoiesis (29, 30). In general, IL-2, IL-7, and IL-15 have been shown to preferentially activate STAT5, while IL-4 and IL-21 preferentially activate STAT6 and STAT3, respectively (31). However, common γ-chain cytokines also play important roles in development, maintenance, and homeostasis of innate-like lymphocyte populations. For example, IL-2, IL-7, and IL-9 have recently been linked to ILC2 survival (32–35). Additionally, $\gamma\delta$ T cell homeostasis requires IL-7 (36), as do certain iNKT cell populations (37). In the current study, we sought to define the lung cell sources of IL-22 after acute exposure to *A. fumigatus* and to determine which, if any, common γ-chain cytokines regulate IL-22 production and host defense.

RESULTS

Common γ-chain cytokines play dichotomous roles in lung IL-22 production and *A. fumigatus* lung clearance. We have previously reported that IL-22 is essential for fungal clearance after acute *A. fumigatus* challenge (25). Although originally identified to be produced by $CD4^+$ T cells, IL-22 is now recognized to be produced by many cell types, including $\gamma\delta$ T cells, NK cells, iNKT cells, LTi cells, and ILC3s (reviewed in references 26 and 27). T and B lymphocytes, as well as innate-like lymphocytes, express the common γ-chain cytokine receptor, and cytokines in this family are critical for generation, development, or homeostasis of these cell types (36, 38). In addition, we have recently identified a role for the common γ-chain cytokine IL-7 in iNKT- and IL-22-associated immunopathogenesis during *A. fumigatus*-associated fungal asthma (39). To this end, we examined the impact of common γ-chain cytokines on lung IL-22 induction and clearance of *A. fumigatus* after acute exposure. In initial studies, we showed that lung IL-22 production is not detected until 24 h after acute *A. fumigatus* exposure and peaks at 48 h postexposure, followed by a reduction at 72 h (Fig. 1A). With respect to IL-7, mice deficient in this mediator demonstrated significantly higher fungal burdens in the lungs 48 h postexposure (Fig. 1B). An increased fungal burden in *Il7*$^{-/-}$ mice correlated with a near complete absence of IL-22 production by lung digest cells at 24 h and 48 h postchallenge (Fig. 1C). As neutrophils are central effector cells against *A. fumigatus* in the lung (40), we assessed their levels in *Il7*$^{-/-}$ mice. Surprisingly, there was no difference in neutrophil numbers between *A. fumigatus*-exposed wild-type (WT) and *Il7*$^{-/-}$ mice (Fig. 1D). As expected based on intact neutrophil

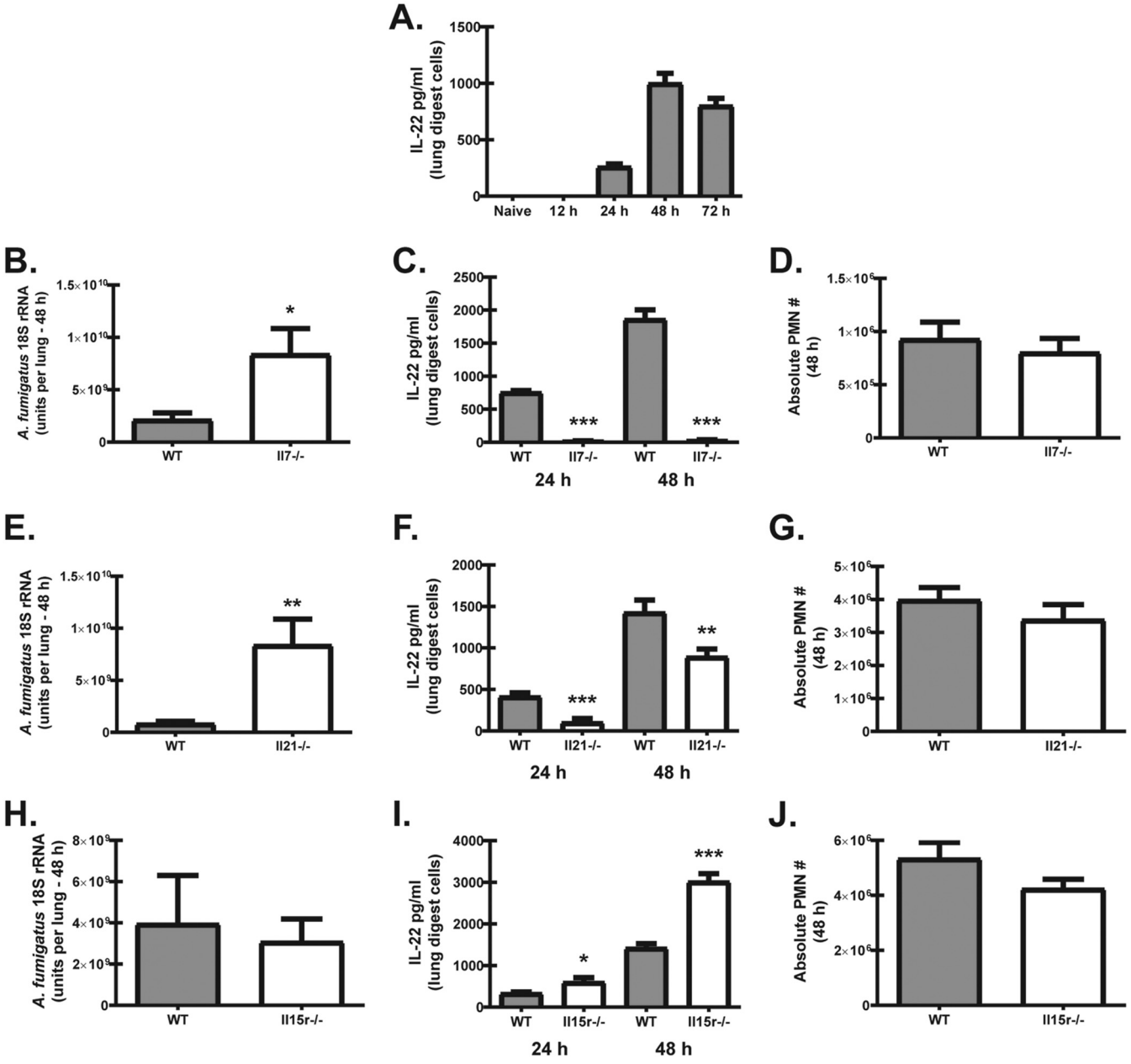

FIG 1 Common γ-chain cytokines play dichotomous roles in lung IL-22 production and *A. fumigatus* lung clearance. (A) C57BL/6 wild-type (WT) mice were challenged with live *A. fumigatus* conidia via intratracheal administration and sacrificed 12, 24, 48, and 72 h postexposure. At each time point, the right lungs were collected and enzymatically digested, and unfractionated lung cells were cultured in triplicate for 24 h. IL-22 levels were quantified in clarified coculture supernatants by ELISA. Cumulative data from three independent experiments ($n = 3$ mice per time point per experiment) are shown. (B) WT and *Il7*$^{-/-}$ mice were challenged intratracheally with *A. fumigatus* conidia, and 48 h after exposure, the lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. Cumulative data from two to three independent experiments ($n = 4$ to 5 mice per group per experiment) are shown. Data are expressed as the mean level of *A. fumigatus* 18S rRNA + standard error of the mean (SEM). (C) WT and *Il7*$^{-/-}$ mice were challenged intratracheally with *A. fumigatus* conidia, and 24 h and 48 h after exposure, the right lungs were collected and enzymatically digested, and unfractionated lung cells were cultured in triplicate for 24 h. IL-22 levels were quantified in clarified coculture supernatants by ELISA. Cumulative data from three independent experiments ($n = 3$ to 5 mice per group per experiment) are shown. (D) Total neutrophil population enumerated from whole-lung digests by flow cytometry. Neutrophils were defined as CD45$^+$ CD11b$^+$ Ly6G$^+$. Cumulative data from two independent experiments ($n = 3$ to 4 mice per group per experiment) are shown. (E) Fungal burden 48 h after challenge in WT and *Il21*$^{-/-}$ mice. Cumulative data from two to three independent experiments ($n = 4$ to 5 mice per group per experiment) are shown. Data are expressed as the mean level of *A. fumigatus* 18S rRNA + SEM. (F) IL-22 production by lung digest cells 48 h after *A. fumigatus* challenge in WT and *Il21*$^{-/-}$ mice. Cumulative data from three independent experiments ($n = 3$ mice per group per experiment) are shown. (G) Total neutrophil numbers in the lung 24 h and 48 h after *A. fumigatus* challenge in WT and *Il21*$^{-/-}$ mice. Cumulative data from two independent experiments ($n = 3$ mice per group per experiment) are shown. (H) Fungal burden 48 h after challenge in WT and *Il15r*$^{-/-}$ mice. Cumulative data from two independent experiments ($n = 4$ to 5 mice per group per experiment) are shown. Data are expressed as the mean level of *A. fumigatus* 18S rRNA + SEM. (I) IL-22 production by lung digest cells 24 h and 48 h after *A. fumigatus* challenge in WT and *Il15r*$^{-/-}$ mice. Cumulative data from three independent experiments ($n = 3$ mice per group per experiment) are shown. (J) Total neutrophil numbers in the lung 48 h after *A. fumigatus* challenge in WT and *Il15r*$^{-/-}$ mice. For all graphs, *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively. PMN, polymorphonuclear leukocytes.

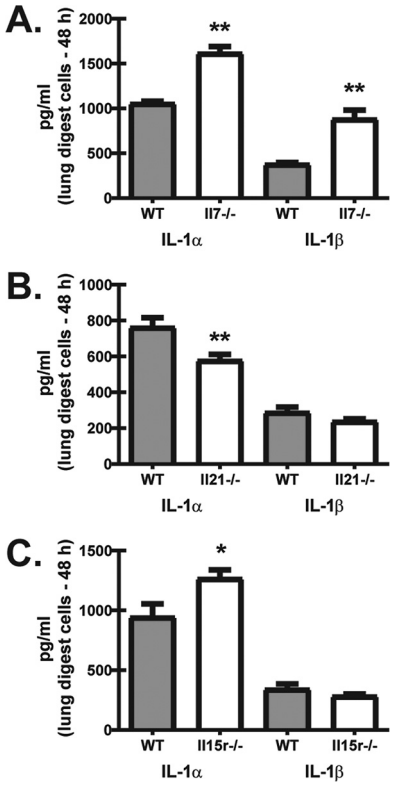

FIG 2 IL-1α and IL-1β levels are differentially affected by common γ-chain cytokines after acute *A. fumigatus* exposure. Mice were challenged intratracheally with *A. fumigatus* conidia, and 48 h after exposure, the right lungs were collected and enzymatically digested, and unfractionated lung cells were cultured in triplicate for 24 h. IL-1α and IL-1β levels in *Il7*$^{-/-}$ mice (A), *Il21*$^{-/-}$ mice (B), and *Il15r*$^{-/-}$ mice (C) were quantified in clarified coculture supernatants using a Bio-Plex system. Cumulative data from three independent experiments ($n = 3$ to 5 mice per group per experiment) are shown. For all graphs, * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

recruitment, *Il7*$^{-/-}$ mice did not succumb to invasive infection (data not shown). Similar to *Il7*$^{-/-}$ mice, mice deficient in IL-21 also demonstrated an impairment in *A. fumigatus* lung clearance (Fig. 1E) and a moderate reduction in IL-22 production at 24 and 48 h (Fig. 1F). Once again, neutrophil numbers were not different in *Il7*$^{-/-}$ mice (Fig. 1G). In contrast to *Il7*$^{-/-}$ and *Il21*$^{-/-}$ mice, mice deficient in IL-15R were not more susceptible to acute *A. fumigatus* exposure (Fig. 1H). Intriguingly, IL-22 was produced at much higher levels at both 24 h and 48 h postchallenge than in similarly exposed WT mice (Fig. 1I). Despite the increase in IL-22, neutrophil levels were not similarly increased (Fig. 1J). Thus, IL-7 and IL-21 are required for optimal IL-22 production and organism clearance after acute *A. fumigatus* exposure, whereas IL-15R signaling functions as a negative regulator of IL-22. Despite variable IL-22 production in these mice, the level of IL-22 did not correlate with the level of neutrophil recruitment to the lungs.

IL-1α and IL-1β levels are differentially affected by common γ-chain cytokines after acute *A. fumigatus* exposure. Renewed interest in the IL-1 family of cytokines has uncovered novel roles for IL-1α and IL-1β in lung defense during acute *A. fumigatus* exposure (41, 42). Although we have previously reported that IL-1α is dependent on IL-22 after acute *A. fumigatus* exposure (25), we have recently shown that signaling through the IL-1 receptor is required for optimal IL-22 production after *A. fumigatus* exposure (43). Despite attenuated IL-22 production, deficiency in IL-7 was associated with elevated levels of IL-1α and IL-1β (Fig. 2A). In contrast, lower IL-22 levels during IL-21 deficiency correlated with lower IL-1α, but not IL-1β, production (Fig. 2B), despite the presence of higher fungal burden. Enhanced IL-22 production in the absence of IL-15 signaling resulted in elevated IL-1α, but not IL-1β (Fig. 2C). Thus, IL-1α and IL-1β

production levels are differentially affected by common γ-chain cytokines during acute *A. fumigatus* exposure.

Multiple innate and innate-like lymphocytes produce IL-22 after acute *A. fumigatus* exposure. We have previously reported that IL-22 was primarily produced by a non-CD4$^+$ T cell source during *A. fumigatus*-associated fungal asthma (28). Antibody-mediated depletion of CD4$^+$ T cells demonstrated no effect on IL-22 production after acute *A. fumigatus* exposure (Fig. 3A), indicating that these cells play little to no role in IL-22 generation (Fig. 3A). We next sought to identify the non-CD4$^+$ T cell source(s) of IL-22 in the lung by employing IL-22^{Cre} R26R^{eYFP} reporter mice (44), in which yellow fluorescent protein (YFP) expression marks IL-22-producing cells. After exposure of mice to *A. fumigatus* for 48 h, gating on eYFP$^+$/IL-22$^+$ cells (where eYFP is enhanced YFP) identified a small population of CD1d tetramer-positive (CD1d tetramer$^+$) cells (representative of iNKT cells) (Fig. 3B) and a much larger population of TCR-δ$^+$ cells (representative of γδ T cells) (Fig. 3B). We did not detect eYFP expression in CD11b$^+$ CD11c$^+$ or CD11b$^+$ CD11c$^+$ cells nor in CD8$^+$ or NK1.1$^+$ cells (Fig. 3B). The population of cells not stained by the CD1d tetramer or TCR-δ was analyzed for expression of lineage markers, with a significant portion of cells noted as negative for all lineage markers queried. Upon further analysis, these cells were found to be lineage-negative CD45$^+$ Thy1$^+$ ILC3s (Fig. 3C). To confirm these observations, we examined IL-22 production in mice deficient in these cell types 48 h after *A. fumigatus* exposure. Surprisingly, despite the identification of iNKT cells as a cell source of IL-22 (Fig. 1A), *Cd1d*$^{-/-}$ mice did not demonstrate a reduction in IL-22 production 48 h postchallenge (Fig. 3D). However, *Cd1d*$^{-/-}$ mice did have a significant reduction (~35%) in IL-22 production at 24 h (Fig. 3D). In contrast, *Tcrd*$^{-/-}$ mice, deficient in γδ T cells, did not demonstrate a significant decrease in IL-22 production at 24 h post-*A. fumigatus* exposure but had an ~80% reduction in IL-22 at 48 h (Fig. 3E). Thus, IL-22 is produced in the lung by multiple innate and innate-like lymphocyte populations in response to fungal exposure, with iNKT cells being an early producer of IL-22 and γδ T cells being a later IL-22 producer.

Common γ-chain cytokines have differential effects on lung IL-22 cell sources after acute *A. fumigatus* exposure. In the above studies, lung IL-22 production was found to be completely dependent on IL-7, partially dependent on IL-21, and negatively regulated by IL-15 signaling. Therefore, we determined the effects of these common γ-chain cytokines on the level of IL-22 cell sources (iNKT cells, γδ T cells, and ILC3s) in the lung. The profound reduction in IL-22 production in *Il7*$^{-/-}$ mice correlated with an ~3-fold reduction in the absolute numbers of iNKT cells (Fig. 4A) and γδ T cells (Fig. 4B) in the lung. Although trending lower, the absolute numbers of ILC3s were not significantly reduced (Fig. 4C). Unlike the near complete absence of IL-22 in *Il7*$^{-/-}$ mice, *Il21*$^{-/-}$ mice exposed to *A. fumigatus* had an ~35% reduction in IL-22, which correlated with an ~2-fold reduction in the absolute numbers of iNKT cells in the lung (Fig. 4D), yet paradoxically an ~2-fold increase in the numbers of γδ T cells (Fig. 4E). ILC3s were also reduced by ~2-fold (Fig. 4F) (45). In direct contrast to *Il7*$^{-/-}$ mice, *Il15r*$^{-/-}$ mice exposed to *A. fumigatus* had a dramatically higher production of IL-22 by lung digest cells at 48 h postchallenge. Surprisingly, the absolute numbers of iNKT cells (Fig. 4G), γδ T cells (Fig. 4H), and ILC3s (Fig. 4I) were not significantly different. Collectively, these data demonstrate that IL-7 is crucial for maintaining iNKT cells and γδ T cells in the lung after fungal exposure, whereas IL-21 is required for maintaining optimal numbers of iNKT cells and ILC3s but appears to be a negative regulator of γδ T cells. In contrast, IL-15R signaling serves as a negative regulator of IL-22 production during lung fungal infection, although the mechanism associated with this is not an increased number of IL-22 cell sources.

iNKT cells and γδ T cells produce more IL-22 per cell in the absence of IL-15 signaling after acute *A. fumigatus* exposure. We next questioned whether there was a difference in the total number of IL-22-producing cells or a difference in a specific population of IL-22-producing cells in *Il15r*$^{-/-}$ mice. Intracellular staining for IL-22 in *Il15r*$^{-/-}$ mice showed no difference in the total number of IL-22-producing cells

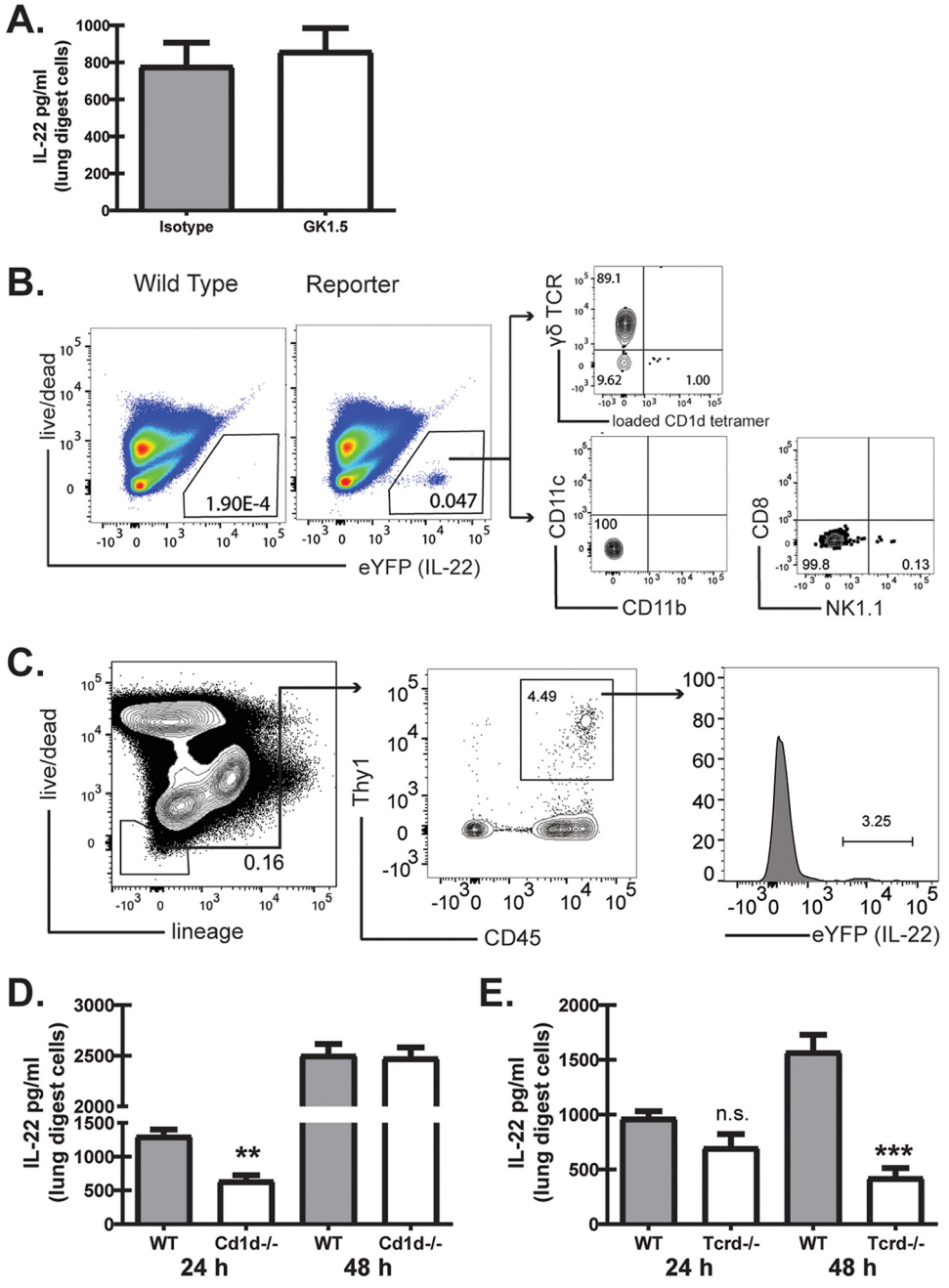

FIG 3 Multiple innate and innate-like lymphocytes produce IL-22 during and after acute *A. fumigatus* exposure. (A) C57BL/6 wild-type mice were administered anti-CD4 depleting antibody GK1.5 or an isotype control 1 day prior to and 1 day after *A. fumigatus* challenge. At 48 h postchallenge, the right lungs were collected and enzymatically digested, and unfractionated lung cells were cultured in triplicate for 24 h. IL-22 levels were quantified in clarified coculture supernatants by ELISA. Cumulative data from three independent experiments ($n = 2$ to 3 mice per group per experiment) are shown. (B) Representative plots from IL-22^{Cre} R26R^{eYFP} and WT mice 48 h after *A. fumigatus* challenge showing live eYFP$^+$ lung digest cells. From the eYFP$^+$ cells, iNKTs were defined as CD1d tetramer$^+$, and γδ T cells were defined as γδ TCR$^+$. CD11b$^+$ CD11c$^+$ and CD11b$^+$ CD11c$^+$ cells were negative for YFP/IL-22, as were CD8$^+$ cells. (C) ILC3s were defined as lineage negative (gate includes CD3, CD4, CD8a, CD11b, CD11c, CD19, Ly6G, NK1.1, FcγR1, αβ TCR, KLRG1, and NKp46), CD1d tetramer$^-$, γδ TCR$^-$, CD4$^-$, CD45$^+$, and Thy1$^+$. (D) C57BL/6 wild-type (WT) and *Cd1d*$^{-/-}$ mice were challenged intratracheally with *A. fumigatus* conidia, and 24 and 48 h after exposure, the right lungs were collected and enzymatically digested and unfractionated lung cells were cultured in triplicate for 24 h. IL-22 levels were quantified in clarified coculture supernatants by ELISA. Representative data from one of two experiments ($n = 3$ mice per group per experiment) are shown. (E) WT and *Tcrd*$^{-/-}$ mice were challenged and lung cells isolated as described for panel B. IL-22 levels were quantified in clarified coculture supernatants by ELISA. Representative data from one of two experiments ($n = 3$ mice per group per experiment) are shown. For all graphs, ** and *** represent $P < 0.01$ and $P < 0.0001$, respectively.

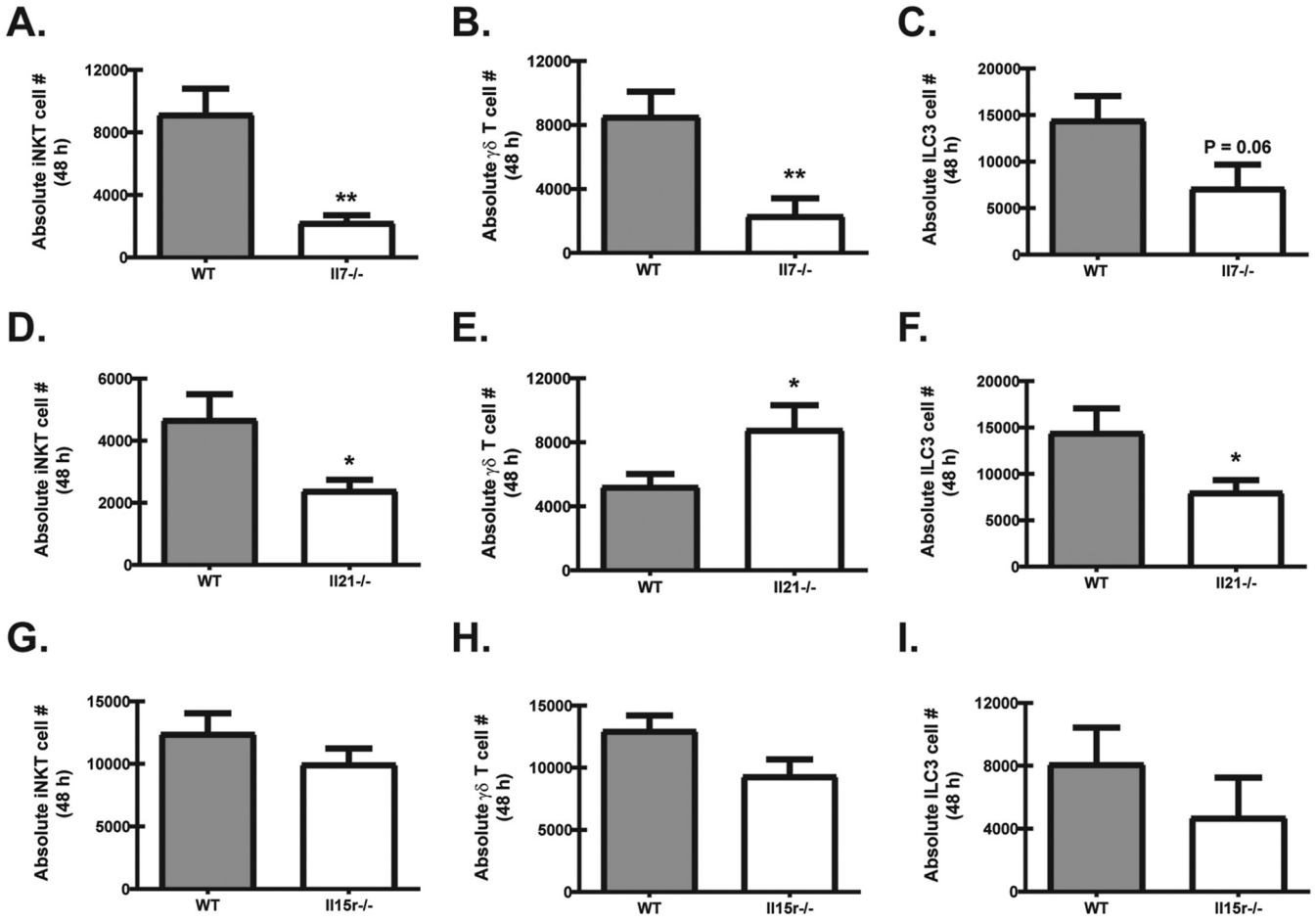

FIG 4 Common γ-chain cytokines have differential effects on lung IL-22 cell sources after acute *A. fumigatus* exposure. WT and *Il7*$^{-/-}$ mice (A to C), WT and *Il21*$^{-/-}$ mice (D to F), and WT and *Il15r*$^{-/-}$ (G to I) mice were challenged intratracheally with *A. fumigatus* conidia, and 48 h after exposure, the right lungs were collected and enzymatically digested, and IL-22-producing cell populations were enumerated. (A, D, and G) iNKTs were defined as CD19^{-} TCRβ^{-} CD1d tetramer^{-}. (B, D, and H) γδ T cells were defined as CD19^{-} CD4^{-} CD3^{+} γδ TCR^{+}. (C, F, and I) ILC3s were defined as Lin^{-} CD45^{+} Thy1^{+} KLRG1^{-}. Cumulative data from three independent experiments (n = 2 to 3 mice per group per experiment) are shown. For all graphs, * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

compared to the number for WT mice (Fig. 5A). Furthermore, we were unable to identify a significant difference in the populations of IL-22^{+} iNKTs (Fig. 5B), IL-22^{+} γδ T cells (Fig. 5C), or IL-22^{+} ILC3s (Fig. 5D) between *Il15r*$^{-/-}$ and WT mice. As iNKT cells, γδ T cells, and ILC3s were not increased in the lungs of *Il15r*$^{-/-}$ mice, yet IL-22 production was significantly higher, we determined whether these cell types produced more IL-22 on a per cell basis. For this, lung digest cells were collected 48 h post-*A. fumigatus* exposure and cultured in the presence of IL-23, followed by permeabilization and staining for intracellular IL-22. This analysis revealed that iNKT cells (Fig. 5E, representative flow; Fig. 5H, geometric mean) and γδ T cells (Fig. 5F, representative flow; Fig. 5I, geometric mean) had higher levels of intracellular IL-22 in *Il15r*$^{-/-}$ mice than WT mice. In contrast, there was no difference in IL-22 production by ILC3s between WT and *Il15r*$^{-/-}$ mice (Fig. 5G, representative flow; Fig. 5J, geometric mean). Thus, IL-15 signaling regulates IL-22 production by iNKT cells and γδ T cells, but not ILC3s, during lung fungal infection.

DISCUSSION

Therapeutics such as granulocyte-macrophage colony-stimulating factor (GM-CSF; sargramostim [Leukine]) and gamma interferon (IFN-γ; Actimmune) are FDA-approved treatments for immunosuppression associated with bone marrow transplantation and for combatting infectious complications associated with chronic granulomatous dis-

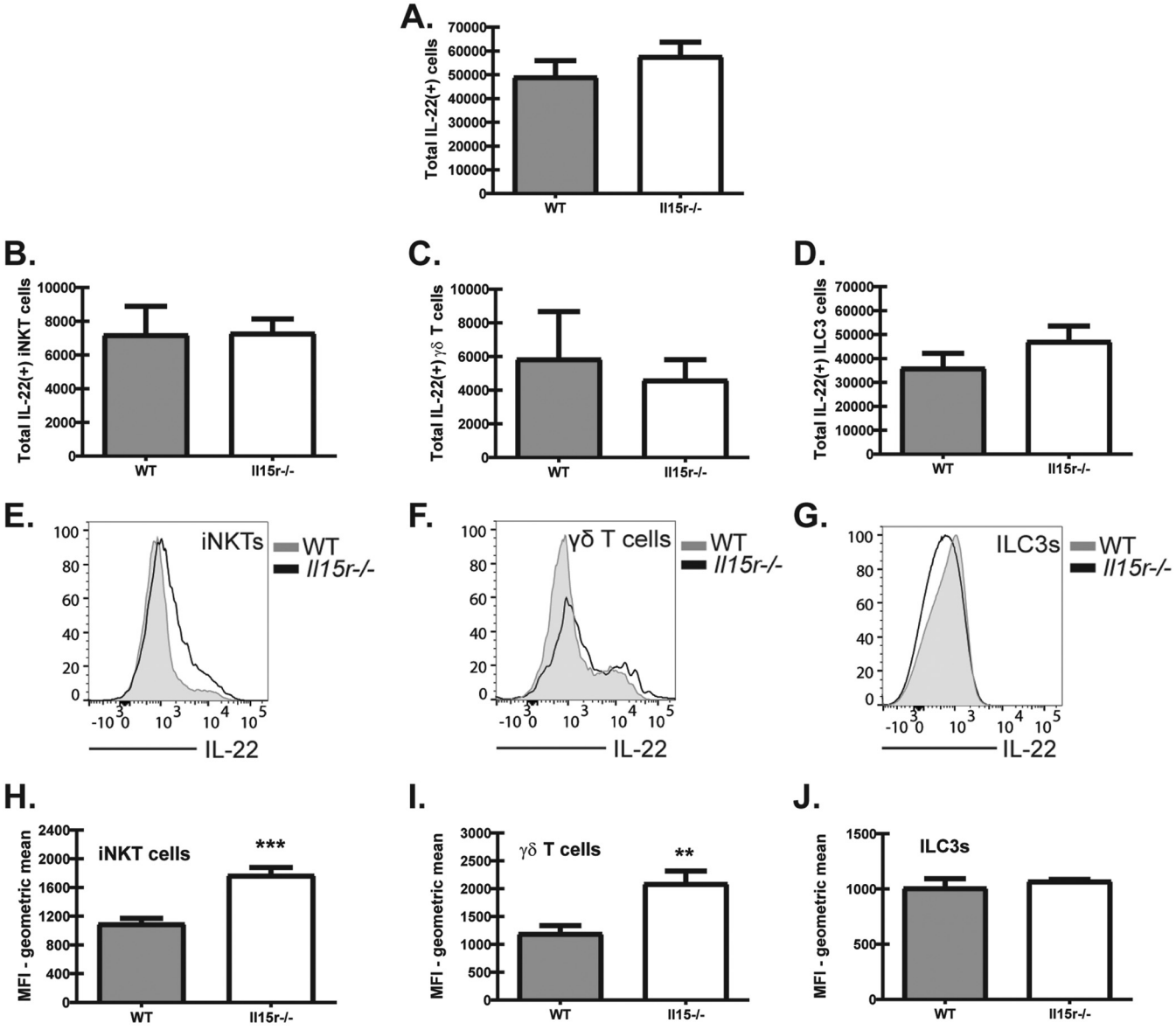

FIG 5 iNKT cells and γδ T cells produce more IL-22 per cell in the absence of IL-15 signaling after acute *A. fumigatus* exposure. (A to D) WT and *Il15r*$^{-/-}$ mouse whole-lung digests were stimulated with IL-23 (4 ng/ml) for 4 h before surface staining, permeabilization, and intracellular staining for IL-22. Populations were defined as described in Materials and Methods. (A) Total IL-22-producing cells enumerated 48 h postexposure by flow cytometry in whole-lung digests from WT and *Il15r*$^{-/-}$ mice. Cumulative data from two independent experiments ($n = 2$ to 3 mice per group per experiment) are shown. (B) Total IL-22-producing iNKT cells enumerated 48 h postexposure by flow cytometry in whole-lung digests from WT and *Il15r*$^{-/-}$ mice. Cumulative data from two independent experiments ($n = 2$ to 3 mice per group per experiment) are shown. (C) Total IL-22-producing γδ T cells enumerated 48 h postexposure by flow cytometry in whole-lung digests from WT and *Il15r*$^{-/-}$ mice. Cumulative data from two independent experiments ($n = 2$ to 3 mice per group per experiment) are shown. (D) Total IL-22-producing ILC3s enumerated 48 h postexposure by flow cytometry in whole-lung digests from WT and *Il15r*$^{-/-}$ mice. Cumulative data from two independent experiments ($n = 2$ to 3 mice per group per experiment) are shown. (E to J) WT and *Il15r*$^{-/-}$ mice were exposed to live *A. fumigatus* conidia via intratracheal administration and sacrificed 48 h postexposure. Cells were enumerated and then stimulated with IL-23 (4 ng/ml) for 4 h before surface staining, permeabilization, and intracellular staining for IL-22. (E to G) Histograms from concatenated samples representing each innate IL-22 cell population. Data are representative of two independent experiments. Gray, WT; black, *Il15r*$^{-/-}$. (H to J) Mean fluorescence intensity (MFI) quantified for iNKTs, γδ T cells, and ILC3s in WT and *Il15r*$^{-/-}$ mice. Cumulative data of two independent experiments ($n = 3$ mice per group per experiment) are shown. For all graphs, ** and *** represent $P < 0.01$ and $P < 0.0001$, respectively.

ease (CGD) (46, 47). Development of these therapies could not have been possible without a better understanding of innate immune mechanisms that are protective against invasive fungal infections. We have previously reported that IL-22 deficiency or neutralization leads to impaired lung clearance of *A. fumigatus* (25). Lack of IL-22 resulted in impaired inflammatory cytokine and chemokine production and impaired antifungal activity of lung lavage fluid (25). The purpose of the current study was to

identify the cell sources of IL-22 and to clarify mediators that may positively or negatively affect these IL-22 cell sources.

To date, CD4 T cells, CD8 T cells, iNKT cells, γδ T cells, and ILC3s have been identified as cell sources of IL-22 (reviewed in reference 48). In addition, neutrophils have been identified as an IL-22 cell source in the gut during experimental colitis (49). Finally, IL-23R reporter mice identified a $CD11b^+$ $CD11c^+$ cell population that produced IL-22 during experimental autoimmune encephalomyelitis (50). Collectively, these studies document the diversity of IL-22 cell sources at multiple sites. We have previously shown that IL-22 protein levels in lung digest cell cultures replicate those observed in whole-lung homogenates (25). Data presented here now show that IL-22 is not detected in or produced by lung digest cell cultures until 24 h post-*A. fumigatus* challenge, which increases at 48 h and begins to decline by 72 h. Based on this observation, we hypothesized that an innate-like lymphocyte or a myeloid cell population was responsible for IL-22 production. γδ T cells have previously been reported to produce IL-17A during *A. fumigatus* exposure, and mice deficient in these cells were susceptible to infection (51). However, this study failed to determine whether γδ T cells also produced IL-22. iNKT cells are thought to be a primary source of IFN-γ during *A. fumigatus* exposure, although small populations may produce IL-17A (52), a phenotype that is associated with reduced fungal clearance in iNKT cell-deficient mice. Here again, IL-22 levels were not investigated. In human peripheral blood mononuclear cells (PBMCs) stimulated with *A. fumigatus*, the primary source of IL-22 has been reported to be $CD4^+$ T cells, with these cells producing IL-22 alone or double-producing IL-22 and either IL-17A or IFN-γ (53). This study however did not further examine other non-$CD4^+$ cell populations, despite showing that ~10% of IL-22 was produced by a non-$CD4^+$, non-$CD8^+$, non-$CD56^+$ population. In the current study, we employed IL-22^{Cre} R26R^{eYFP} fate reporter mice, which have previously demonstrated that the majority of IL-22^+ cells in the naive lung are γδ T cells (~65%), followed by $CD4^+$ cells (~20%) and ILC3s (~10%) (44). At 48 h post-*A. fumigatus* exposure, we observed three YFP^+ cell populations that we subsequently identified as iNKT cells, γδ T cells, and ILC3s. We did not observe $CD11b^+$ or $CD11c^+$ cells that were also YFP^+. An intriguing finding from our work is the observation that iNKT cells appear to be the "first line" of IL-22 production, as mice deficient in iNKT cells had a blunted IL-22 response 24 h after challenge but intact IL-22 48 h after challenge. In contrast, mice deficient in γδ T cells had the opposite phenotype, in that these cells were more critical for IL-22 production 48 h after exposure. Although we are unaware of mice with a specific deletion of ILC3s, we speculate that these cells produce IL-22 at both time points.

IL-7 is a well-studied common γ-chain cytokine with a known role in the development, homeostasis, or expansion of many cell types that also produce IL-22 (36, 37, 54–56). Previous studies have shown that IL-7 is required for the development of γδ T cells in multiple peripheral and mucosal tissues (57). In contrast, although numbers of iNKT cells in peripheral lymphoid tissues are reduced in *Il7ra*$^{-/-}$ mice, they are not completely absent, implicating a role for IL-7 in iNKT cell expansion rather than in their development (58). Likewise, a recent study has shown that ILC3s in the small intestine lamina propria are lower in number in *Il7ra*$^{-/-}$ mice yet are competent to produce IL-22 during *Citrobacter rodentium* infection (59). Moreover, our data indicate that IL-7 was required for optimal levels of iNKT cells and γδ T cells in the lung during acute fungal exposure, with each population reduced by 3-fold in *Il7*$^{-/-}$ mice. In contrast to *C. rodentium* infection (59), however, IL-7 was absolutely required for IL-22 production, as lung cells from *Il7*$^{-/-}$ mice produced less than 10% of that observed for WT mice. These results are in line with reports documenting IL-7 involvement in IL-17A production in iNKT (58) and γδ T cell *in vitro* cultures (60).

IL-21 is recognized as a critical mediator driving effector and memory CD8 T cell responses (61) and T follicular helper cell differentiation (62) and as an autocrine signal for CD4 T cells producing IL-17 and IL-22 (63). Regarding iNKT cells, IL-21 may be produced by, as well as activate, iNKT cells (64). Support for IL-21 in iNKT cell maintenance comes from studies in humans showing that a loss-of-function mutation in *Il21r*

results in blunted iNKT cell numbers in peripheral blood (65). Our data also support a role for the IL-21/IL-21R axis in maintaining iNKT cell levels in the lung, as these cells were reduced by a little more than half after acute fungal exposure. γδ T cells may also be a source of IL-21 in response to IL-1β and IL-23 stimulation (66). However, mice deficient in IL-21R have an increase in IL-17A-producing γδ T cells during influenza lung infection (67). We extend this observation here by demonstrating that *Il21*$^{-/-}$ mice also have increased numbers of γδ T cells in the lung during acute fungal exposure. To our knowledge, however, we are the first to demonstrate that optimal ILC3 levels in the lung during infection require IL-21. The finding that IL-22 is only partially reduced during IL-21 deficiency is putatively due to higher γδ T cell numbers (some of which are producing IL-22) in the presence of the lower numbers of iNKT cells and ILC3s.

Homeostasis of peripheral iNKT cells was previously found to require IL-15 (68), although IL-15 is dispensable for the development of IL-17A-producing iNKT cells in the spleen (58). IFN-γ-producing, but not IL-17A-producing, splenic γδ T cells require IL-15 (58). However, data suggest that IL-17A-producing γδ T cells in lymph nodes are restricted by IL-15R signaling (45), although some reports suggest a role for IL-15 in γδ T cell development in the lymph nodes (36) and skin (69). IL-15 was recently demonstrated to sustain IL-7R-independent small intestine lamina propria ILC3 development (59). However, IL-15 may function in ILC survival in general, as reports suggest a role for IL-15 in intestinal ILC1 and ILC2 differentiation (70). Overall, these observations suggest that the role of IL-15 is diverse based on the type of cell and its cytokine-producing phenotype. In our study, the absence of IL-15 signaling (i.e., IL-15R deficiency) did not affect the numbers of iNKT cells, γδ T cells, and ILC3s in the lung after fungal exposure, suggesting that in a mucosal tissue (lung) during an infectious process (fungal exposure), IL-15 is not essential for the presence of these cell types. Rather, the lack of IL-15 signaling in iNKT cells and γδ T cells resulted in higher IL-22 production. In contrast to findings for the small intestine lamina propria during *C. rodentium* infection (59), IL-15 signaling did not positively or negatively affect IL-22 production by ILC3s.

The receptor for IL-1 was originally shown to have little to no role in *A. fumigatus* host defense (71); however, renewed interest in the IL-1 family of cytokines has uncovered novel roles for IL-1α and IL-1β in early neutrophil recruitment and macrophage antifungal responses (41). More recently, IL-1α was required for the elimination of highly virulent, highly germinating *A. fumigatus* strains (42). We have previously demonstrated that IL-1α is dependent on IL-22 after *A. fumigatus* lung exposure (25). However, we have also recently reported that IL-22 production is highly dependent on IL-1R signaling (43, 72). Therefore, IL-1α and IL-1β exist as both upstream promoters of IL-22 production and downstream effectors of IL-22-mediated antifungal defense. Our data here demonstrate that IL-1α and IL-1β were increased in the absence of the common γ-chain cytokine IL-7, which we hypothesize is a result of increased fungal burden in these mice rather than a direct effect of IL-7 on their production. Nevertheless, as neutrophil recruitment was intact in *Il7*$^{-/-}$ mice, this suggests that susceptibility to *A. fumigatus* is a specific result of impaired IL-22-induced soluble antifungal activity. It is not clear why IL-1α was lower in the absence of IL-21, as fungal burden was also increased in *Il21*$^{-/-}$ mice, and in theory, so might IL-1α. However, IL-1α was recently reported to be primarily produced by radiosensitive myeloid cells, including macrophages (42). As macrophages express the receptor for IL-21 (73), it is possible that the lack of this common γ-chain cytokine directly affects the production of IL-1α by this cell type. Future studies will probe the relationship between IL-21 and IL-1α generation. IL-1α was observed to be increased in the absence of IL-15R, and we speculate that this is a result of the increased IL-22 response.

In summary, we have shown that IL-22 is primarily produced by three innate-like lymphocyte subsets, iNKT cells, γδ T cells, and ILC3s, in the lung after acute fungal exposure. The common γ-chain cytokine IL-7 was indispensable for IL-22 production, whereas the common γ-chain cytokine IL-21 was only partially required for IL-22 production. Signaling through the receptor for the common γ-chain cytokine IL-15 had

a profound negative effect on IL-22 production. Collectively, these results provide insight into the parameters of IL-22 induction in the lung during fungal exposure.

MATERIALS AND METHODS

Mice. WT BL/6 mice, 6 to 8 weeks of age, were obtained from The Jackson Laboratory (Bangor, ME) or Taconic (Hudson, NY). *Il7*$^{-/-}$ mice were a kind gift from Chris Klug (UAB). *Il21*$^{-/-}$ mice were provided in collaboration with Allan Zajac (UAB). *Cd1d*$^{-/-}$, *Tcrd*$^{-/-}$, *Il15r*$^{-/-}$ B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$/J and C57BL/6-*Il22*$^{tm1.1(icre)Stck}$/J mice (IL-22 reporter mice, which were developed using a sequence encoding Cre recombinase cloned into the *Il22* locus and crossed with reporter mice expressing enhanced yellow fluorescence protein [eYFP] under the control of the endogenous Rosa26 promoter [44]) were obtained from The Jackson Laboratory. All animals were housed in a specific-pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care-certified facility and handled according to Public Health Service Office of Laboratory Animal Welfare policies after review by the UAB Institutional Animal Care and Use Committee.

Preparation of *A. fumigatus*, *in vivo* challenge, and lung fungal burden assessment. *A. fumigatus* isolate 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5 to 7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile phosphate-buffered saline supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40-μm nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. For challenge, mice were lightly anesthetized with isoflurane and administered 7×10^7 A. *fumigatus* conidia in a volume of 50 μl intratracheally as previously described (24, 25). Briefly, mice are held in a vertical, upright position, and the tongue is withdrawn from the mouth using forceps. A pipette is used to deliver the 50-μl inoculum to the caudal oropharynx in which normal breathing results in fluid aspiration into the lungs (74). For lung fungal burden analysis, the left lungs were collected at 48 h postexposure and homogenized in 1 ml of phosphate-buffered saline (PBS). Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI), which includes a DNase treatment step to eliminate genomic DNA as previously reported (75). Total RNA was also extracted from serial 1:10 dilutions of live *A. fumigatus* conidia (10^1 to 10^9) and DNase treated to form a standard curve. The lung *A. fumigatus* burden was analyzed with a real-time PCR measurement of the *A. fumigatus* 18S rRNA (76) and quantified using a standard curve of *A. fumigatus* conidia, as previously described (75). As a validation of the real-time PCR method, heat-killed *A. fumigatus* conidia did not yield a signal by real-time PCR and were unable to grow on potato dextrose agar plates (75). In addition, no amplification controls (i.e., no reverse transcriptase included in the cDNA reaction) yielded a signal of $<$0.001% by real-time PCR, indicating that the DNase treatment step efficiently eliminated contaminating *A. fumigatus* DNA (as DNA is not predicative of organism viability [77]).

Lung cell isolation, flow cytometry, and analysis of IL-22. Mice were anesthetized with intraperitoneal ketamine/xylazine and sacrificed by exsanguination 12, 24, or 48 h postinfection. Both lungs were collected and minced in IMDM medium (Sigma, St. Louis, MO) supplemented with 1% pen-strep-glut (Mediatech, Herndon, VA), 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), and 0.4 mg/ml polymyxin B (Thermo Fisher), followed by incubation for 50 min with tissue culture-grade type IV collagenase (1 mg/ml; Sigma, St. Louis, MO) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through a sterile 70-μm nylon filter, and red blood cells were lysed with ACK buffer and finally filtered through sterile 40-μm nylon (Lonza, Walkersville, MD) to create lung cell preparations. For assessing IL-22 production, cells were enumerated on a hemacytometer and plated at 1×10^6 cells in a volume of 0.2 ml. Supernatants were collected after 24 h, clarified by centrifugation, and stored at −80°C. IL-22 levels were quantified in supernatants by ELISA (R&D Systems) (24). For intracellular IL-22 staining, lung digest cells were stimulated with 4 ng/ml IL-23 (R&D Systems) for 4 h, and brefeldin A (eBioscience) was then added per the manufacturer's instruction for an additional 3 h. For surface and intracellular staining, cells were incubated with Mouse BD Fc Block (clone 2.4G2; BD Pharmingen), followed by viability assessment (LIVE/DEAD fixable aqua; Life Technologies). Cell populations were identified with a combination of the following markers: anti-CD45R allophycocyanin (APC)–Cy7 (cloneRA3-6B2; BioLegend), anti-CD4 fluorescein isothiocyanate (FITC) (clone GK1.5; BioLegend), anti-CD3 phycoerythrin (PE) (clone17A2; BD Pharmingen), anti-TCRβ PE (clone H57-597; eBioscience), anti-$\gamma\delta$ TCR APC (clone eBioGL3; eBioscience), PBS 57 loaded CD1d tetramer APC (NIH tetramer core), anti-CD19 biotin (clone eBio1D3; eBioscience), anti-CD3 biotin (clone 17A2; eBioscience), anti-CD4 biotin (clone GK1.5; eBioscience), anti-CD8a biotin (clone 53-6.7; eBioscience), anti-$\gamma\delta$ TCR biotin (clone UC7-13DS; eBioscience), anti-CD11b biotin (M1/70; eBioscience), anti-CD11c biotin (clone N418; eBioscience), anti-Ly6G biotin (clone RB6-8C5; eBioscience), anti-FcεR1 biotin (clone MAR1; eBioscience), anti-NK1.1 biotin (clone PK136; eBioscience), anti-KLRG1 biotin (clone2F1; eBioscience), anti-CD127 PE (clone A7R34; eBioscience), anti-Thy1.2 PE–Cy7 (clone 53-2.1; BD Pharmingen), anti-CD45 BV785 (clone 30-F11; BioLegend), anti-RORγt BV421 (cloneQ31-378; BD Horizon), BV421 streptavidin, and/or BV650 streptavidin. After surface staining for appropriate cell markers, cells were fixed and permeabilized via the Foxp3/transcription factor staining buffer set (eBioscience) and stained for IL-22 PE (clone 1H8PWSR; eBioscience). All staining was carried out at 4°C. Data were acquired using a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). Data acquisition and analysis were supported by the UAB Rheumatic Disease Core Center NIH P30 AR048311.

Statistics. Data were analyzed using GraphPad Prism version 5.0 statistical software (GraphPad Software, San Diego, CA). Comparisons between groups when data were normally distributed were made with the two-tailed unpaired Student *t* test. Significance was accepted at a *P* value of $<$0.05.

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