Activation of Type 3 Innate Lymphoid Cells and Interleukin 22 Secretion in the Lungs During *Streptococcus pneumoniae* Infection

Laurye Van Maele,[1,2,3,4] Christophe Carnoy,[1,2,3,4] Delphine Cayet,[1,2,3,4] Stoyan Ivanov,[1,2,3,4] Rémi Porte,[1,2,3,4] Emeric Deruy,[1,2,3,4] José A. Chabalgoity,[9] Jean-Christophe Renauld,[10,11] Gérard Eberl,[5,6] Arndt G. Benecke,[7,8] François Trottein,[1,2,3,4] Christelle Faveeuw,[1,2,3,4] and Jean-Claude Sirard[1,2,3,4]

[1]Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille, [2]Institut National de la Santé et de la Recherche Médicale, U1019, [3]Centre National de la Recherche Scientifique, UMR 8204, and [4]Univ Lille Nord de France, Lille, [5]Lymphoid Tissue Development Unit, Institut Pasteur, and [6]Centre National de la Recherche Scientifique, URA 1961, Paris, and [7]Institut des Hautes Études Scientifiques and [8]Centre National de la Recherche Scientifique, Bures-sur-Yvette, France; [9]Laboratory for Vaccine Research, Department of Biotechnology, Instituto de Higiene, Facultad de Medicina, Universidad de la Republica, Montevideo, Uruguay; and [10]Ludwig Institute for Cancer Research, Brussels Branch, and [11]de Duve Institute, Université catholique de Louvain, Brussels, Belgium

Mucosal sites are continuously exposed to pathogenic microorganisms and are therefore equipped to control respiratory infections. Type 3 innate lymphoid cells (ILC3) are key players in antimicrobial defense in intestinal mucosa, through interleukin 17 and interleukin 22 (IL-22) production. The present study aimed at analyzing the distribution and function of ILC3 in the respiratory tract. We first observed that lung mucosa harbors a discrete population of ILC3 expressing CD127, CD90, CCR6, and the transcriptional factor RORγt. In addition, lung ILC3 were identified as a major source of IL-22 in response to interleukin 23 stimulation. During *Streptococcus pneumoniae* infection, ILC3 rapidly accumulated in the lung tissue to produce IL-22. In response to *S. pneumoniae*, dendritic cells and MyD88, an important adaptor of innate immunity, play critical functions in IL-22 production by ILC3. Finally, administration of the Toll-like receptor 5 agonist flagellin during *S. pneumoniae* challenge exacerbated IL-22 production by ILC3, a process that protects against lethal infection. In conclusion, boosting lung ILC3 might represent an interesting strategy to fight respiratory bacterial infections.

Keywords. Innate lymphoid cells; interleukin-22; Toll-like receptor 5; *Streptococcus pneumoniae*.

Mucosa are tailored to fight constant exposure to environmental insults such as allergens, pollutants, or microorganisms. Innate lymphoid cells (ILC) are prevalent at mucosal sites and represent a family of immune cells that promptly react in response to various stimuli, thereby playing an early crucial role in mucosal immunity. Currently, 3 ILC groups that parallel CD4^{+} T-helper lymphocyte subsets have been described [1]. ILC1 such as natural killer (NK) cells can produce large amounts of the T-helper type 1 (Th1) cytokine interferon γ (IFN-γ), which is critical for the eradication of tumor and virus-infected cells [2]. ILC2 secrete the Th2 cytokines interleukin 5 and interleukin 13 (IL-13) in response to interleukin 25 or interleukin 33 (IL-33) stimulation and are associated with mucosal defenses and repair mechanisms against helminths or during asthma [3, 4]. ILC3 are able to promptly produce the Th17/Th22 cytokines interleukin 17A (IL-17A), IL-17F, and interleukin 22 (IL-22) [5–8]. However, the role of ILC3 in the early regulation of antimicrobial functions, inflammation, and the maintenance of tissue homeostasis remains poorly understood in the respiratory tract.

ILCs are characterized by (1) the lack of expression of lineage (Lin) markers associated with T and B lymphocytes, monocytes/macrophages, neutrophils, and dendritic cells (DCs); (2) the independence of recombination activating gene (*Rag*); and (3) the requirement for the common cytokine receptor γ-chain (γc) [1].

DOI: 10.1093/infdis/jiu106

ILC3 are specifically defined by the expression of the nuclear hormone receptor RORγt (retinoid-related orphan receptor) that is mandatory for their development and function [9]. In addition, ILC3 express the interleukin 7 (IL-7) receptor (formed by the α-chain CD127 and the γc chain), CD90 (alloantigen Thy-1), and CCR6 (the receptor for the epithelial chemokine CCL20) but lack the NK marker NK1.1 [1, 10]. The ability of ILC3 to produce IL-17 and IL-22 is assumed to be the driving force for protection against mucosal infection [5–8, 11–14]. Indeed, IL-17 and/or IL-22 orchestrate inflammation and antimicrobial peptide production and thereby participate to mucosal defenses against the extracellular pathogens *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, or *Candida albicans* [15–18]. Receptors of IL-17 and IL-22 are essentially expressed on epithelial cells [17, 19]. Whereas both receptors enhance production of antimicrobial peptides like RegIIIγ or S100A9, as well as chemokines specific for phagocytes, only IL-22R signaling is associated with activation of tissue repair molecules that regenerate the mucosal epithelial barrier damaged during infections [13]. In response to pathogens and commensals, DCs secrete interleukin 23 (IL-23; and, in some conditions interleukin 1β [IL-1β]) to stimulate the production of IL-17 and IL-22 by ILC3 [20–22]. Recent studies in the mouse model showed that flagellin, the agonist for Toll-like receptor 5 (TLR5), enhances the production of IL-17 and IL-22 by ILC3 via DC activation in spleen and mucosa [20, 21]. Most studies on ILC3 were conducted on intestinal mucosa and tonsils, and the characterization of lung ILC3 remains poorly defined. Recent studies introduced some controversies regarding the presence and the function of ILC3 in the respiratory tract. Taube et al identified lung ILC3 producing IL-22 in a model of experimental asthma in mice [23]. In contrast, Sonnenberg et al found that lung Lin^neg cells that are stimulated by IL-23 do not produce IL-22 [4]. There is thus an urgent need to define the unappreciated impact of lung ILC3 on innate defenses and tissue repair in the context of respiratory infections.

Streptococcus pneumoniae is a gram-positive bacterium that colonizes the nasopharynx and causes respiratory and invasive infections. Immunity against pneumococcus strongly depends on innate defenses. Thus, signaling via TLR and the TLR-specific adaptor MyD88 is crucial to prevent pneumonia and invasive pneumococcal diseases in animal models and in humans [24, 25]. Moreover, several studies have reported that IL-17 is an important factor for the regulation of *S. pneumoniae* colonization and infection [26, 27]. Protection against infection has been characterized by the rapid recruitment of neutrophils into the airways or the prompt production of IL-17, IL-22, and IFN-γ [28–30].

In this study, we identified that lung mucosa harbors ILC3 at steady state that can be activated (via DCs and MyD88-dependent signaling) to produce IL-22 during acute pulmonary infection with *S. pneumoniae*. Furthermore, our results show that systemic treatment with flagellin overstimulates the production of IL-22 by lung ILC3 and that this activity is associated with protection against *S. pneumoniae* infection.

MATERIALS AND METHODS

Mice
Six-to-ten-week-old female wild-type C57BL/6J, *Myd88*^−/−^, *Il22*^−/−^ (ref JCR), *Rag2*^−/−^*Il2rg*^−/−^, *Rag2*^−/−^, *Rorc(γt)-Gfp*^TG^, or *Cd11c-DTR-EGFP* (*Itagx-DTR/EGFP*) (on C57BL/6J background) were purchased from Janvier or were bred and maintained in a specific pathogen-free or biosafety level 2 facility (protocol A59–350009, Institut Pasteur de Lille) [10, 21, 31, 32]. Depletion of CD11c^+^ cells was achieved by intraperitoneal injection of diphtheria toxin, as described elsewhere [21, 28]. All experiments complied with national and institutional regulations and ethical guidelines.

Bacteria
S. pneumoniae serotype 1 (clinical isolate E1586) were grown as described previously [28–30]. For infection, frozen working stocks were diluted in phosphate-buffered saline (PBS). Mice were anesthetized by intraperitoneal injection of ketamine-xylazine, and 20 μL of the inoculum were administered by intranasal route. For quantification of bacteria, lungs were collected 24 hours after infection and homogenized in PBS. Viable counts (colony forming unit [CFU]) were determined by plating serial dilutions onto 5% blood-agar plates.

Proteins
Native flagellin (FliC) from *Salmonella enterica* serovar Typhimurium was prepared as described previously [21]. Flagellin (5 μg in 200 μL of PBS) was injected by the intravenous or intraperitoneal route into mice. When indicated, mice were injected intravenously 5 minutes before infection with 250 μg of neutralizing anti-IL-22 (AM22) or with control isotype (AH953A; mouse IgG2a) antibodies [33].

Flow Cytometry Analysis
Lungs were digested with collagenase IA (Sigma, 1 mg/mL) and DNase I (Sigma, 40 μg/mL) for 10 minutes at 37°C. Cells were separated on Percoll 20% and stained for NKp46-, CD3-, NK1.1-, and Gr1–fluorescein isothiocyanate; CCR6-, B220-, CD11b-, and CD11c-phycoerythrin [PE]; NK1.1-, CD3-, Gr1–peridinin chlorophyll-Cy5.5; CD127-PE-Cy7; CCR6- and IL-22-allophycocyanin [APC]; CD4-APC-H7; CD90.2-AF700; or CD25-V450 (Becton Dickinson, Biolegend, and eBioscience). Cells were incubated 4 hours with brefeldin A (10 μg/mL) and processed for intracellular staining using the kit Intracellular Fixation and Permeabilization (eBiosciences) and IL-22–APC or control isotype as described elsewhere [32]. Data were collected on a BD LSR Fortessa and analyzed with BD FACSDiva software. When specified, cells were sorted on a BD FACSAria.

Gene Expression

Total RNA was extracted with Nucleospin kit (Macherey Nagel) and reverse transcribed with the High-Capacity complementary DNA (cDNA) Archive Kit (Applied Biosystems). cDNA from sorted cells was preamplified (12 cycles) before real-time polymerase chain reaction using the PreAmp kit (Applied Biosystems). cDNA was amplified using SYBR or TaqMan assays. Relative messenger RNA (mRNA) levels ($2^{-\Delta\Delta Ct}$) were determined as described previously [21].

Cytokines Production

The concentrations of IL-22, IL-13, IL-17A, and IL-17F were determined in serum, lung extracts, or cells supernatants by enzyme-linked immunosorbent assay (ELISA; eBioscience). Lung homogenates was prepared with T-PER Reagent (Pierce) supplemented with protease inhibitors (Roche).

In Vitro Assay

ILC were negatively enriched from lungs by magnetic sorting (BD Biosciences) with biotinylated antibodies specific for the Lin markers Ter-119, CD3, CD11b, GR1, B220, NK1.1, and CD11c. The enriched ILC fraction (Lin^{neg} cells) were cultured for 3 days in Roswell Park Memorial Institute 1640 medium with 5% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol, 100 U/100 μg penicillin-streptomycin, recombinant mouse IL-7 (10 ng/mL), and interleukin 2 (IL-2; 40 ng/mL) plus IL-33 (30 ng/mL) or IL-23 (10 ng/mL). Cytokines were measured on cell supernatants.
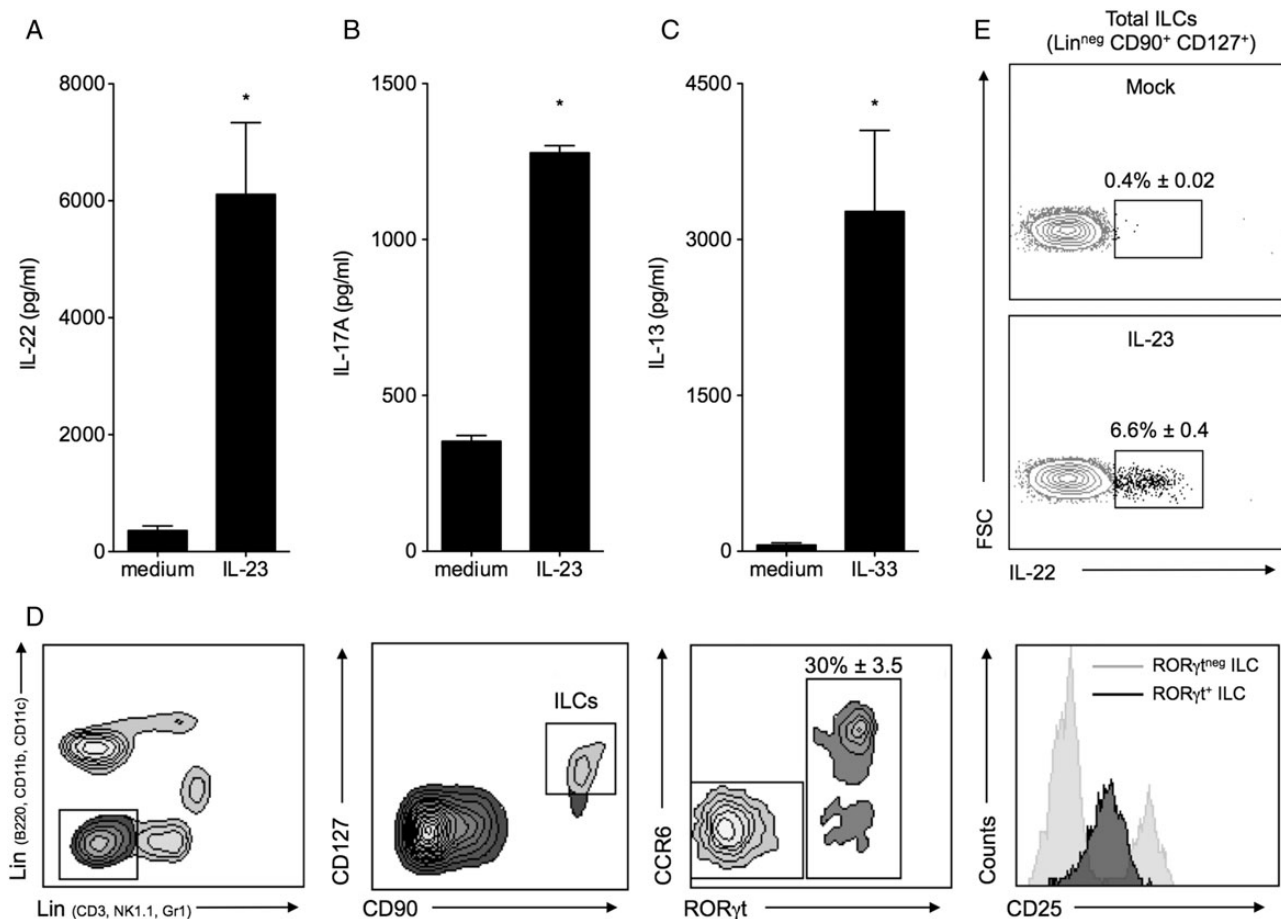

Figure 1. Type 3 innate lymphoid cells (ILC3) are present in lung. *A–C*, Production of interleukin 22 (IL-22), interleukin 17A (IL-17A), and interleukin 13 (IL-13) by lung lineage-negative (Lin^{neg}) cells. Lung Lin^{neg}-enriched cells from C57BL/6 mice (n = 3) were cultured for 3 days with interleukin 2 and interleukin 7, with or without interleukin 23 (IL-23; *A* and *B*) or interleukin 33 (IL-33) (*C*). IL-22, IL-17A, or IL-13 secretion was detected by enzyme-linked immunosorbent assay. Results are given as means ± standard errors of the mean (SEMs). Statistically significant differences from the medium group (*P < .05) were assessed by the Mann–Whitney test. *D*, Characterization of lung ILCs. Lung cells from *Rorc(gt)-Gfp^{TG}* mice (n = 3) were analyzed by flow cytometry. Lin^{neg} cells were selected with regard to lack of expression of CD3, NK1.1, and Gr1 (horizontal axis) and B220, CD11b, and CD11c (vertical axis). ILCs were defined as Lin^{neg}CD90$^+$CD127$^+$ cells and analyzed for expression of CCR6, RORγt, and CD25. *E*, Production of IL-22 by lung Lin^{neg}CD90$^+$CD127$^+$ ILCs. Lung cells from C57BL/6 mice were stimulated for 4 hours with IL-23 and brefeldin, and IL-22 production was assessed by intracellular cytokine staining. The percentage of cells (±SEM) expressing IL-22 is shown.

Conditioned Dendritic Cells

Bone marrow–derived DCs (BMDCs) from C57BL/6J or $Il22^{-/-}$ mice were differentiated with granulocyte macrophage colony-stimulating factor as described previously [28]. At day 14, BMDCs were infected for 1 hour with *S. pneumoniae* as described elsewhere [34] at a multiplicity of infection of 2 and were washed with medium containing antibiotics. Conditioned DCs were then cocultured 72 hours with enriched ILC, and supernatants were analyzed by ELISA.

Statistical Analysis

Results are expressed as means ± standard errors of the mean or medians. The statistical significance was assessed using the Mann–Whitney test (GraphPad Prism 5.0). *P* values of <.05 were considered statistically significant.

RESULTS

ILC3 Populate the Lung Tissue

To characterize ILC3 in the respiratory tract, lung Lin^{neg} cells were enriched by magnetic sorting and stimulated for 72 hours with IL-23. Stimulation by IL-23 triggered the production of IL-22 and IL-17A (Figure 1*A* and 1*B*). These data indicated that lung potentially contains ILC3. In addition, lung Lin^{neg} cells produced IL-13 in response to IL-33 (Figure 1*B*). This is consistent with a previous study demonstrating the presence of ILC2 in the lung [4]. Pulmonary Lin^{neg} cells were next characterized by flow cytometry. The various types of ILCs are known to lack expression of markers specific for T cells (CD3), B cells (B220), NK cells (NK1.1), neutrophils (Gr1), macrophages (CD11b) and DCs (CD11c). Among the Lin^{neg} population, lung cells expressing CD90 (alloantigen Thy-1) and CD127 (α chain of IL-7 receptor) were defined as ILCs and further analyzed for expression of other markers (Figure 1*C*). Among lung ILCs, approximately 30% expressed the transcriptional factor RORγt, the surrogate marker of ILC3. We also found that approximately 70% of RORγt-positive ILC3 coexpressed the CCR6 receptor. Remarkably, all $CCR6^+$ ILCs express RORγt, suggesting that all $Lin^{neg}CD90^+CD127^+CCR6^+$ are ILC3. We evaluated that the lung tissue, at steady state, contains approximately approximately 10 000 ILCs, including approximately 2000 ILC3. On the basis of CD25 expression, pulmonary ILCs were constituted of various populations, including $CD25^+$ ILCs that may correspond to the previously described ILC2 and $CD25^{low}$ that contained all RORγt-positive ILCs (Figure 1*C*). Since RORγt expression is associated with the capacity to produce Th17
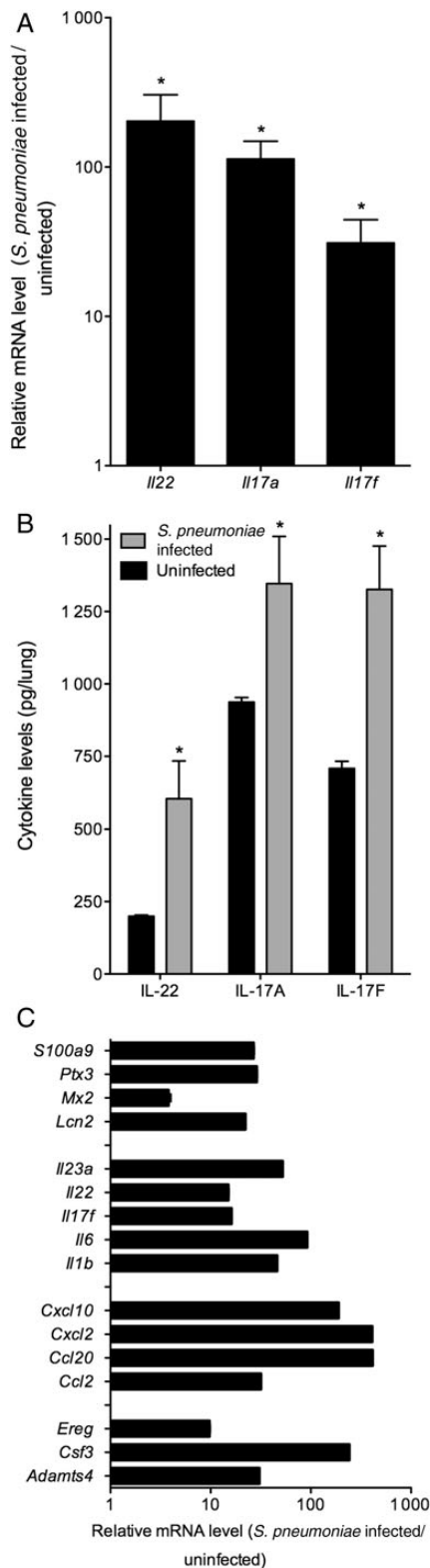
Figure 2. *Streptococcus pneumoniae* enhances production of interleukin 17 (IL-17) and interleukin 22 (IL-22). C57BL/6 mice (n = 3–5) were infected intranasally with 2×10^6 *S. pneumoniae*. Lungs were collected 12 hours (*C*) or 24 hours (*A* and *B*) after infection for analysis of transcript levels by quantitative polymerase chain reaction (*A* and *C*) or protein production by enzyme-linked immunosorbent assay (*B*). Transcriptional analysis at 24

Figure 2 continued. hours (*A*) or 12 hours (*C*). Results are given as means ± standard errors of the mean. Messenger RNA levels are expressed relative to those of the mock group, arbitrarily set to 1. Statistically significant differences from the mock group (**P*< .05) were assessed by the Mann–Whitney test.

cytokines, we addressed whether some Lin^{neg}CD90$^+$CD127$^+$ lung ILCs were functionally related to ILC3. To this end, IL-22–specific intracellular staining was performed. About 7% of the whole ILCs (Lin^{neg}CD90$^+$CD127$^+$) produced IL-22 in response to IL-23 stimulation in contrast to mock conditions, thereby suggesting that 35% ILC3 were activated (Figure 1D). These data demonstrated that lungs harbor ILC3 that express RORγt and CCR6 and promptly respond to IL-23 by secreting IL-17 and IL-22.

S. pneumoniae Enhances IL-17 and IL-22 Production in Lungs

Previous studies showed that *S. pneumoniae* promotes the early expression of Th17-related cytokines in the lung [29]. We investigated whether Th17 cytokines were produced by ILC3. Intranasal inoculation of a lethal dose of *S. pneumoniae* enhanced in the lung the transcription of *Il22*, *Il17a*, and *Il17f* genes 24 hours after infection (Figure 2A). The levels of IL-22, IL-17A, and IL-17F proteins were also increased in the lung tissue (Figure 2B). The production of IL-22 was detected in the serum at later times and correlated with translocation of bacteria in the systemic compartment (data not shown). *S. pneumoniae* infection also induced the swift expression of genes associated with IL-17R and IL-22R signaling pathways (Figure 2C). Indeed, genes encoding modulators of IL-17 and IL-22 responses (*Il1b*, *Il6*, and *Il23a*), antimicrobial molecules (*Lcn2*, *Ptx3*, and *S100a9*), tissue remodeling and differentiation factors (*Adamts4*, *Csf3*, and

Ereg), as well as chemokines (*Cxcl2*, *Cxcl10*, *Ccl2*, and *Ccl20*), were induced in the lung tissue early after infection. In addition, this IL-17/IL-22 signature induced by *S. pneumoniae* is MyD88 dependent (Supplementary Figure 1). Indeed, the lung mRNA levels of *Il22* and *Il17* genes were not upregulated at 6 hours in *Myd88$^{-/-}$* mice, in contrast to conventional animals (Supplementary Figure 1A). We next performed mouse whole-genome microarrays to dissect changes in MyD88-dependent lung gene expression. Analysis of differentially expressed genes revealed that the proinflammatory lung signature in response to infection was significantly impaired in *Myd88$^{-/-}$* animals for numerous genes (Supplementary Figure 1B). As described previously [25], *Myd88$^{-/-}$* mice were more susceptible to infection, because doses as low as 2×10^4 bacteria were lethal for >70% of animals, whereas conventional animals all survived. The transcript levels of target genes of Th17 cytokines were consistently lower in *Myd88$^{-/-}$* mice (Supplementary Figure 1C and 1D). Altogether, these data suggest that *S. pneumoniae* enhances IL-17 and IL-22 production in lungs in a MyD88-dependent manner.

S. pneumoniae Enhances IL-17 and IL-22 Production by Lung ILC3

Since NK cells, NK T cells, γδ T lymphocytes, and ILC3 are potential early producers of IL-22 [13], we addressed their respective contribution in IL-22 production after *S. pneumoniae* infection. To this end, the transcriptional response of infected
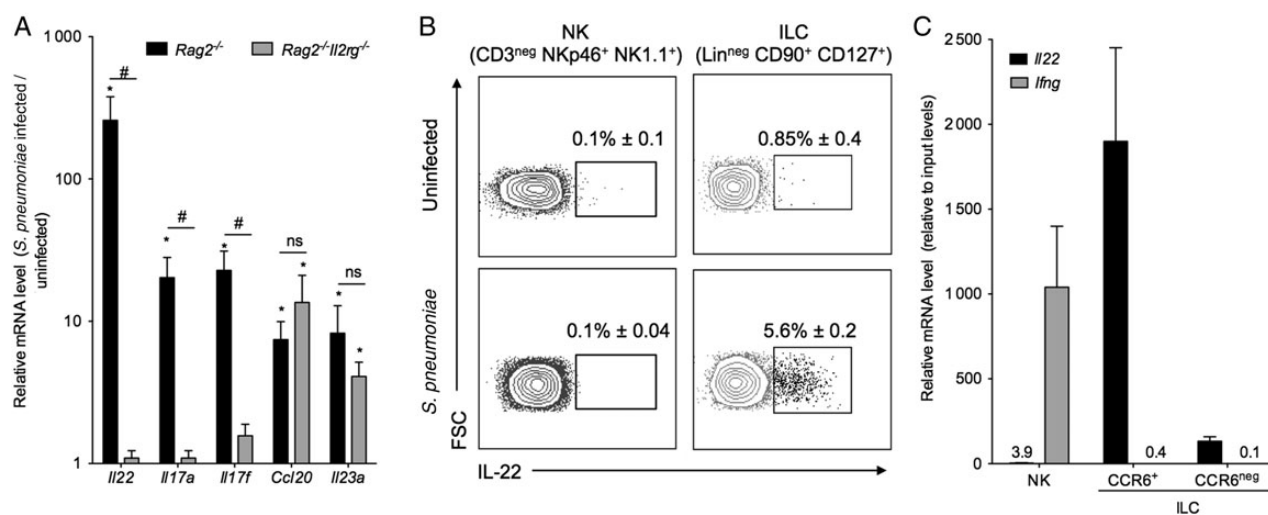

Figure 3. *Streptococcus pneumoniae* activate the production of interleukin 17 (IL-17) and interleukin 22 (IL-22) by type 3 innate lymphoid cells (ILC3). C57BL/6, *Rag2$^{-/-}$*, or *Rag2$^{-/-}$ Il2rg$^{-/-}$* mice (n = 3–5) were infected intranasally with 2×10^6 *S. pneumoniae*. Lungs were collected after infection for analysis of transcript levels by quantitative polymerase chain reaction or protein production by intracellular cytokine staining. *A*, Transcriptional analysis in *Rag2$^{-/-}$* and *Rag2$^{-/-}$ Il2rg$^{-/-}$* mice 24 hours after infection. Results are given as means ± standard errors of the mean (SEMs). Messenger RNA (mRNA) levels are expressed relative to those of the mock group, arbitrarily set to 1. Statistically significant differences from the mock group (*$P < .05$) or the *Rag2$^{-/-}$ Il2rg$^{-/-}$* group ($^\#P < .05$) were assessed by the Mann–Whitney test. *B*, IL-22 production by ILCs or natural killer (NK) cells. Lung cells from mock and infected C57BL/6 mice were sampled at 24 hours and incubated for 4 hours with brefeldin. ILCs (Lin^{neg}CD90$^+$CD127$^+$) and NK cells (CD3^{neg}NK1.1$^+$NKp46$^+$) were analyzed for IL-22 production by intracellular cytokine staining. The percentage of cells (±SEM) expressing IL-22 is shown. *C*, Transcriptional analysis of innate immune cells sorted from lungs 16 hours after infection. Lung NK cells (TCRβ^{neg}NK1.1$^+$) and ILCs (Lin^{neg}CD90$^+$CD127$^+$CCR6$^{+/-}$) were sorted for quantification of mRNA levels. mRNA levels are expressed relative to those of the input group, arbitrarily set to 1. Results are given as means ± SEMs.

$Rag2^{-/-}$ and $Rag2^{-/-}Il2rg^{-/-}$ mice was compared (Figure 3A). As observed in wild-type animals, $Rag2^{-/-}$ mice that lack conventional and $\gamma\delta$ T lymphocytes, as well as NK T cells, upregulated $Il22$ and $Il17a$ and $Il17f$ transcripts in response to infection. In contrast, their expression was profoundly impaired in $Rag2^{-/-}Il2rg^{-/-}$ animals that are also devoid of NK cells and ILCs. The unresponsiveness was not due to a general impairment of responses in $Rag2^{-/-}Il2rg^{-/-}$ mice, because expression of $Ccl20$ and $Il23a$, which are surrogate activation markers of epithelial cells and DCs, respectively, was equally induced in
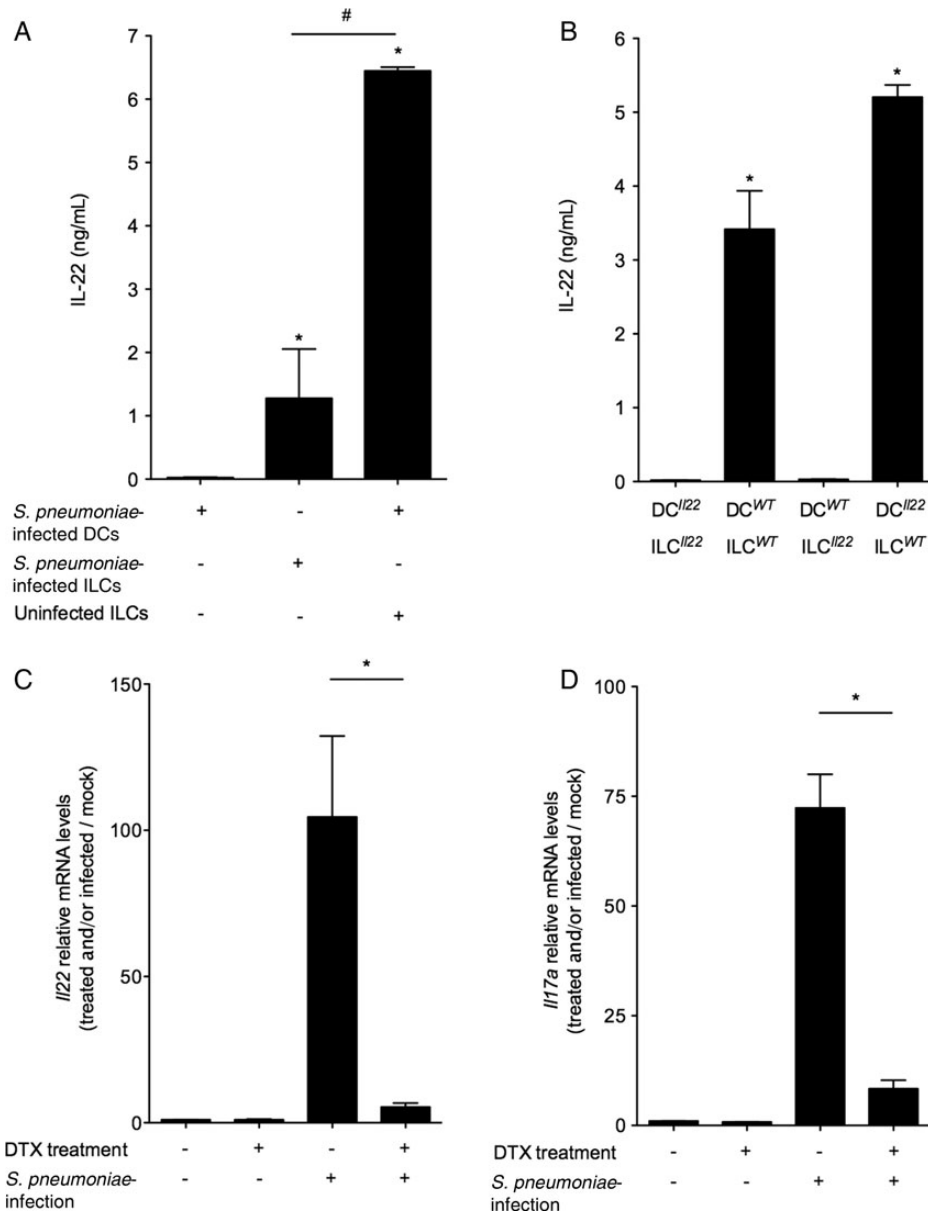
Figure 4. Dendritic cells (DCs) are required for pneumococcus-mediated innate lymphoid cell (ILC)–dependent interleukin 22 (IL-22) secretion. *A* and *B*, Bone marrow–derived DCs (BMDCs) and/or lung ILC were infected for 1 hour with *Streptococcus pneumoniae*, washed with antibiotics, and then cultured separately or together for 72 hours. ILCs were enriched from lung as lineage-negative cells, using magnetic beads. Production of IL-22 was determined in supernatants by enzyme-linked immunosorbent assay. *A*, Optimal IL-22 production requires both DCs and ILCs. Cells were derived or isolated from C57BL/6 (wild-type) mice. *B*, ILCs but not DCs are the source of IL-22 in coculture. Cells were derived or isolated from WT or $Il22^{-/-}$ mice. Statistically significant differences ($P < .05$) were assessed by the Mann–Whitney test compared to DC (*A*) and DC^{Il22}/ILC^{Il22} (*B*) group (*) or compared to infected ILCs (#). *C* and *D*, *Cd11c-DTR-EGFP* mice (n = 4) were injected intraperitoneally with 100 ng of diphtheria toxin (DTX) or untreated 24 hours before intranasal infection with 2×10^6 *S. pneumoniae*. Lungs were sampled after 24 hours for the quantification of transcripts specific for T-helper type 17–related cytokine *Il22* (*C*) and *Il17a* (*D*) genes. Messenger RNA (mRNA) levels are expressed relative to those of the mock group, arbitrarily set to 1. Statistically significant differences from the *S. pneumoniae*–infected group (*$P < .05$) were assessed by the Mann–Whitney test. Results are given as means ± standard errors of the mean.

$Rag2^{-/-}$ and $Rag2^{-/-}Il2rg^{-/-}$ mice. These results suggested that NK cells and/or ILCs are the main source of IL-22 in the context of infection. We next monitored the production of IL-22 by intracellular staining in lung cells isolated from wild-type animals (Figure 3B). ILCs ($Lin^{neg}CD90^+CD127^+$) but not NK cells ($CD3^{neg}NK1.1^+NKp46^+$) produced IL-22. In agreement with this finding, sorted NK cells did not express $Il22$ and $Il17a$ mRNA in response to S. pneumoniae but upregulated the level of $Ifng$ transcripts (Figure 3C and data not shown). In contrast, $CCR6^+$ ILCs upregulated transcription of $Il22$ but not $Ifng$.

We next examined whether S. pneumoniae directly activates ILC3 or whether respiratory DCs are required. BMDCs and ILCs (cells enriched by magnetic sorting in the Lin^{neg} population) were infected by S. pneumoniae and then cocultured to assess IL-22 production. As Figure 4A and 4B show, S. pneumoniae–infected DCs triggered the production of IL-22 by ILCs. ILCs were assigned as the source of IL-22 since DCs exposed to bacteria failed to produce IL-22. Moreover, when ILCs were isolated from $Il22^{-/-}$ mice and cocultured with S. pneumoniae–infected IL-22–proficient DCs, no IL-22 was detected (Figure 4B). We next performed diphtheria toxin treatment in Cd11c-DTR-EGFP animals to deplete selectively $CD11c^+$ cells before infection (Figure 4C and 4D). The depleted animals were significantly impaired in $Il17a$ or $Il22$ transcription in response to infection. In conclusion, S. pneumoniae infection activates lung ILC3 to produce IL-22 in a DC-dependent mechanism.

Flagellin Administration Exacerbates Activation of ILC3 During S. pneumoniae Infection

IL-17 and IL-22 were promptly induced by the infection, but levels were not sufficient to trigger antimicrobial defense. However, protection against pneumococcus is associated with the production of important levels of IL-17, IL-22, and IFN-γ [28, 29]. We hypothesized that flagellin administration to S. pneumoniae–infected mice might boost ILC3 and production of associated cytokines to confer some protection. Indeed, previous studies showed that systemic injection of flagellin activates ILCs and stimulates production of IL-22 and IL-17 in the respiratory and gut mucosa [20, 21]. Mice were intranasally infected with a lethal dose of S. pneumoniae and concomitantly treated with flagellin. Strikingly, flagellin treatment further increased S. pneumoniae–induced $Il22$ and $Il17a$ transcript levels (Figure 5A and 5B). Of importance, the transcriptional activation was not only exacerbated but also accelerated, with a response detected as soon as 2 hours after treatment. In parallel, genes with a Th17 signature, including those for chemokines and antimicrobial peptides, were significantly increased by flagellin treatment (Figure 5C). While S. pneumoniae infection resulted in a higher number of $CCR6^+RORγt^+$ ILC3s in the lung as compared to the mock group, flagellin treatment did not further increase the number of cells (Figure 5D). In contrast, the frequency of lung ILC3 expressing IL-22 was enhanced >3-fold in flagellin-treated mice (Figure 5E). In conclusion, these data support the idea that flagellin accelerates and overstimulates lung ILC3 to produce IL-22 in the context of pneumococcal infection.

Flagellin Treatment Induces IL-22–Dependent Protection Against S. pneumoniae

We next analyzed the effect of flagellin on antipneumococcal defenses and evaluated the contribution of IL-22. Flagellin protected animals against S. pneumoniae infection, as assessed by an improved survival rate and reduced bacterial load in the lung (Figure 6A and 6B). To determine the role of IL-22, wild-type and $Il22^{-/-}$ mice were treated with flagellin and infected with S. pneumoniae. Although flagellin significantly reduced the bacterial load in IL-22–proficient (ie, wild-type) mice, this protective effect was not observed in $Il22^{-/-}$ animals (Figure 6C). To confirm this finding, an IL-22–specific neutralizing antibody was used. Whereas flagellin protected isotype control–injected mice against infection, mice treated with the IL-22 neutralizing antibody were impaired in their capacity to clear S. pneumoniae (Figure 6D). Together, our data support a major role for IL-22 in flagellin-mediated protection against lethal pneumococcal infection.

DISCUSSION

This study demonstrated that lung ILC3 are the main cellular source of IL-22 during respiratory infection with S. pneumoniae. In addition, our data suggest that boosting the activity of ILC3 and the production of IL-22 by flagellin treatment promotes protection against lethal S. pneumoniae infection.

Previous studies addressed the activity of ILC3 in the respiratory tract [4, 23]. Monticelli et al proposed that ILC3 are not present in the lungs. In contrast, Taube et al identified RORγt-positive Lin^{neg} cells that produce IL-22 after culture of lung cells of asthmatic animals [35, 36]. Here, we performed a detailed analysis of mouse lung ILC3 cells, using multiparametric flow cytometry and reporter and immunodeficient animals. We found that lung steady-state ILC3 can promptly produced IL-22 (and IL-17) upon IL-23 stimulation (Figure 1). During S. pneumoniae infection, lung ILC3 but not NK or NK T cells were the only source of early IL-22 production (Figures 2 and 3). We finally demonstrated that most lung ILC3 coexpress RORγt and CCR6, a hallmark of spleen, tonsil, and gut ILC3 [10, 37]. These observations match with a recent study reporting that C. albicans primes lung ILC3 and IL-22 production to promote protection against infection with Pseudomonas aeruginosa [38]. ILC3 activation by C. albicans was also observed in the oral/tongue compartment [14]. Interestingly, CCL20, the CCR6 ligand, is expressed at steady state but is strongly upregulated by airway epithelial cells in response to inflammatory signals and pathogens [39]. The increase in the ILC3 number in lungs could be the result of CCL20 upregulation upon infection
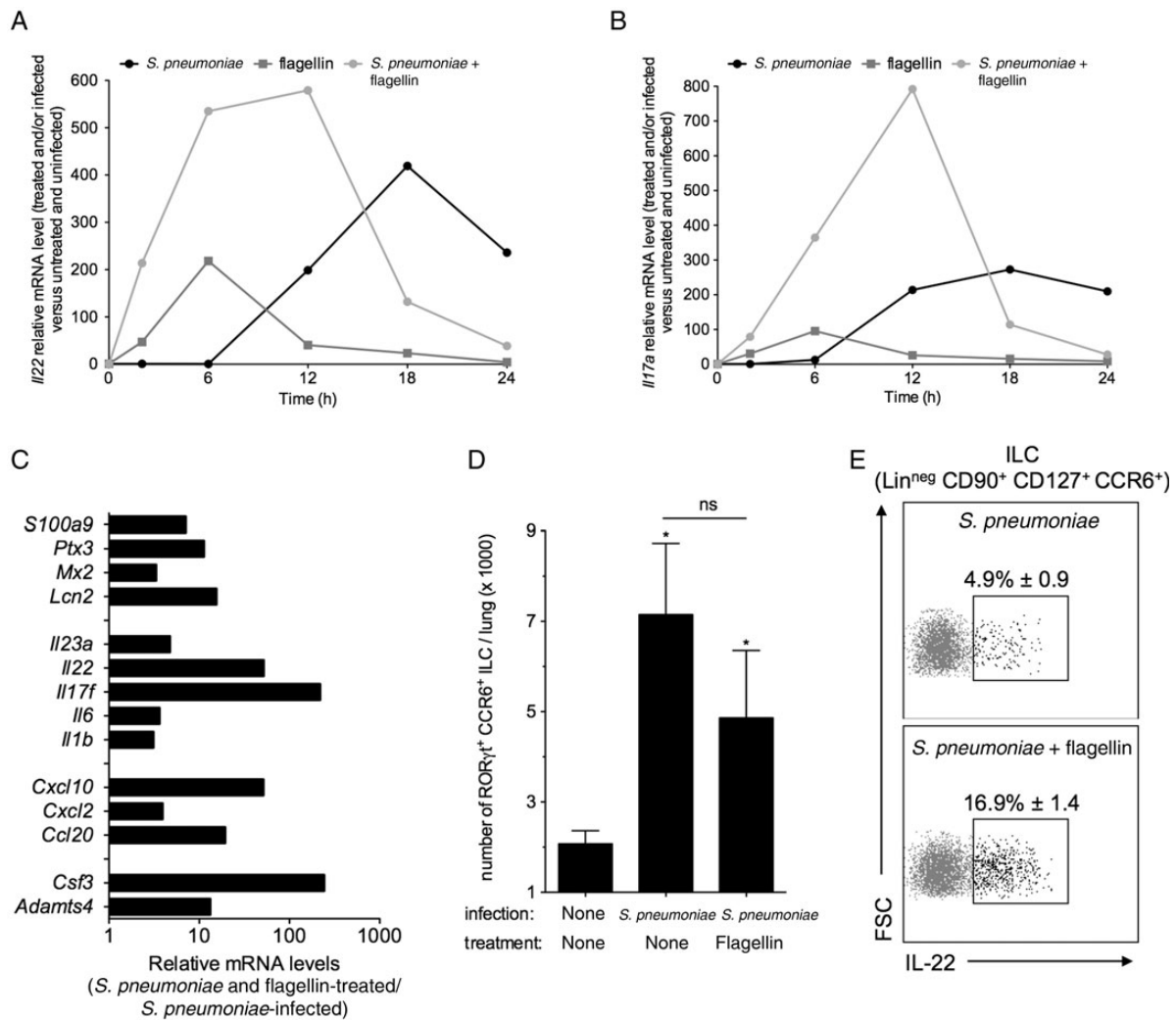
Figure 5. Flagellin treatment overstimulates type 3 innate lymphoid cells (ILC3)–mediated *Il22* expression in lung of infected mice. Mice (n = 3–4) were left untreated or were infected intranasally with 2×10^6 *Streptococcus pneumoniae*. When indicated, untreated and infected animals were concomitantly treated intravenously with flagellin (5 µg). Lungs were collected at indicated times after infection and analyzed by quantitative polymerase chain reaction or flow cytometry. *A* and *B*, Time course analysis of *Il22* and *Il17a* expression in C57BL/6 animals. *C*, Transcriptional analysis at 6 hours in C57BL/6 mice. Transcript levels were expressed relative to those of the untreated group, arbitrarily set to 1. *D*, Impact of infection on the lung ILC3 numbers. *Rorc(γt)-Gfp^{TG}* mice were sampled at 16 hours, and lung ILC3 were identified by flow cytometry as Lin^{neg}CD90$^+$CD127$^+$RORγt$^+$CCR6$^+$. *E*, Interleukin 22 (IL-22) production by ILC3. Lungs from C57BL/6 mice were sampled at 16 hours and Lin^{neg}CD90$^+$CD127$^+$CCR6$^+$ cells were assessed for IL-22 production by intracellular cytokine staining. The percentage of IL-22–producing ILCs is shown. Results are represented as means *A–C* or means ± standard error of the mean (*D* and *E*). Statistically significant differences from the mock group (*$P < .05$) were assessed by the Mann–Whitney test.

(Figures 2 and 3). ILC3 are heterogeneous in the expression of several markers, including CD4 and NKp46 [5–8, 10–14, 37]. We found that lung ILC3 do not express CD4, and only low levels of CD25 were detected. Such phenotype might explain the discrepancy between our data and those from the study by Monticelli et al, who used CD25 to sort out total ILCs from the lung [4]. In general, ILC3 are known to respond to DC-derived IL-23 and IL-1β [1, 13]. We found that pneumococcus upregulates transcription of genes encoding IL-23 and IL-1β, suggesting their contribution to ILC3 activation. Moreover, the activation was shown to require DCs and MyD88 (Figure 3

and Supplementary Figure 1). As reported for Th17 lymphocytes [40–42], the contribution of MyD88 to ILC3 activation may depend on IL-1β and/or IL-23 signaling.

The production of IL-17 and IL-22 by innate immune cells and Th17/Th22 lymphocytes contributes to early defenses and adaptive immunity to infections [1, 13]. This work hypothesized that flagellin administration to pneumococcus-infected mice influences ILC3 and modulates lung defenses, as described elsewhere for intestinal infections [20, 43]. Flagellin was effective in boosting the production of IL-22 (and IL-17), thereby contributing to protection at the onset of infection. The
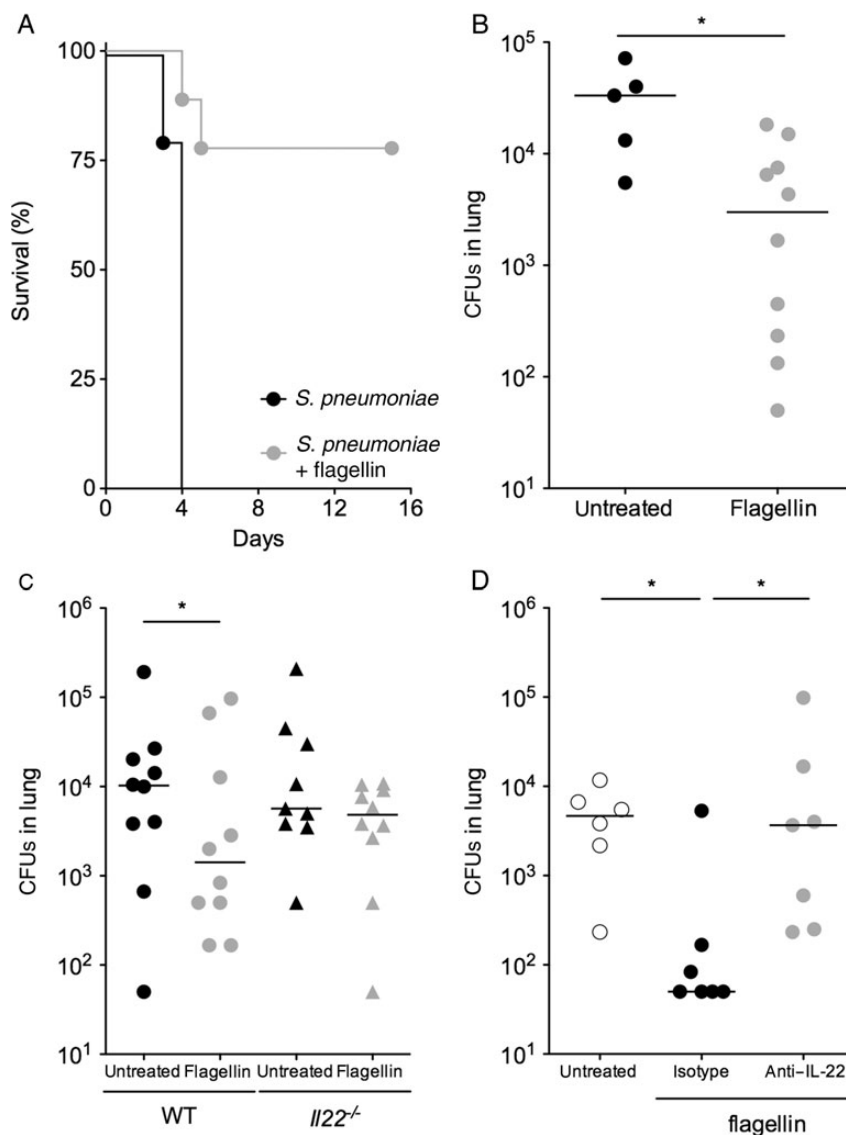
Figure 6. Interleukin 22 (IL-22) is required for flagellin-mediated protection. C57BL/6 (wild-type [WT]) mice or $Il22^{-/-}$ mice (n = 6–10) were infected intranasally with 2×10^6 *Streptococcus pneumoniae* and concomitantly treated intravenously with flagellin (5 µg). Protection was assessed by monitoring survival or counting bacteria in lung at 24 hours. *A* and *B*, Flagellin-mediated protection in WT animals. *C* and *D*, Flagellin-mediated protection is IL-22 dependent. *C*, Protection is abolished in $Il22^{-/-}$ mice. *D*, IL-22 neutralization impairs protection. To this end, WT animals were treated intravenously with IL-22–specific neutralizing antibodies or control isotype. Colony-forming unit (CFU) counts of individual mice are shown, and results are expressed as medians. Statistical significance (*$P < .05$) was assessed by the Mann–Whitney test.

observations that ILC3 respond to flagellin and that IL-22 inhibition abrogates protection supported a role for ILC3 in flagellin-mediated defenses. Studies of gut and oral infections with *Citrobacter rodentium* and *C. albicans* demonstrated that ILC3, through IL-17/IL-22 production, are instrumental to eradicate mucosal microbial colonization [12, 14]. Mear et al also reported that ILC3 regulate lung infection [38]. Similar to findings in the gut, several defense mechanisms may be proposed for the lung. Thus, in response to IL-17– and IL-22, regionalized production of antimicrobial peptides

and phagocyte-specific chemokines could trigger the containment of pathogens within the conducting airways, preventing dissemination and invasive disease. Our data showed that expression of chemokines and antimicrobial molecules such as lipocalin 2, S100A9, and pentraxin 3 are induced by pneumococcus and further upregulated by flagellin (Figures 2 and 5). All these factors are targets of IL-17 and IL-22 signaling in the epithelial compartment. In the gut, ILC3 are key players for the regulation of the commensal bacterial communities [12, 20, 44]. One can assume that ILC3 could also influence

rhinopharyngeal colonization by commensal and opportunistic bacteria, including *S. pneumoniae.* Finally, IL-22 was recently found to play a role in *S. pneumoniae* superinfection during influenza by downregulating lung inflammation and reinforcing epithelial integrity [32]. Such a mechanism may participate in the effect of IL-22 in our model of primary infection. This study focused on IL-22 during infection, although the production of IL-17A, IL-17F, and IFN-γ is also strongly boosted with regard to time and magnitude of expression when animals are resistant to infection [28, 29]. Because IL-17 and IL-22 are reciprocally regulating each other [33, 45], it will be required to address their relative contributions to bacterial defense.

In conclusion, this study provides substantial understanding of lung ILC3 and IL-22 in the context of respiratory infection. Beyond the effect on innate defenses, ILC3 have been shown to participate to the onset of gut Th22 responses [46]. Further investigations addressing the longstanding impact of the early lung ILC3 activation may reveal unexpected effects on the mucosal adaptive immunity and microbial colonization of the respiratory tract, especially in individuals with inflammatory diseases, including asthma and chronic obstructive pulmonary disease. Altogether, our data provide evidence that lung ILC3 and IL-22 are interesting targets for immune-based therapies against respiratory infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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