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Interleukin-22 Reduces Lung Inflammation during Influenza A Virus Infection and Protects against Secondary Bacterial Infection

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Interleukin-22 (IL-22) has redundant, protective, or pathogenic functions during autoimmune, inflammatory, and infectious diseases. Here, we addressed the potential role of IL-22 in host defense and pathogenesis during lethal and sublethal respiratory H3N2 influenza A virus (IAV) infection. We show that IL-22, as well as factors associated with its production, are expressed in the lung tissue during the early phases of IAV infection. Our data indicate that retinoic acid receptor-related orphan receptor-γt (RORγt)-positive αβ and γδ T cells, as well as innate lymphoid cells, expressed enhanced *Il22* transcripts as early as 2 days postinfection. During lethal or sublethal IAV infections, endogenous IL-22 played no role in the control of IAV replication and in the development of the IAV-specific $CD8^+$ T cell response. During lethal infection, where wild-type (WT) mice succumbed to severe pneumonia, the lack of IL-22 did not accelerate or delay IAV-associated pathogenesis and animal death. In stark contrast, during sublethal IAV infection, IL-22-deficient animals had enhanced lung injuries and showed a lower airway epithelial integrity relative to WT littermates. Of importance, the protective effect of endogenous IL-22 in pulmonary damages was associated with a more controlled secondary bacterial infection. Indeed, after challenge with *Streptococcus pneumoniae*, IAV-experienced *Il22*$^{-/-}$ animals were more susceptible than WT controls in terms of survival rate and bacterial burden in the lungs. Together, IL-22 plays no major role during lethal influenza but is beneficial during sublethal H3N2 IAV infection, where it limits lung inflammation and subsequent bacterial superinfections.

Interleukin-22 (IL-22) plays a dual role in autoimmune and inflammatory diseases (for reviews, see references 1 and 2). It is produced by conventional lymphocytes and by lymphoid cells displaying "innate-like" functions (3–11). Among the later group are certain subsets of retinoic acid receptor-related orphan receptor-γt (RORγt)-positive αβ and γδ T lymphocytes and innate lymphoid cells (termed as ILC3) (4, 7–12). IL-22 solely acts on nonhematopoietic cells, including hepatocytes and epithelial cells, to exert both proinflammatory and tissue-protective properties depending on the context and the tissue in which it is expressed (2, 13–16). In experimental noninfectious systems, IL-22 exerts a potent protective effect on hepatocytes and epithelial cells at barrier surfaces, particularly in the intestine and in the thymus (17–21). IL-22 is also a key factor controlling mucosal immunity and the dissemination of commensal bacteria from the intestinal tract (22). In the lungs, IL-22 protects against experimental lung fibrosis (23) and ventilator-induced lung injury (24). IL-22 also limits Th2-mediated airway inflammation and tissue damage during asthma (25–27). On the other hand, uncontrolled IL-22 production favors dermal inflammation and acanthosis, bleomycin-induced airway inflammation, collagen-induced arthritis, and lipopolysaccharide shock, in part by enhancing tissue inflammation in concert with inflammatory factors such as tumor necrosis factor alpha (TNF-α) and IL-17 (4, 28–31).

During infection, IL-22 production by innate cells or effector conventional T cells plays a dual role depending on the pathogen and the tissue. The early production of IL-22 by innate immune cells is crucial for host protective immunity to extracellular bacteria, including *Klebsiella pneumoniae* in the lung and *Citrobacter rodentium* in the intestine (32, 33). On the other hand, IL-22 has no substantial role in host defense against *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *M. avium*, *Listeria monocytogenes*, *Candida albicans*, or *Schistosoma mansoni* (34–37). Of importance, IL-22 can provide protective innate immunity when the adaptive immune system is impaired. This redundant function has been described during infection with *C. albicans* and *Eimeria falciformis* (38–40). Finally, a deleterious role for IL-22 on intestinal inflammation was reported after oral infection with *Toxoplasma gondii* (37, 41).

The potential role of IL-22 during viral infection has recently been addressed. IL-22 might participate in resistance to human immunodeficiency virus infection in subjects who do not sero-

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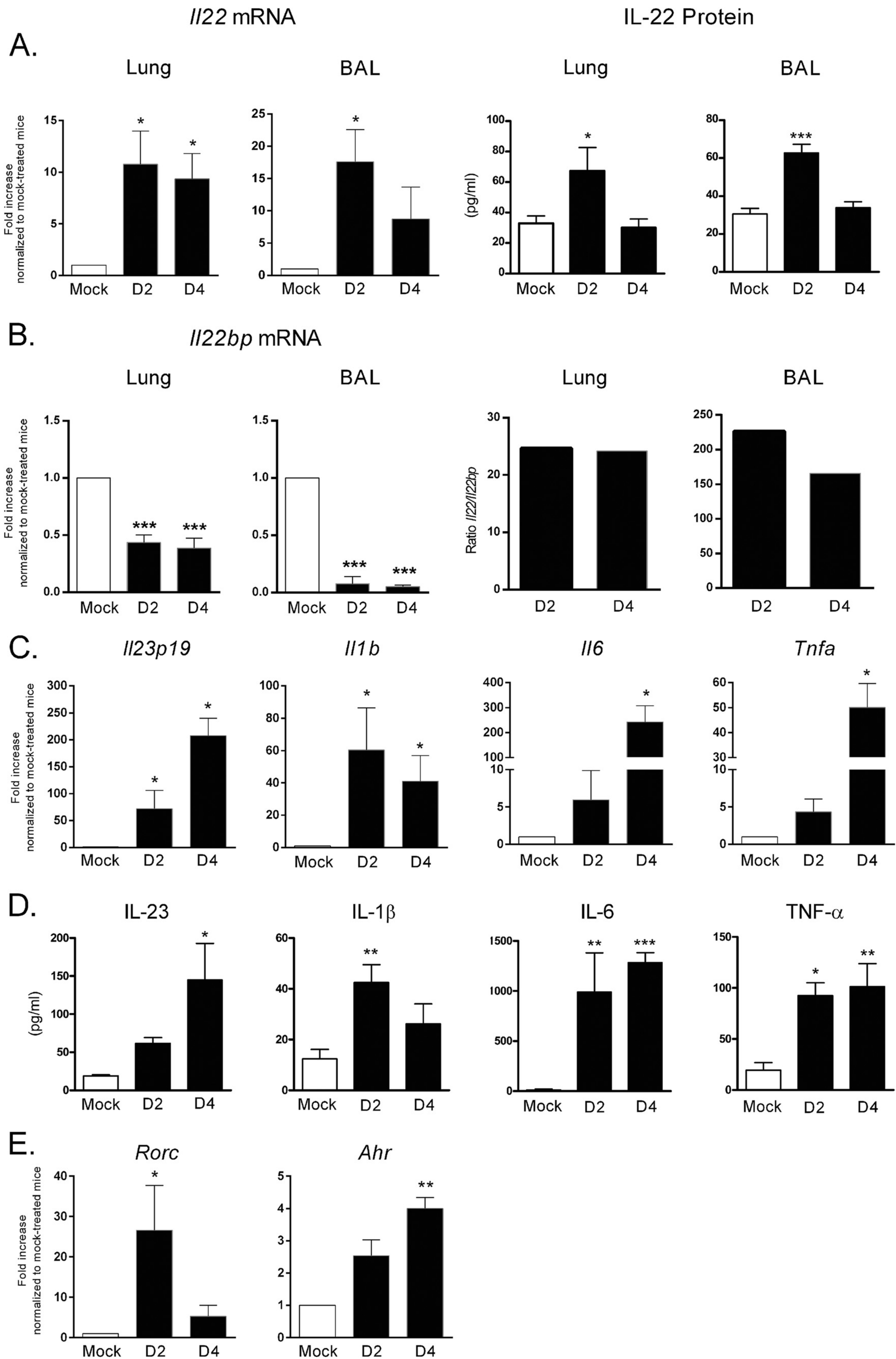


Il22 mRNA
IL-22 Protein
A.
Lung
BAL
Lung
BAL
Fold increase normalized to mock-treated mice
(pg/ml)
Mock
D2
D4
B.
Il22bp mRNA
Ratio Il22/Il22bp
C.
Il23p19
Il1b
Il6
Tnfa
D.
IL-23
IL-1β
IL-6
TNF-α
E.
Rorc
Ahr

convert despite multiple exposures to the virus (42, 43). Recent data also indicated that reduced IL-22 production in the gut mucosa is important in human immunodeficiency virus mucosal immunopathogenesis (44). On the other hand, although hepatic IL-22 expression is upregulated in viral hepatitis, IL-22 lacks direct antiviral activity against hepatitis B and C viruses (45, 46). Of interest, IL-22 aggravates tissue inflammation during experimental hepatitis B virus infection (47) and West Nile virus encephalitis (48). The potential role of IL-22 in antiviral host defense and virus-associated inflammation has recently been studied during experimental influenza A virus (IAV) infection. Using neutralizing antibodies (Abs), Guo et al. (49) initially showed that IL-22 has little role during acute H1N1 IAV infection, as assessed by IAV-associated morbidity and mortality. In parallel, Monticelli et al. recently reported that during mild, resolving H1N1 IAV infection, neutralization of IL-22 had no impact on the morbidity, on the decreased lung function and on respiratory tissue remodeling (50). In contrast, based on *in vitro* assays and using IL-22-deficient mice, we suggested that during the early phase of H3N2 IAV infection (day 4), IL-22 protects bronchial epithelial cells against damages caused by IAV (51). In this report, invariant natural killer T (iNKT) cells were characterized as cellular sources of IL-22. In line with our observation, IL-22 was next reported to participate in airway epithelial regeneration during the resolution phase of H1N1 IAV infection (days 7 to 18) (52). Surprisingly, enough, in this later report, RORγt-negative conventional NK cells were shown to be the main producers of IL-22 (52). The aims of the present study were to characterize the cellular sources of IL-22 during the early phase of H3N2 IAV infection and to study its potential role on the development of viral pathogenesis and host responses during infection. To this end, an acute lethal model of IAV infection, where animals succumbed to pneumonia, and a model of infection where animals ultimately resolved inflammation, were used. Since bacterial superinfections can occur after primary IAV infection, a phenomenon accounting for a high rate of morbidity and mortality worldwide (53–60), we also examined the role of endogenous IL-22 in secondary bacterial infection following influenza virus infection. We show that IL-22 is produced by several subsets of lung RORγt-positive, but not RORγt-negative (including NK cells), cells early after infection. In both lethal and sublethal conditions, IL-22 plays no role in the control of virus replication and in the promotion of the virus-specific $CD8^+$ T cell response. Of interest, during sublethal influenza, but not lethal influenza, IL-22 plays a protective function against respiratory tissue damages caused by IAV, a phenomenon associated with a higher resistance to secondary bacterial infections.

MATERIALS AND METHODS

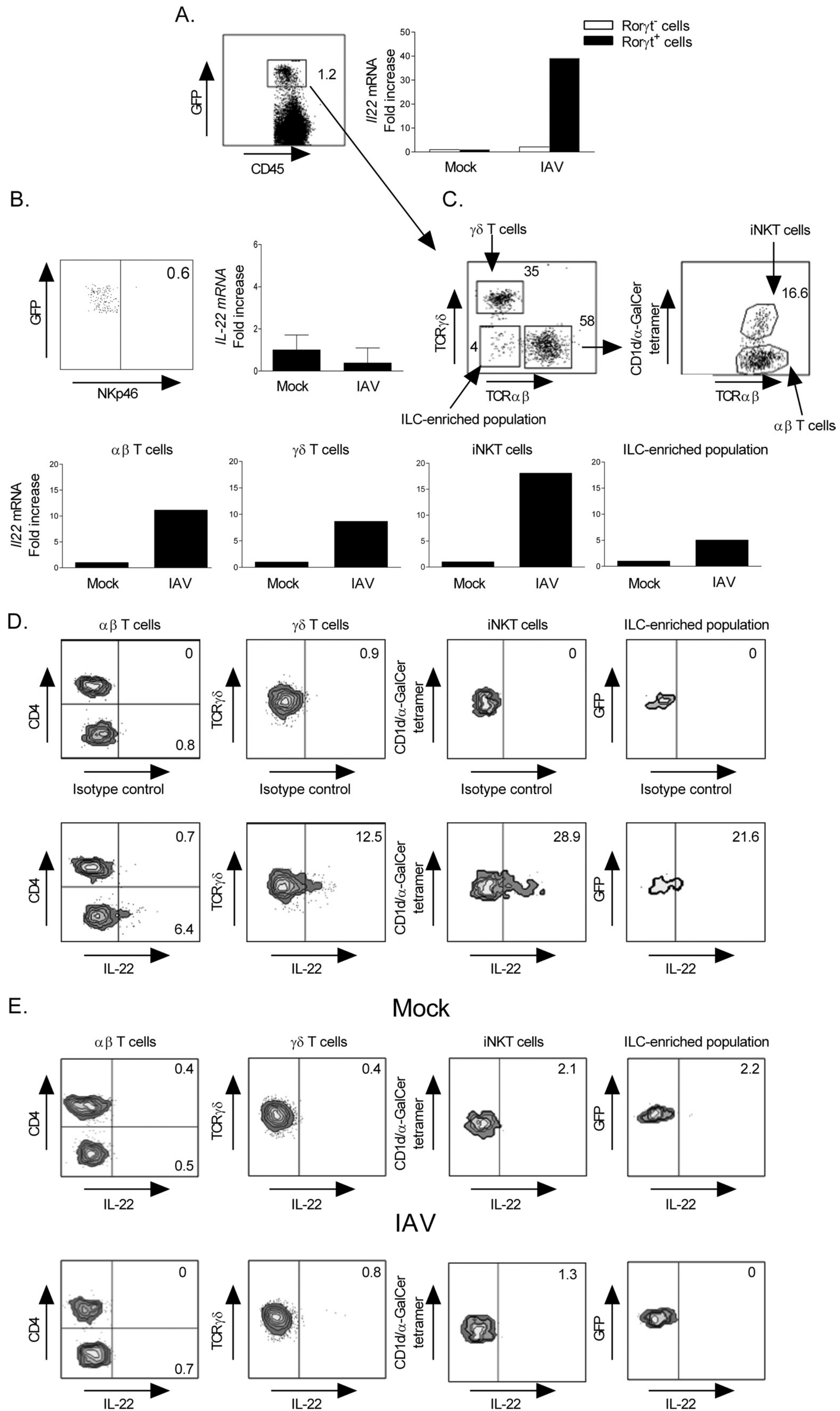
Virus, bacteria, and mice. The highly pathogenic human-origin H3N2 IAV strain Scotland/20/74 and *S. pneumoniae* serotype 1 clinical isolate E1586 sequence type ST304 have been described (61–64). *Interleukin-$22^{-/-}$* mice, backcrossed at least 10 times in C57BL/6 (65), as well as WT littermate controls, were bred in the Ludwig Institute (Brussels, Belgium). RORγt- green fluorescent protein (GFP) mice were described earlier (66, 67). Mice (8- to 10-week-old male) were maintained in a biosafety level 2 facility in the Animal Resource Center at the Pasteur Institute, Lille, France. All animal work conformed to Pasteur Institute, Lille, Animal Care and Use Committee guidelines (agreement AF 16/20090 from the Comité d'Ethique en Expérimentation Animale Nord Pas-De-Calais).

Abs and reagents. Monoclonal Abs against mouse TCRβ (allophycocyanin [APC] and V450 conjugated), TCRγδ (PerCP-Cy5.5 conjugated), CD45 (eFluor605NC, Pacific Blue, or APC-H7 conjugated), NKp46 (phycoerythrin [PE] conjugated), CD127 (PE-Cy7 conjugated), CD90.2 (Alexa Fluor 700 conjugated), streptavidin (Alexa Fluor 700 or PE conjugated), and CD4 (APC-H7-conjugated) were purchased from BD Biosciences (Le Pont de Claix, France). The biotin mouse lineage panel was from BD Biosciences. APC-conjugated and PE-conjugated phosphate-buffered saline (PBS)-57-loaded CD1d tetramers were, respectively, obtained from ProImmune (Oxford, United Kingdom) and the NIAID Tetramer Facility (Emory University, Atlanta, GA). The monoclonal Ab against mouse IL-22 (clone 3F11) was kindly provided by W. Ouyang (Genentech, San Francisco, CA). Recombinant IL-22 was produced as described previously (68).

IAV infection and assessment of gene expression by quantitative RT-PCR. Mice were anesthetized and administered intranasally (i.n.) with 50 μl of PBS containing different dose (50 or 600 PFU) of virus (Scotland/20/74, H3N2). Total RNA from whole lungs or from cells recovered from the bronchoalveolar lavages (BAL) of mock-treated or IAV-infected mice was extracted and cDNA was synthesized by classical procedures. Quantitative reverse transcription-PCR (RT-PCR) was carried out as described previously (51). Primers specific for the *gapdh*, *Ifng*, *Il17a*, *mx1*, *Ifnb*, *Il22*, and IAV *M2* genes have been described (51). In addition, the following primers were used: *Il22bp*, 5′-ACTCTGCCTGGACCAGGACA-3′ and 5′-GAGAAGCACCCGCAAAGATG; *Il23p19*, 5′-AATCTCTGCATGCTAGCCTGG-3′ and 5′-GATTCATATGTCCCGCTGGTG-3′; *Il1b*, 5′-TCCCCAACTGGTACATCAGCA-3′ and 5′-ACACGGATTCCATGGTGAAGTC-3′; *Rorgt*, 5′-TGAAAGCAGGAGCAATGGAAGT-3′ and 5′-ACAGCTCCACACCACCGTATTT-3′; *Ahr*, 5′-ATCGACATAACGGACGAAATCC-3′ and 5′-TTAGGTGCTGAGTCACAGGCTG-3′; *Il6*, 5′-AGCCTCCGACTTGTGAAGTG-3′ and 5′-CTGATGCTGGTGACAACCAC-3′; *Il17f*, 5′-TGTCCTCCCCTGGAGGATAAC-3′ and 5′-GAACTGGAGCGGTTCTGGAA-3′; *Il21*, 5′-AAACTCAAGCCATCAAACCCTG-3′ and 5′-TGTTTCTTTCCTCCCCTCCTG-3′; and *Tnfa*, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′ and 5′-TGGGAGTAGACAAGGTACAACCC-3′. ΔC_T values were obtained by deducting the raw cycle threshold (C_T values) obtained for *gapdh* mRNA, the internal standard, from the C_T values obtained for investigated genes.

Analysis of IL-22 transcript levels in RORγt-positive cells during IAV infection. RORγt-GFP mice were infected, or not, with IAV, and lung MNCs were prepared 60 h postinfection (p.i.). RORγt-positive αβ T lymphocytes ($CD45^+$ $TCR\beta^+$), γδ T lymphocytes ($CD45^+$ $TCR\gamma\delta^+$), and $TCR\alpha\beta^-$ $TCR\gamma\delta^-$ ($CD45^+$ $TCR\beta^-$ $TCR\gamma\delta^-$) cells were sorted from naive and IAV-infected mice. The expression of IL-22 transcript was performed by quantitative RT-PCR.

FIG 1 IL-22 production in the lungs during the early steps of IAV infection. C57BL/6 WT animals were infected with 600 PFU of IAV Scotland/20/74/H3N2 strain. The lungs and the BAL fluids were collected 2 and 4 days p.i. (A) *Il22* mRNA copy numbers were determined by quantitative RT-PCR. The data are normalized to the expression of *gapdh* and are expressed as the fold increased over the average gene expression in mock-treated mice (left panels). IL-22 protein production was measured by ELISA (right panels). (B) *Il22bp* mRNA levels in the lungs and BAL fluids were measured (left panels). The ratio of *Il22* to *Il22bp* are depicted (right panels). (C) *Il23p19*, *Il1b*, *Il6*, and *Tnfa* mRNA levels in the BAL fluids were measured by quantitative RT-PCR. (D) IL-23, IL-1β, IL-6, and TNF-α levels were measured in the BAL fluids by ELISA. (E) RORγt and aryl hydrocarbon receptor (AhR) mRNA levels in the BAL fluids were measured by quantitative RT-PCR. The values in panels A to E represent the means ± the SEM of four independent experiments. For quantitative RT-PCR of BAL samples, BAL fluids were pooled (5 mice/group). For quantitative RT-PCR of lung samples and for ELISA (BALs and lungs), $n = 12$ to 15. Significant differences were determined using a one-way analysis of variance, followed by a Bonferroni post test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).


A.
GFP
CD45
1.2
Il22 mRNA Fold increase
Rorγt⁻ cells
Rorγt⁺ cells
Mock
IAV
B.
GFP
NKp46
0.6
IL-22 mRNA Fold increase
Mock
IAV
C.
γδ T cells
iNKT cells
35
58
4
16.6
TCRγδ
TCRαβ
CD1d/α-GalCer tetramer
ILC-enriched population
αβ T cells
γδ T cells
iNKT cells
ILC-enriched population
Il22 mRNA Fold increase
Mock
IAV
D.
αβ T cells
γδ T cells
iNKT cells
ILC-enriched population
CD4
TCRγδ
CD1d/α-GalCer tetramer
GFP
Isotype control
0
0.8
0.9
0
0
0.7
6.4
12.5
28.9
21.6
IL-22
E.
Mock
0.4
0.5
0.4
2.1
2.2
IAV
0
0.7
0.8
1.3
0

Analysis of IL-22-producing cells. Analysis of IL-22-producing cells was assessed 2 days post-IAV infection. As a possible control, lung MNCs were cultured at 10^7 cells/ml in complete medium containing 10 ng of recombinant mouse IL-1β/ml and IL-23 plus 10 μg of brefeldin A (Sigma-Aldrich, Steinheim, Germany)/ml at 37°C for 4 h. After activation, cells were washed and stained with Live/Dead fixable dead cell stain kit (Life Technologies, Carlsbad, CA) in PBS for 30 min. The cells were washed and incubated with appropriate dilutions of eFluor605NC-conjugated CD45, PE-conjugated PBS-57-loaded CD1d tetramer, V450-conjugated anti-TCRβ Ab, and PerCP-Cy5.5-conjugated anti-TCRγδ Ab for 30 min in PBS containing 2% fetal calf serum (FCS). Cells were washed and fixed using IC fixation buffer (eBioscience, CliniSciences, Montrouge, France). Fixed cells were then permeabilized in permeabilization buffer (eBioscience), according to the manufacturer's instructions. Cells were stained with APC-conjugated MAb against IL-22 or control mouse IgG2a MAb and analyzed on a LSR Fortessa (BD Biosciences). To analyze the proportions of innate lymphoid cells (ILCs) in RORγt-positive $TCR\alpha\beta^-$ $TCR\gamma\delta^-$ cells, lung MNCs from RORγt-GFP mice were labeled with appropriate dilutions of APC-H7-conjugated anti-CD45, Percp-Cy5.5-conjugated anti-TCRγδ, V450-conjugated anti-TCRβ, a biotin lineage-specific Ab cocktail (TER119, CD11b, Gr1, B220, CD3, CD11c, and NK1.1) plus a PE-conjugated streptavidin, PE-Cy7-conjugated anti-CD127, and Alexa Fluor 700-conjugated CD90.2. Cells were analyzed on a LSR Fortessa.

Assessment of the mortality rate and of the pathology. After IAV infection (600 or 50 PFU), mice were monitored daily for illness and mortality for a period of 17 days. Disease was assessed by measuring lung inflammation, viral load in the lungs, and lethality. Mice found to be moribund were euthanized and considered to have died on that day. Mice were also sacrificed at day 7 p.i. to recover the whole lung and the BALs. For histopathologic examination, lungs were fixed by inflation and immersion in PBS–3.2% paraformaldehyde and then embedded in paraffin. To evaluate airway inflammation, we subjected fixed lung slices (5-μm sections) to hematoxylin and eosin (H&E) staining. Evaluators who were blinded to genotype scored lung sections (0 [none] to 3 [extreme]) according to criteria described by Paget et al. (69).

Analysis of the viral load and of the IAV-specific $CD8^+$ T cell response. Lungs were homogenized and virus titers determined using a standard plaque assay on Mardin-Darby canine kidney cells. *Ifnb* and *mx1* mRNA expression levels were determined by quantitative RT-PCR as described previously (69). The number of IAV-specific $CD8^+$ T cells was determined as reported (69). For this, cells specific for an immunodominant D^b-restricted $CD8^+$ T epitope derived from the viral polymerase 2 protein ($PA_{224-233}$) (70) were analyzed. Briefly, lung MNCs were incubated with appropriate dilutions of APC-conjugated anti-CD19, fluorescein isothiocyanate-labeled anti-CD8 and PE-conjugated Pro5 major histocompatibility complex pentamer H-$2D^b$ SSLENFRAYV. To assess the functionality of virus-specific $CD8^+$ T cells, lung cells and cells isolated from the lung draining (mediastinal) lymph nodes (dLNs) were stimulated with the peptide SSLENFRAYV (10 μg/ml), and cytokine production was assessed by enzyme-linked immunosorbent assay (ELISA).

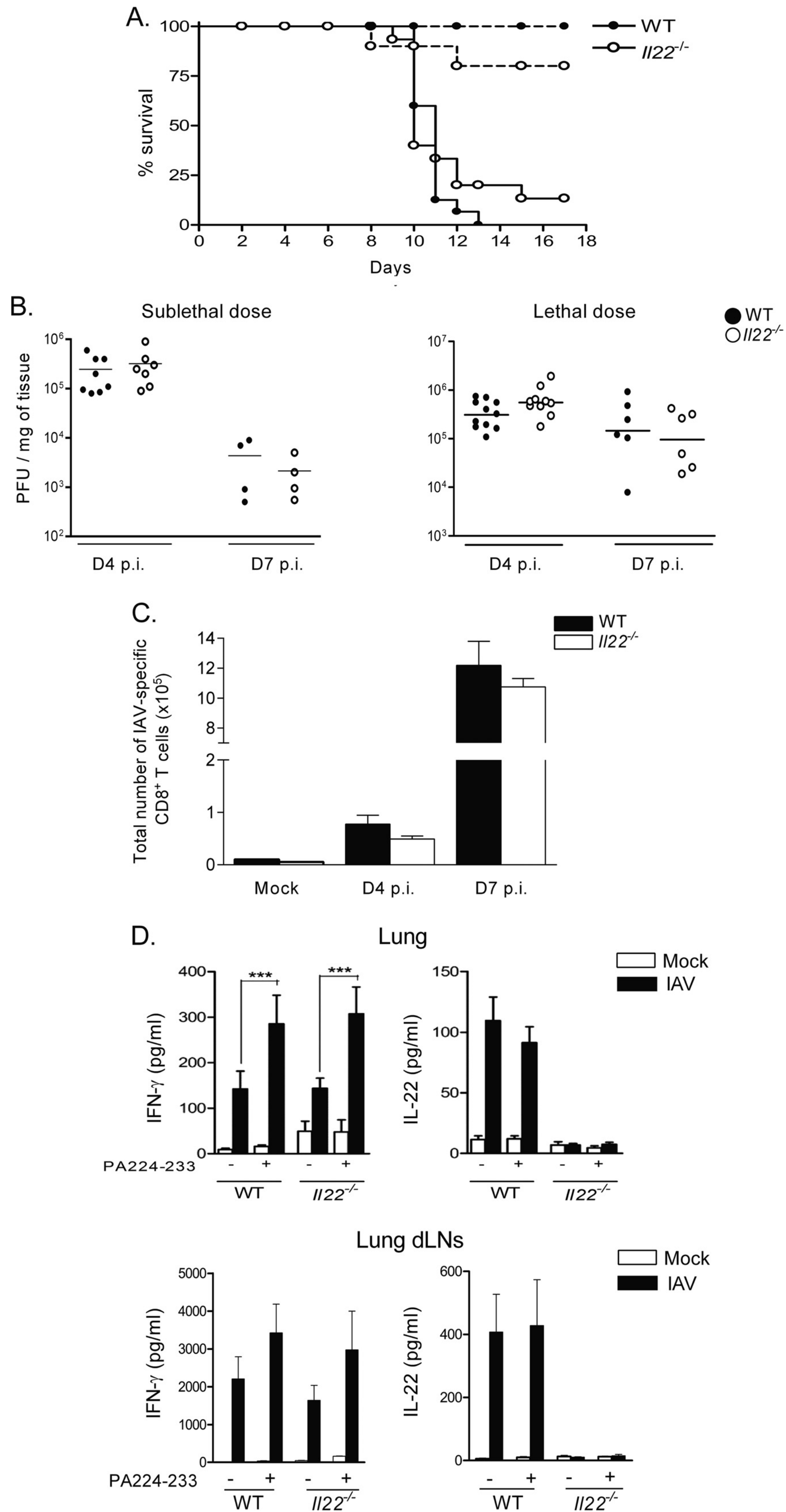
Secondary infection with *S. pneumoniae*. Mice, infected or not with IAV (50 PFU) 7 days earlier, were i.n. inoculated with 10^4 *S. pneumoniae* serotype 1. In naive animals, this dose is self-limiting since mice cleared the bacteria within 24 h. In some circumstances, *Il22*$^{-/-}$ mice received by the i.n. route recombinant IL-22 (5 μg/mouse) or vehicle (PBS) just before IAV infection and 2 days p.i. Mice were monitored daily for illness and mortality for a period of 13 days. The number of viable bacteria in the lungs was determined 24 h after *S. pneumoniae* challenge. This was measured by plating lung homogenates onto blood agar plates (71). CFU were enumerated 24 h later. A morphology-based differential cell count was conducted on cytospin preparations from the BAL fluid and stained with Diff-Quik solution (Sigma).

Statistical analysis. Results are expressed as the means ± the standard deviations (SD) or ± the standard errors of the mean (SEM). The statistical significance of differences between experimental groups was calculated by a one-way analysis of variance, followed by a Bonferroni posttest (Prism 4 Software; GraphPad, San Diego, CA). The possibility to use these parametric tests was assessed by checking whether the population is Gaussian and the variance is equal (Bartlett's test). Results with a P value of <0.05 were considered significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS

IL-22 is produced in the lungs during the early stages of IAV infection. The kinetics of IL-22 expression during the early phases of IAV infection are ill-defined, although we and others have shown that natural killer (NK) cells and invariant natural killer (iNKT) cells are primary sources of IL-22 2 days after H1N1 or H3N2 IAV infection, respectively (49, 51). As shown in Fig. 1A (left panels), and relative to mock-treated animals, a higher level of *Il22* gene transcript was detected in the lung tissue and alveolar spaces of IAV-infected mice 2 and 4 days p.i. (~10-fold enhancement). An enhanced concentration of IL-22 protein was also detected 2 days, but not 4 days, p.i. (Fig. 1A, right panels), a phenomenon probably due to its rapid consumption. IL-22 activity can be neutralized *in vivo* by its specific opponent IL-22 binding protein (IL-22BP) (72). As seen in Fig. 1B, *Il22bp* messengers were slightly downregulated (~2-fold) at day 2 and 4 p.i. in the lung tissue and the decrease was more important (~20-fold) in the BALs. The ratio of *Il22* to *Il22bp* was ~10-fold higher in the BALs compared to the lung tissue and did not significantly differ at days 2 and 4 p.i. *Il17a* and *IL17f* transcripts were also found to be upregulated 2 and 4 days p.i. in the BAL cells, but not in pulmonary cells, whereas *Il21*, another member of the IL-17 family, was not modulated (not shown). Augmented IL-17A protein was also detected at day 2 p.i., but only in the BAL fluids (not shown).

FIG 2 Expression of IL-22 transcript by pulmonary RORγt-positive cells during IAV infection. (A) RORγt-positive and RORγt-negative cells were sorted from mock-treated or IAV-infected RORγt-GFP mice (60 h p.i., 600 PFU). RNAs were prepared, and IL-22 mRNA copy numbers were measured by quantitative RT-PCR as described in Fig. 1A. Of note, the frequency of RORγt-positive cells remained stable 2 days p.i. (B) *Il22* mRNA copy numbers were determined from NK cells sorted from mock-treated or IAV-infected RORγt-GFP mice. (C) RORγt-positive αβ T lymphocytes ($TCR\beta^+$) without iNKT cells, iNKT (PBS57-loaded CD1d tetramer$^+$ $TCR\beta^+$) cells, which represent ~15% of total RORγt-positive $TCR\beta^+$ cells, γδ T lymphocytes ($TCR\gamma\delta^+$), and the ILC-enriched population ($TCR\beta^-$, $TCR\gamma\delta^-$) were sorted from mock-treated or IAV-infected RORγt-GFP mice (60 h p.i.) and analyzed for IL-22 mRNA expression. For panels A to C, the data are normalized to the expression of *gapdh* and are expressed as the fold increased over the average gene expression in mock-treated mice. Each group is a pool of cells from six to eight mice and is representative of two independent experiments. (D) Intracellular staining of IL-22 in lung RORγt-positive lung cells. Lung MNCs from RORγt-GFP mice were treated with IL-1β and IL-23 (10 ng/ml) for 4 h in the presence of brefeldin A, and gated RORγt-positive αβ T lymphocytes, γδ T lymphocytes, an ILC-enriched population, and iNKT cells were analyzed for intracellular IL-22 production. (E) RORγt-GFP mice were infected with IAV (600 PFU), and lung MNCs were prepared 60 h p.i. Cells were cultured for 4 h in the presence of brefeldin A without restimulation, and gated RORγt-positive cell subpopulations were analyzed for intracellular IL-22 production. (D and E) Gates were set based on the isotype control. The percentage of cells expressing IL-22 is shown. The results of representative experiments out of two (D) or five (E) are depicted. (E) Similar data were obtained at 50 PFU (data not shown).


A.
100
75
50
25
0
% survival
0 2 4 6 8 10 12 14 16 18
Days
WT
Il22-/-
B.
Sublethal dose
Lethal dose
WT
Il22-/-
PFU / mg of tissue
D4 p.i.
D7 p.i.
C.
Total number of IAV-specific CD8+ T cells (x10^5)
WT
Il22-/-
Mock
D4 p.i.
D7 p.i.
D.
Lung
Mock
IAV
IFN-γ (pg/ml)
IL-22 (pg/ml)
***
PA224-233
WT
Il22-/-
Lung dLNs
Mock
IAV
IFN-γ (pg/ml)
IL-22 (pg/ml)
PA224-233
WT
Il22-/-

IL-23, IL-1β, IL-6, and TNF-α have been described to participate in early IL-22 production in some settings (for reviews, see references 2, 8, and 9). As seen in Fig. 1C, *Il23*, *Il1b*, *Il6*, and *Tnfa* gene transcripts were strongly upregulated in the BAL cells and in the lungs (data not shown) by 2 and 4 days p.i. Enhanced IL-23, IL-1β, IL-6, and TNF-α protein levels were also evident at these time points (Fig. 1D). Of note, RORγt, and to a lesser extent, aryl hydrocarbon receptor, transcription factors known to be crucial in IL-22 synthesis (2, 8, 9), were also upregulated at the transcript level (Fig. 1E). Collectively, IL-22, as well as factors known to regulate its expression, are produced in the lungs during the early stages of H3N2 IAV infection.

RORγt-expressing cells produce enhanced IL-22 transcripts early after IAV infection. We took advantage of RORγt-GFP mice to analyze the early source(s) of IL-22 during IAV infection. As seen in Fig. 2A, RORγt-positive cells purified from IAV-infected mice express an enhanced level of *Il22* gene transcripts compared to RORγt-positive cells isolated from noninfected animals. In contrast, influenza virus infection did not trigger *Il22* messenger expression in RORγt negative cells. Of note, NKp46^{+} cells did not express RORγt in the lung tissue and failed to produce *Il22* messenger in response to IAV (Fig. 2B).

The different pulmonary RORγt-expressing cell populations were next sorted from mock-treated and IAV-infected animals and analyzed for *Il22* gene transcript expression. In agreement with earlier studies (66, 73), αβ T lymphocytes and γδ T lymphocytes represented the two major populations expressing RORγt (Fig. 2C, upper panel). Invariant NKT cells were discarded from the αβ T lymphocyte pool and were analyzed separately. A fourth population of RORγt^{+} cells was also identified. This population, which represents a minor fraction of RORγt^{+} cells (~4% of the total pool), contains ~70% of Lin^{-} CD127^{+} CD90^{+} CD4^{-} cells (data not shown) and was thus termed the ILC3-enriched population (12). As shown in Fig. 2C (lower panel), αβ T lymphocytes, γδ T lymphocytes, and iNKT cells, and to a lesser extent the ILC3-enriched population, produced a higher level of *Il22* mRNAs in the context of IAV infection.

Analysis of IL-22 protein expression by RORγt-expressing cells was next assessed in response to a cocktail of IL-1β and IL-23, used here as a positive control. As represented in Fig. 2D, αβ T lymphocytes (mainly CD4^{neg}), γδ T lymphocytes, and iNKT cells and, to a lesser extent cells within the ILC-enriched population, produced IL-22. Of note, lung NKp46^{+} cells failed to express IL-22 in response to IL-1β and IL-23 (not shown). In the context of IAV infection, and without *ex vivo* restimulation, IL-22 protein expression was not evidenced by intracellular staining using fluorescence-activated cell sorting, regardless of the cell population analyzed, the kinetics (60 h and 4 days), and the dose of IAV used (Fig. 2E and data not shown). The low level of expression and/or the rapid secretion of IL-22 might explain these data.

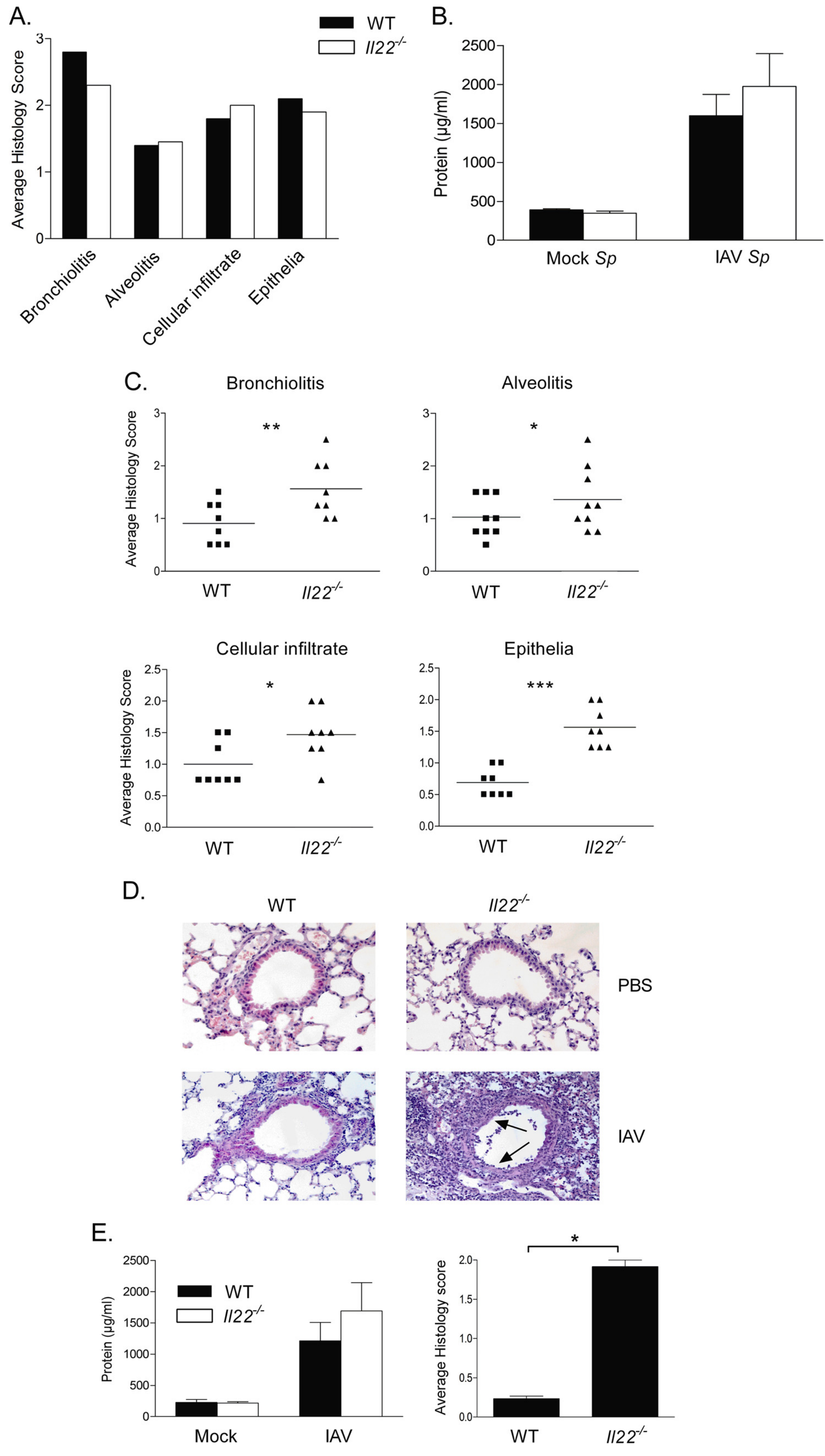
IL-22 deficiency has no impact on mouse survival, viral clearance, and IAV-specific CD8^{+} T cell response. Severe lung immunopathology strongly contributes to influenza virus-related morbidity and mortality (74). It is known that IL-22 is a versatile controller of immunopathology (2, 8, 9). To address its role during influenza, WT and *Il22*$^{-/-}$ mice were used in survival studies. To rule out potential bias due to genetic background, WT and *Il22*$^{-/-}$ littermates were used. Upon a lethal dose (600 PFU) of IAV, WT animals demonstrated severe sickness ending in death at day 13 (Fig. 3A). Although not significant, 10 to 15% of *Il22*$^{-/-}$ mice survived the infection out to day 18 p.i. In contrast, using a sublethal dose (50 PFU), *Il22*$^{-/-}$ mice displayed slightly, although not significantly, decreased resistance compared to WT animals.

To determine whether endogenous IL-22 plays a part in viral clearance, the viral load was monitored at day 4 and at day 7 p.i. by plaque assay. Whatever the dose of IAV used, *Il22*$^{-/-}$ mice controlled virus replication in their lungs with kinetics similar to those for WT animals (Fig. 3B).

IL-22 has been shown to attenuate antigen (Ag)-induced pulmonary immune responses in some settings (23, 25–27). We thus compared the virus-specific CD8^{+} T cell response in IAV-infected WT and *Il22*$^{-/-}$ mice in terms of cell number and cytokine production. Whatever the dose of IAV (shown is 50 PFU), the number of lung CD8^{+} D^{b}PA$_{224\text{-}233^{+}}$ cells were not different between WT and *Il22*$^{-/-}$ mice at 4 and 7 days p.i (Fig. 3C and data not shown). Similarly, upon restimulation with PA$_{224\text{-}233}$, the amount of IFN-γ released by lung cells and by cells from the lung dLNs was identical (Fig. 3D). Of note, whereas pulmonary and lung dLN cells from IAV-infected mice spontaneously released IL-22 at day 7, IL-22 production was not amplified after IAV peptide restimulation. This indicates the lack of IAV-specific IL-22-producing CD8^{+} T cells in the context of early IAV, at least in response to the immunodominant peptide used.

Endogenous IL-22 plays a positive role in the control of sublethal, but not lethal, IAV-associated pneumonia. We next addressed the potential role of endogenous IL-22 in the development and/or the control of IAV-associated pulmonary pathogenesis. To do so, lungs from WT and *Il22*$^{-/-}$ mice infected with a lethal or a sublethal dose of IAV were harvested for histology during the acute-phase response. In conditions where IAV causes acute pneumonia ending by the death of the animals (600 PFU), the lack of IL-22 did not significantly modulate the intensity of the pulmonary inflammation. As shown in Fig. 4A, the cellular infiltration in the lungs, as the well as the alveolitis and bronchiolitis scores, were not significantly different between infected WT and *Il22*$^{-/-}$ mice. In both infected animal groups, bronchial epithelia were strongly damaged, and this was associated with an impaired epithelial integrity, as measured by the high total protein concentration in the BAL fluid (Fig. 4B).

FIG 3 Impact of IL-22 deficiency on mouse survival, on the control of viral clearance, and on the development of IAV-specific CD8^{+} T cells in the lungs. (A) Age-matched WT or *Il22*$^{-/-}$ mice were infected with a lethal (600 PFU, solid line) or a sublethal (50 PFU, dotted line) dose of IAV and then observed until death. The percentages of survival are represented (n = 10 to 15/group, at least two independent experiments). A log-rank test for comparisons of Kaplan-Meier survival curves indicated no significant difference in the mortality of *Il22*$^{-/-}$ mice compared to WT animals. (B) Viral loads in the lungs of WT and *Il22*$^{-/-}$ mice infected with 50 PFU (sublethal) or 600 PFU (lethal) of IAV were assessed at 4 and 7 days p.i. The data are given as PFU/mg of tissue (n = 4 to 11). (C) At 4 and 7 days p.i. (50 PFU), lung cells from infected WT and *Il22*$^{-/-}$ mice were recovered and analyzed for CD8^{+} T cell responses. The number of PA$_{224\text{-}233}$-specific CD8^{+} T cells in the lung tissue was determined by flow cytometry. The results of one representative experiment out of three are shown. Values represent the means ± the SD (n = 4 to 6). (D) At day 7 p.i. (50 PFU), lung and lung dLN cells were restimulated with PA$_{224\text{-}233}$ peptide (10 μg/ml) for 72 h. IFN-γ and IL-22 production was measured by ELISA. The results of one representative experiment out of three (lung) or two (lung dLNs) are shown. Values represent the means ± the SD (n = 4 to 6). Significant differences were determined using a one-way analysis of variance, followed by a Bonferroni post test (***, $P < 0.001$).


A.
WT
Il22-/-
Average Histology Score
Bronchiolitis
Alveolitis
Cellular infiltrate
Epithelia
B.
Protein (µg/ml)
Mock Sp
IAV Sp
C.
Bronchiolitis
Alveolitis
Cellular infiltrate
Epithelia
**
*
*
***
WT
Il22-/-
D.
WT
Il22-/-
PBS
IAV
E.
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Mock
IAV
Average Histology score
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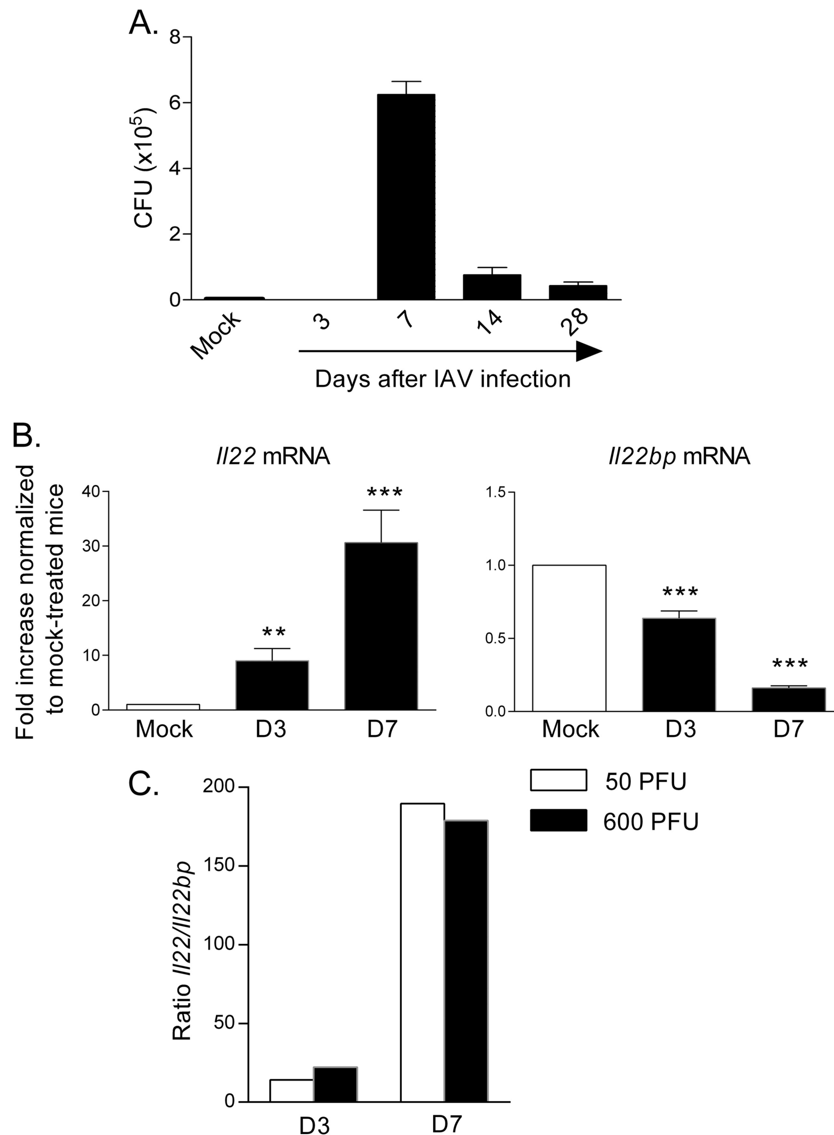
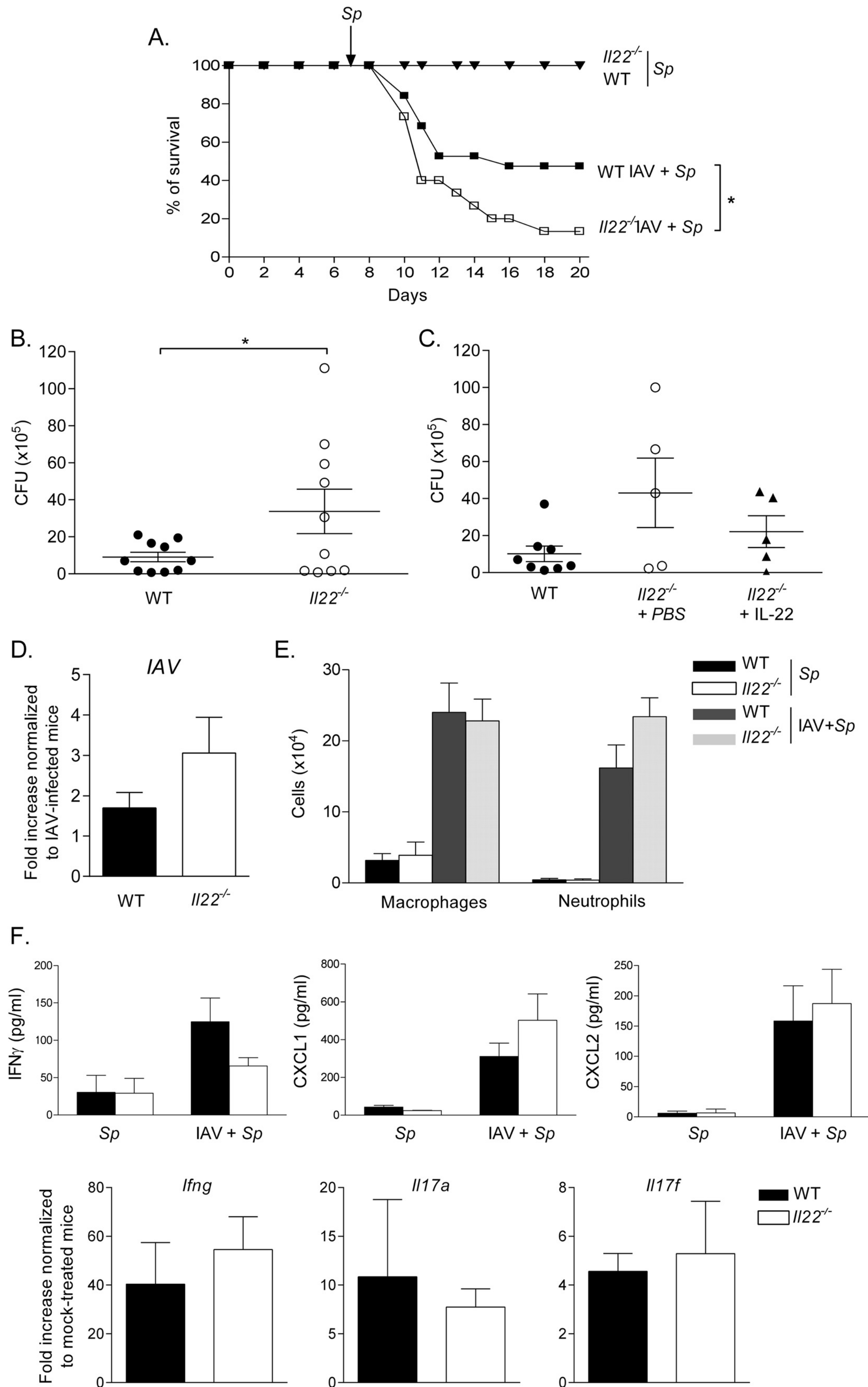


FIG 5 Establishment of the superinfection model and *Il22/Il22bp* expression analysis. (A) Wild-type mice were infected with IAV (50 PFU) and, 3, 7, 14, or 28 days later, mice were challenged with *S. pneumoniae* (10^4 CFU). The numbers of live bacteria in the lungs were determined 24 h after *S. pneumoniae* infection. The results of one representative experiment out of two are shown (means ± the SEM, $n = 5$). (B and C) WT animals were infected with 50 PFU (B) or with 600 PFU (B and C) of IAV. The lungs were collected at 3 and 7 days p.i. (B) *Il22* and *Il22bp* mRNA copy numbers were determined by quantitative RT-PCR. The data are expressed as the fold increased over the average gene expression in mock-treated mice ($n = 6$). (C) The ratios of *Il22* to *Il22bp* (50 PFU versus 600 PFU) are depicted at days 3 and 7 p.i.

In stark contrast, upon a sublethal challenge (50 PFU), a significant enhancement of airway inflammation was noticed in *Il22*$^{-/-}$ mice relative to WT mice as reflected by the higher histopathology scores in the former group (Fig. 4C). In *Il22*$^{-/-}$ mice, alveolitis and bronchiolitis were more pronounced relative to WT littermates (Fig. 4C and D). In addition, epithelia surrounding the bronchi from *Il22*$^{-/-}$ mice were more damaged (Fig. 4D). Signs of severe injury, characterized by augmented loss of intercellular cohesion and denuded epithelium were observed in *Il22*$^{-/-}$ animals (scored in Fig. 4C). This apparent loss of epithelial integrity was associated with a slight enhancement of protein concentration in the BALs (Fig. 4E, left panel). Of note, the number of red blood

FIG 4 Analysis of the pathology of WT or *Il22*$^{-/-}$ mice infected with a lethal or sublethal dose of IAV. Age-matched WT or *Il22*$^{-/-}$ mice were infected with 600 PFU (A and B) or 50 PFU (C to E) IAV and sacrificed 7 days postchallenge. The lungs were harvested, and histopathological analysis and scoring (A and C) were performed exactly as described previously (51, 69). (B) Lung permeability of infected mice as assessed by the quantification of protein concentrations in BAL fluids from each mouse. (D) Representative H&E-stained tissue sections (magnification, ×200) are shown. Arrows indicate denuded epithelia. (E) The left panel shows the protein concentrations in BAL fluids. The right panel shows the scoring for the alveolar leakage, and lesional edema was based on the number of red blood cells in the alveoli. The data represent the means ± the SEM (B and E, left panel) or SD (E, right panel). All results are representative of two independent experiments ($n = 8$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ [two-tailed Student t test]).


A.
Sp
100
80
60
40
20
0
% of survival
0 2 4 6 8 10 12 14 16 18 20
Days
Il22-/-
WT
Sp
WT IAV + Sp
Il22-/-IAV + Sp
*
B.
*
120
100
80
60
40
20
0
CFU (x10^5)
WT
Il22-/-
C.
CFU (x10^5)
WT
Il22-/- + PBS
Il22-/- + IL-22
D.
IAV
Fold increase normalized to IAV-infected mice
WT
Il22-/-
E.
Cells (x10^4)
Macrophages
Neutrophils
WT
Il22-/-
Sp
WT
Il22-/-
IAV+Sp
F.
IFNγ (pg/ml)
CXCL1 (pg/ml)
CXCL2 (pg/ml)
Sp
IAV + Sp
Ifng
Il17a
Il17f
Fold increase normalized to mock-treated mice
WT
Il22-/-

cells in the luminal side of the alveoli was dramatically enhanced in *Il22*$^{-/-}$ mice indicative of alveolar leakage and lesional edema (Fig. 4E, right panel).

IL-22 deficiency leads to an enhanced susceptibility to secondary bacterial infection following influenza virus infection. Epithelial damages post-influenza virus infection has been proposed to play a part in secondary bacterial infections such as *S. pneumoniae* (53, 55–57, 59, 60, 75). We thus investigated the role of endogenous IL-22 in bacterial superinfection following influenza virus infection. Before this, we determined the optimal timing between exposure to the virus and exposure to the bacteria to achieve bacterial superinfection. For this, animals were infected with a sublethal dose of IAV (50 PFU) and were then superinfected with a self-limiting dose of *S. pneumoniae* (10^4 CFU) at different time points after virus infection. As seen in Fig. 5A, mice challenged with *S. pneumoniae* 7 days after influenza virus infection had a high number of live bacteria in the lungs, while animals infected after 3 days completely clear the bacteria 24 h after the challenge. Of note, mice infected 2 and 4 weeks after IAV had a lower capacity to control bacterial replication, although the numbers of bacteria in the lungs were much lower relative to those in mice receiving IAV 7 days earlier. Thus, *S. pneumoniae* was administered 7 days post-IAV infection, the peak of bacterial susceptibility.

At the dose of IAV used (50 PFU), the levels of *Il22* mRNA were increased, whereas those of *Il22bp* were decreased at day 3 and particularly at day 7 p.i. (Fig. 5B). As a consequence, the ratio of *Il22* to *Il22bp* was higher at day 7 relative to day 3. Of note, this ratio was comparable at 50 and 600 PFU (Fig. 5C). As expected, preceding IAV infection induced animal death after *S. pneumoniae* challenge (Fig. 6A, IAV+*Sp*). Remarkably, while 50% of IAV-experienced WT mice succumbed following *S. pneumoniae* infection, almost all coinfected *Il22*$^{-/-}$ mice died from bacterial infection. This effect was associated with an increased number of viable bacteria in the lung tissue (Fig. 6B). Of importance, the observed effect was probably not due to a direct antibacterial effect of endogenous IL-22 *per se*. Indeed, both non-IAV experienced WT and *Il22*$^{-/-}$ mice survived (Fig. 6A) and equally cleared bacteria in the lungs after sublethal *S. pneumoniae* challenge (data not shown). To confirm our findings, *Il22*$^{-/-}$ mice were administered with recombinant IL-22. Compared to IAV-infected *Il22*$^{-/-}$ animals treated with vehicle alone, the administration of recombinant IL-22 reduced the number of CFU in the lungs (Fig. 6C). Thus, the lack of IL-22 worsens the outcome of secondary *S. pneumoniae* pneumonia. This effect was not associated with a significantly higher IAV burden in the lungs (Fig. 6D) or a differential recruitment of macrophages and neutrophils in the BALs of doubly infected mice, cells known to be crucial in the clearance of *S. pneumoniae* (76, 77) (Fig. 6E). Furthermore, the production of factors known to exert antipneumococcal effects, including IFN-γ, IL-17, and the neutrophil-attracting factors CXCL1 and CXCL2, were not different between WT and *Il22*$^{-/-}$ doubly infected mice (Fig. 6F and not shown). ICAM-1 plays a role in the control of *S. pneumoniae* early after infection (78). However, the transcript level of *Icam1* remained unchanged between WT and *Il22*$^{-/-}$ doubly infected mice (data not shown). In the same line, REGIIIγ, a known target of IL-22 able to exert an antibacterial effect in the intestine (33) and the lung (34), was not modulated at the transcript level in doubly infected *Il22*$^{-/-}$ mice, relative to doubly infected WT animals (data not shown). Collectively, endogenous IL-22 limits bacterial superinfection post-influenza virus infection, a phenomenon probably independent from direct enhanced host defense mechanisms.

DISCUSSION

IL-22 participates in host defense against bacteria at mucosal surfaces and plays a part in the maintenance of the barrier integrity, with predominant effects on epithelial cells. However, this cytokine can also serve pathogenic functions in many experimental and clinical settings (1, 2, 13, 29). In the present study, we show that during nonlethal IAV-induced pathogenesis, IL-22 reduces lung inflammation and maintains pulmonary epithelial integrity, an effect associated with a more controlled secondary bacterial infection following influenza.

During H3N2 infection, and at the transcript level, IL-22 was induced as early as day 2 in the lung tissue and BALs, whereas IL-22BP, the endogenous IL-22 antagonist, was downmodulated. Of note, whatever the dose of IAV, the *Il22*/*Il22bp* ratio was enhanced in a similar extent and peaked at day 7 p.i. Along with RORγt-positive iNKT cells, other subsets of RORγt-positive cells, including αβ T lymphocytes, γδ T lymphocytes, and ILC3, express enhanced *Il22* transcript during the early phases of IAV infection. In contrast, RORγt-negative cells failed to do so. The absence of IL-22 production by lung NKp46^{+} cells, either after IL-1β plus IL-23 stimulation or in the context of IAV infection, can be explained by the lack of RORγt expression by these cells and indicates that among ILC3, lymphoid tissue inducer cells, rather than "NK-like" cells, are a source of IL-22 during early IAV infection. Our data are in contrast to other studies (49, 52), which reported IL-22 protein production by lung conventional NK cells during the early (day 2) and the later (days 4 and 7) stages of IAV infection. The reasons for this discrepancy may be explained by differences in the virus strains used.

Whatever the dose of IAV, the overall pulmonary viral burdens

FIG 6 Role of endogenous IL-22 on secondary bacterial infection following influenza infection. (A) WT or *Il22*$^{-/-}$ mice were infected, or not, with IAV (50 PFU). Seven days later, IAV-infected or naive animals were challenged with *S. pneumoniae* (10^4 CFU) and then observed until death. The percentages of survival are represented ($n = 15$, three independent experiments). A log-rank test for comparisons of Kaplan-Meier survival curves indicated a significant difference in the mortality of *Il22*$^{-/-}$ mice compared to WT animals. (B) Doubly infected WT or *Il22*$^{-/-}$ mice were sacrificed 24 h after *S. pneumoniae* challenge, and the number of CFU was determined in the lungs. Of note, WT and *Il22*$^{-/-}$ mice only infected with *S. pneumoniae* (10^4 CFU) had no CFU in the lungs at 1 day p.i. (data not shown). The data represent pooled results from two independent experiments (*, $P < 0.05$). (C) *Il22*$^{-/-}$ mice were administered with recombinant IL-22 or vehicle (PBS) at the time of IAV infection and at day 2 p.i. (D to F), The lungs of doubly infected WT or *Il22*$^{-/-}$ mice were collected 1 day after the *S. pneumoniae* challenge. (D) IAV M2 mRNA copy numbers were determined by quantitative RT-PCR. The data are normalized to the expression of *gapdh* and are expressed as the fold increased over the average gene expression in singly infected WT mice (day 7 post-IAV). (E) The numbers of macrophages and neutrophils in the BALs were determined. (F) For the upper panel, the concentrations of IFN-γ, CXCL1, and CXCL2 were determined in BAL fluids 24 h after bacterial challenge. For the lower panel, *Ifng*, *Il17a*, and *Il17f* mRNA copy numbers were determined by quantitative RT-PCR. The data are normalized to the expression of *Gapdh* and are expressed as the fold increased over the average gene expression in naive mice. Shown are the results of a representative experiment out of two performed (means ± the SEM, $n = 5$). For panels E and F, the data represent the means ± the SEM ($n = 3$ [*Sp*] or $n = 10$ [IAV+*Sp*]).

of $Il22^{-/-}$ mice showed no difference from those of control mice. These data are in line with other studies reporting no direct or indirect antiviral activity of IL-22 (45–49). Furthermore, in agreement with a previous finding showing that IL-22 has no impact on Ag-driven lymphocyte priming and expansion (65), $Il22^{-/-}$ mice had a normal IAV-specific $CD8^+$ T cell response. Thus, IL-22 does not favor or interfere with mechanisms leading to T cell priming in the dLNs. Likewise, IL-22 plays no major role on the synthesis of epithelial-derived chemokines necessary for the recruitment of expanded T cells in the lung compartment.

In response to a highly immunopathogenic IAV dose (600 PFU), $Il22^{-/-}$ mice did not exhibit reduced or enhanced mortality rates. In parallel, compared to WT mice, $Il22^{-/-}$ mice showed no improvement, or conversely dysregulation, of pneumonia. This indicated that during excessive, nonresolving, IAV-associated pneumonia, IL-22 plays no major function. In stark contrast, during sublethal IAV infection where inflammation is less intense and more controlled, endogenous IL-22 reduced lung injury. This did not significantly influence the survival outcome, although a trend toward enhanced mortality was observed in $Il22^{-/-}$ mice. The reason why IL-22 is redundant at a high dose of IAV and beneficial at a sublethal dose is not due to a difference in the levels of IL-22 and IL-22BP expression (at least at the transcript level). A difference in the production of IL-17 and TNF-α, known to control IL-22 activities (5, 29, 79), as well as of other inflammatory factors, might explain the different role of IL-22 during lethal and sublethal influenza. The positive role of IL-22 during sublethal IAV infection is in agreement with observations reported by Kumar et al. (H1N1) (52). During the early phase of IAV infection, the loss of epithelial integrity plays a major role in the pathology (50, 75). It leads to the loss of homeostasis and the development of pulmonary inflammation, features found to be enhanced in $Il22^{-/-}$ mice. Epithelial damages are caused by IAV-induced cytopathology and/or the lysis of virus-infected epithelial cells by recruited NK cells, cytotoxic T cells, apoptosis-inducing cytokines, and the activation of death receptors. Our recent data showed that IL-22 protects epithelial cells from cell death after infection with IAV *in vitro* (51). In line with this finding, we observed an enhanced level of epithelial damages in infected $Il22^{-/-}$ mice, relative to WT littermates. Although the mechanisms sustaining the protective effect of IL-22 are still unknown, we speculate that its *in vivo* beneficial effect is due to its role on the maintenance of epithelial integrity. In this setting, IL-22 might play a positive role in the prevention of injury (51) and, as recently demonstrated (52), in the acceleration of the epithelial repair. Recently, a population of lung RORγt-negative ILCs that resembles natural helper cells has been described to play a protective role, through amphiregulin release, on airway epithelia during IAV infection (50). It is possible that the positive role played by IL-22-producing cells during the early stages of IAV infection complements natural helper cell functions to ensure airway epithelial integrity and lung tissue homeostasis during IAV infection.

It is well documented that IAV infection enhances susceptibility to secondary bacterial infections, leading to increased morbidity and mortality (53–60). The mechanisms include alterations of mechanical and immunological defenses. For instance, epithelial cell damages caused by influenza virus improve bacterial adhesion to the mucosa and its subsequent crossing through the lung epithelium. In parallel, impairment of the host innate response is an important feature of bacterial superinfections (53, 55, 59). In the present study, we report that IAV-experienced $Il22^{-/-}$ mice challenged with *S. pneumoniae* have decreased survival compared to similarly infected WT animals. We favor the hypothesis that the protective role of IL-22 in bacterial superinfection post-IAV infection is rather due to its positive early effect on lung, including epithelial, injury due to IAV infection rather than to an antibacterial effector mechanisms *per se*. Indeed, molecular and cellular effectors associated with early innate defense mechanisms against bacteria were not significantly decreased in the absence of IL-22. Thus, by limiting the alteration of the epithelial barrier instigated by IAV, IL-22 might decrease the level of bacterial invasion into the lungs. This hypothesis needs to be confirmed using different IAV subtypes, including H1N1. Since in humans, dysregulation of IL-22 or IL-22 receptor production has been reported in certain pathologies, including psoriasis, Crohn's disease, and allergic diseases (80), it might be interesting to determine the potential correlation, if any, between susceptibility to bacterial superinfections and IL-22 or IL-22 receptor production in patients. Recent findings suggest that, in combination with antibiotic and antiviral therapies, treatments that protect lung epithelium and/or stimulate lung repair responses could be beneficial in improving survival in patients during influenza virus and bacterial coinfection (50, 75). Whether IL-22 supplementation in order to improve lung epithelial integrity and to prevent microbial translocation leads to less severe outcome resulting from bacterial complications during influenza virus infection is under intense investigation.

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