Interleukin-22 Is Produced by Invariant Natural Killer T Lymphocytes during Influenza A Virus Infection

POTENTIAL ROLE IN PROTECTION AGAINST LUNG EPITHELIAL DAMAGES*

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Background: Invariant natural killer T (iNKT) cells play a beneficial role during experimental influenza A virus (IAV) infection.

Results: iNKT cells produce IL-22 during infection, and IL-22 prevents the IAV-triggered cell death of pulmonary epithelium.

Conclusion: IL-22 produced by iNKT cells might be important during IAV infection.

Significance: Understanding how iNKT cells function during IAV infection might be instrumental to control IAV-associated pathogenesis.

Invariant natural killer T (iNKT) cells are non-conventional lipid-reactive αβ T lymphocytes that play a key role in host responses during viral infections, in particular through the swift production of cytokines. Their beneficial role during experimental influenza A virus (IAV) infection has recently been proposed, although the mechanisms involved remain elusive. Here we show that during in vivo IAV infection, mouse pulmonary iNKT cells produce IFN-γ and IL-22, a Th17-related cytokine critical in mucosal immunity. Although permissive to viral replication, IL-22 production by iNKT cells is not due to IAV infection per se of these cells but is indirectly mediated by IAV-infected dendritic cells (DCs). We show that activation of the viral RNA sensors TLR7 and RIG-I in DCs is important for triggering IL-22 secretion by iNKT cells, whereas the NOD-like receptors NOD2 and NLRP3 are dispensable. Invariant NKT cells respond to IL-1β and IL-23 provided by infected DCs independently of the CD1d molecule to release IL-22. In vitro, IL-22 protects IAV-infected airway epithelial cells against mortality but has no role on viral replication. Finally, during early IAV infection, IL-22 plays a positive role in the control of lung epithelial damages. Overall, IAV infection of DCs activates iNKT cells, providing a rapid source of IL-22 that might be beneficial to preserve the lung epithelium integrity.

Invariant natural killer T (iNKT)8 cells represent a unique subpopulation of αβ T lymphocytes expressing markers associated with the NK lineage and recognizing lipid antigens (Ag) (for reviews, see Refs. 1 and 2). These cells express an invariant TCRα chain that pairs with a limited number of Vβ chains. Unlike conventional T lymphocytes that recognize peptide-MHC complexes, the TCR of iNKT cells detects self and foreign (i.e. microbial-derived) (glyco)lipids presented by the non-polymorphic MHC class I-like protein CD1d expressed by Ag-presenting cells (APCs), including dendritic cells (DCs) (3, 4). Exposure of iNKT cells with the synthetic, non-physiological, glycolipid α-galactosylceramide (5) promptly induces the production of large amounts of both Th1-, Th2-, and Th17-associated cytokines that lead to downstream activation of DCs, NK

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8 The abbreviations used are: iNKT cells, invariant natural killer T cells; Ag, antigen; APC, Ag presenting cell; DC, dendritic cell; IAV, influenza A virus; TLR, Toll-like receptor; RIG-I, retinoic acid inducible gene-I; Myd88, myeloid differentiation 88; IPS-1, IFN-β promoter stimulator-1; NOD, nucleotide binding oligomerization domain; NLRP3, NOD-like receptor, pyrin domain containing 3; p.i., post-infection; m.o.i., multiplicity of infection; AEC, airway epithelial cells; REG3β, regenerating protein 3β; NLR, NOD-like receptor; BAL, bronchoalveolar lavage; ANOVA, analysis of variance; TCR, T cell receptor.
cells, B cells, and conventional T cells (6, 7). This enables iNKT cells to influence the outcome of developing or ongoing immune reactions with, in general, beneficial effects on infection and cancer (1, 2). Activation of iNKT cells can also occur during stressful conditions, including infection (8, 9). Several in vivo models demonstrated that upon natural activation, iNKT cells are flexible in nature and can either suppress or enhance the outcome of infectious diseases (1, 8, 10, 11). Although the contribution of iNKT cells in different immune responses as regulators has been acknowledged, the exact mechanisms polarizing their effector functions are only poorly understood.

Invariant NKT cells have, in general, a positive role in antiviral immunity, in particular through their ability to rapidly release IFN-γ (12–18). However, they can also display a detrimental role in virus-induced immunopathogenesis. For instance, using an experimental mouse model of chronic lung disease triggered by infection with Sendai virus, Kim et al. (19) showed that IL-13 production by iNKT cells is important in the pulmonary pathology. More recently, Stout-Delgado et al. (20) demonstrated that IL-17 production by iNKT cells from aged mice infected with herpes simplex virus 2 is sufficient to promote liver damage and death. Finally, we recently reported that iNKT cells produce IFN-γ during dengue virus infection and participate in the cytokine storm and vascular leakage, two major pathological features associated with severe dengue virus infection (21). Thus, the nature of the iNKT cell response is variable according to the virus and possibly the organ targeted.

To control iNKT cell functions in the future for therapeutic purposes, it is important to better understand how these cells become activated. It is likely that the recruitment of innate sensors by viruses in sentinel cells, such as DCs, is important for the indirect activation of iNKT cells (8, 9). In this setting, activation of iNKT cells might be triggered by cytokines produced from DCs, by TCR ligation with self lipids, or both (8, 9). In light of recent demonstrations that iNKT cells play a natural role during experimental influenza A virus (IAV) infection (22–24), we herein attempted to study the nature of the iNKT cell cytokine response during IAV infection. Using a mouse-adapted H3N2 IAV strain, we show for the first time that iNKT cells produce IL-22 during infection, a critical cytokine in mucosal immunity. We further present detailed mechanisms by which iNKT cells release IL-22 in the context of IAV infection. Finally, we show that IL-22 protects the pulmonary epithelium against damages during IAV infection.

EXPERIMENTAL PROCEDURES

Mice and Viruses—The IAV strain used in this study (Scotland/20/74, H3N2) was grown in 10-day embryonated hens eggs by standard procedures and titrated on Madin-Darby canine kidney cells as previously described (25). Six- to eight-week-old male wild type (WT) C57BL/6 mice were purchased from Janvier (Le Genest-St-Isle, France). Mice were bred in the SPF facilities of Pasteur Institute, Lille and Transgenose Institute, Pas-de-Calais). DRs. S. Akira and O. Takeuchi (Osaka University, Osaka, Japan), Dr. R. Flavell (Yale University, New Haven, CT), and the late Dr. J. Tschopp (University of Lausanne, Switzerland), respectively (27–32). Interleukin-22−/− C57BL/6 mice (33) were bred in the Ludwig Institute (Brussels, Belgium). For IAV infection, mice were maintained in a biosafety level 2 facility in the Animal Resource Center at the Pasteur Institute, Lille. All animal work conformed to the Pasteur Institute, Lille animal care and use committee guidelines (agreement no. AF 16/2009 from the Comité d’Ethique en Expérimentation Animale Nord Pas-De-Calais).

Reagents and Abs—Monoclonal Abs against mouse CD5 (APC-conjugated), NK1.1 (PerCP-Cy5.5-conjugated), TCRβ (FITC-conjugated), and isotype controls were purchased from BD Pharmingen. APC-conjugated and phosphatidyethanolamine-conjugated PBS-57-loaded CD1d tetramers were, respectively, obtained from Prolimmune (Oxford, UK) and the NIARD (National Institutes of Health) Tetramer Facility (Emory University, Atlanta, GA). The monoclonal Ab against mouse IL-22 (clone 3F11), a kind gift from Dr. W. Ouyang (Genentech, San Francisco, CA), was labeled with the APC conjugation kit from AbD Serotec (Colmar, France). APC-conjugated control mouse IgG2a mAb was from eBioscience. The neutralizing goat IgGs directed against mouse IL-1β, IL-23, TNF-α, and IL-6 were from R&D Systems. Recombinant mouse IL-23 was from Clinisciences, and recombinant mouse IL-1β and IL-22 were from Peprotech (Neuilly-sur-Seine, France).

IAV Infection and Assessment of Gene Expression by Quantitative RT-PCR—Mice were anesthetized and administered intranasally with 50 μl of PBS containing 600 plaque forming units of virus (Scotland/20/74, H3N2) or PBS alone (mock). Total RNA from cell-sorted pulmonary iNKT cells of mock-treated or IAV-infected mice was extracted, and cDNA was synthesized by classical procedures. Quantitative RT-PCR was carried out in an ABI 7500 Thermocycler (Applied Biosystems, Foster City, CA) using 0.5 μM concentrations of specific primers and QuantiTect SYBR Green PCR Master Mix (Qiagen). Primers specific for gapdh (5′-TGCCCAAGAACATCATCCC-TG-3′) and (5′-TCAGATCCAGCGCCAGACAC-3′), Ifng (5′-GTCCTGAATAACTTATTTTAACTCAAG-3′) and (5′-GGTGTTGACCTCAAACTTGGC-3′), Tal1 (5′-CAGAGCTATTGATGG-3′), Il-17A (5′-AGAATTCATGTGGTGGTCCAGC-3′), Il-21 (5′-AGAATTCATGTGGTGGTCCAGC-3′) and (5′-TCAGATCCAGCGCCAGACAC-3′), Il-23 (5′-TGAGCCGCA-CAGACGCTTTGAG-3′) and (5′-TCCTCTTCTCTGTCACCTCGTT-3′), Il-17A (5′-CGCAGATGAAGACCTGTAGA-3′) and (5′-AGAATTCATGTGGTGCTACCAGC-3′), Il-17F (5′-TGTCCTCCTCTGAGGATAAC-3′) and (5′-GACTG AGGGGTCTGCTGAA-3′), Il-21 (5′-AAACTCAACGCGCCACCTGCTGCA-3′) and (5′-TGAGGCGCCCTCTCCGAGGATAAC-3′), Il-22 (5′-TGATCCTCTCTGAGGATAAC-3′) and (5′-TGTCCTCCTCTGAGGATAAC-3′), Il-23 (5′-GGTGTTGACCTCAAACTTGGC-3′), and (5′-TTCCAGCCAGACGCGGACTTGAG-3′) and (5′-TCCTCTTCTCTGTCACCTCGTT-3′) were designed by the Primer Express Program (Applied Biosystems) and used for amplification in triplicate assays. PCR amplification of gapdh was performed to control for sample loading and to allow normalization between samples. Quantitative comparison was obtained through the ΔΔCt method. For graphical representation, data are expressed as -fold mRNA level increase compared with the expression level in lung iNKT cells from mock-treated mice.
iNKT Cells as Producers of IL-22 during IAV Infection

mice. Primers specific for IAV M2 gene 5′-AAGACCAATCC-
CTGTCACTCCTGA-3′ and 5′-CAAGGGTGCTACGCTG-
CAGTCC-3′ and for mouse hprt 5′-CAGGCCAGCTTTGT-
TGGAT-3′ and 5′-TTCGCGCTCATTTGCGTTT-3′ were
used to quantify viral load in iNKT cells. IAV and hprt copi-
s for each experimental sample were quantified in duplicate
using the standard curves obtained by PCR amplification on serial
dilutions of purified PCR products. Viral load is expressed as
viral RNA normalized to hprt expression level.

Preparation of Pulmonary and Liver iNKT Cells—Pulmonary
and hepatic mononuclear cells from mock-treated or IAV-
infected mice were prepared by classical procedures. Briefly,
lungs were perfused with PBS, excised, and finely minced fol-
lowed by enzymatic digestion for 20 min at 37 °C in PBS con-
taining 1 mg/ml collagenase type VIII and 1 μg/ml DNase type
I (Sigma). After washing, lung and liver homogenates were
resuspended in a 35% Percoll™ gradient, carefully layered onto
I (Sigma). After washing, lung and liver homogenates were
further purified according to the expression, or not, of the
CD45, phosphatidylethanolamine-conjugated PBS-57-loaded
CD1d tetramer and FITC-conjugated anti-TCR (Sigma). To purify
iNKT cells, mononuclear cells were labeled with
phosphatidylethanolamine-conjugated PBS-57-loaded CD1d
tetramer and FITC-conjugated anti-TCR/β Ab. After cell
surface labeling, cells were sorted using a FACSAria (BD Bio-
sciences). PBS57-loaded CD1d tetramer + TCR/β cell purity
after sorting was consistently >98%. In some cases iNKT cells
were further purified according to the expression, or not, of the
NK1.1 molecule using PerCP-Cy5.5-conjugated anti-NK1.1
Ab.

Collection of Bronchoalveolar Lavage Fluids—Bronchoalveo-
lar lavage (BAL) fluids were collected 2 or 4 days post-infection
(p.i.) by classical procedures in a 0.5-ml wash. Cytokines
were quantified using kits from R&D Systems.

Intracellular FACS Staining—Lung mononuclear cells were
cultured at 1 x 105 cells/ml in complete medium containing 10
ng/ml recombinant mouse IL-1β and IL-23 plus 10 μg/ml
brefeldin A (Sigma) at 37 °C for 4 h. After activation, cells were
washed and stained with LIVE/DEAD® Fixable Dead Cell Stain
kit (Invitrogen) in PBS for 30 min. The cells were washed and
incubated with appropriate dilutions of eFluor-conjugated
CD45, phosphatidylethanolamine-conjugated PBS-57-loaded
CD1d tetramer, V450-conjugated anti-TCR/β Ab, and PerCP-
Cy5.5-conjugated anti-NK1.1 Ab for 30 min in PBS containing
2% FCS and 0.01% NaN3. Cells were washed and fixed using IC
Fixation Buffer (eBioscience, CliniSciences, Montrouge,
France). Fixed cells were then permeabilized in permeabiliza-
tion 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).

Generation of Murine Bone Marrow-derived DCs, IAV Infection,
and Co-culture—Bone marrow-derived DCs were gener-
ated exactly as described in Paget et al. (34). DCs (1 x 105 cells)
were challenged with IAV at 1 multiplicity of infection (m.o.i.)
for 1 h at 37 °C without FCS. After incubation, cells were exten-
sively washed to remove unbound virus and incubated at 37 °C
in medium containing FCS. Cell-free supernatants were har-
vested after 24 h for cytokine analysis. For co-culture experi-
ments, treated DCs were cultured for 2 days with sorted iNKT
cells (106 DCs + 105 iNKT cells/well) in round-bottom 96-well
plates in RPMI supplemented with 5% FCS. In some cases
neutralizing or control Abs were added during the co-culture.
Co-culture supernatants were collected, and IFN-γ, IL-4, IL-17A/F,
IL-21, and IL-22 concentrations were measured by ELISA
(R&D systems, Abington, UK (IFN-γ, IL-2, IL-21, and IL-22)),
eBiosciences, San Diego, CA (IL-17A and IL-17F), and BD
Pharmingen (IL-4). Cytokines present in DC supernatants were
quantified using commercial ELISA kits distributed by R&D
systems (IL-12p40, IL-23, IL-1β, TNF-α), BD Pharmingen (IL-
6), and PBL Biomedical Laboratories (Piscatway, NJ) (IFN-β).

Preparation and Infection of Murine Airway Epithelial Cells—
Mouse primary airway epithelial cells (AEC) were prepared as
follows. Briefly, lungs were digested for 25 min in dissociation buffer
containing Pronase (1 mg/ml; Streptomyces griseus protease; Sigma).
After macrophage depletion (adherence onto polypropy-
lene plates), cells were resuspended on culture plates precoated
with BD Matrigel (BD Biosciences) in airway epithelial cell growth
medium (Promocell). After 2 weeks, more than 95% of the cells
were cytokeratin positive (Abcam, Paris). Before use, cells were
starved overnight in airway epithelial cell basal medium and pre-
incubated with recombinant murine IL-22 (Peprotech) for 4 h.
Cells were then infected with IAV at 1 m.o.i. for 1 h. Viral infection
was stopped by intensive changes of culture medium and addition
of serum. Expression of regenerating protein 3β (Reg3β) mRNA
was performed by quantitative RT-PCR using 5′-CGCATTGT-
TGCCCCAAGG-3′ and 5′-TCCAGGCTCTTTTGGCAG-3′.
Cell viability was evaluated 24 and 48 h after infection using Reso-
rufin (CellTiter-Blue Reagent, Promega).

IAV Infection and Assessment of Pathology—Four days p.i.
(100 plaque forming units), the BAL fluid and the whole lungs
were collected from WT and IL-22−/− mice. Analysis of cell
number in the BALs was performed as described (24). For his-
topathologic examination, fixed lung slices (5 μm sections)
were subjected to hematoxylin and eosin staining (24). Evalua-
tors who were blinded to genotype scored lung sections (0
(none)–3 (extreme) on the basis of hyperplasia of bronchial
epithelium and intercellular cohesion.

Analysis of Virus Load by Plaque Assay—Cell or whole lung
supernatants were diluted before being applied to 95% confluent
Madin-Darby canine kidney cells. Virus was adsorbed onto the
cells for 1 h at room temperature before being washed off four
times with serum-free DMEM. Cells were then covered with
serum-free DMEM containing trypsin and 0.8% agarose and incu-
bated without FCS at 37 °C for 4 h. After incubation, cells were
removed, and the monolayer was stained with 1% gentian violet to
allow for the visualization and counting of plaques.

Statistical Analysis—Results are expressed as the mean ±
S.D. or ± S.E. The statistical significance of differences between
experimental groups was calculated by one-way ANOVA with
a Bonferroni post-test or an unpaired Student’s t test (GraphPad
Prism 4 Software, San Diego, CA). The possibility of using these
parametric tests was assessed by checking if the population is
Gaussian and the variance is equal (Bartlett’s test). Results with
a p value of less than 0.05 were considered significant. The

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percentages of inhibition are averages of at least three independent experiments indicated under “Results.”

RESULTS

Pulmonary iNKT Cells Produce IL-22 Early after IAV Infection—Ourselves and others have provided evidence that (i)NKT cells are important controllers of host pulmonary responses during experimental IAV infection by H1N1 (22, 23, 35) and H3N2 (24) strains in the mouse system. To determine the nature of the cytokines produced by iNKT cells, which may be important for their biological activities, the transcript levels of cytokines expressed by iNKT cells were monitored during H3N2 IAV infection by quantitative RT-PCR. As shown in Fig. 1A and relative to iNKT cells purified from mock-treated animals, iNKT cells from IAV-infected mice expressed a higher level of transcripts for IFN-γ, a cytokine routinely produced by activated iNKT cells (~35- and ~12-fold enhancement at days 2 and 4 p.i.). In contrast, IL-4 and IL-17A/F mRNAs were not up-regulated. Of interest was the finding that, relative to control cells, FACS-sorted iNKT cells expressed a higher level of IL-22 messenger (~38-fold increase at day 2 p.i.), a cytokine produced mostly by terminally differentiated Th17 cells. This induction was transient as no enhanced IL-22 transcript level was detected at day 4 p.i. Notably, the expression level of IL-21 transcript, another member of the IL-17 family (for reviews, see Refs. 36 and 37)) remained near base line. To confirm these findings at the protein level, pulmonary iNKT cells were sorted

FIGURE 1. Production of IFN-γ and IL-22 by lung iNKT cells during in vivo IAV infection. A, shown is analysis of cytokine mRNA levels in sorted iNKT cells during IAV infection. Upper panel, iNKT cells were gated on lymphocytes expressing TCR-β and positive for the PBS57-loaded CD1d tetramer (shown are mock-treated and IAV-infected mice, 2 days p.i.). Despite a slight and not significant decrease in iNKT cell frequency, the number of detectable iNKT cells in the lung tissue remained stable 2 and 4 days p.i. Lower panel, invariant NKT cells were purified from the lungs of mock-treated and IAV-infected mice days 2 and 4 p.i. Sorted iNKT cells were analyzed for cytokine mRNA levels (lower panel). RNAs were prepared and IFN-γ (Ifng), IL-4 (Il4), IL-17A (Il17A), IL-17F (Il17F), IL-21 (Il21), and IL-22 (Il22) mRNA copy numbers were measured by quantitative RT-PCR. Data are normalized to expression of Gapdh and are expressed as -fold increase over average gene expression in iNKT cells isolated from mock-treated mice. Genes varying at least 2-fold were considered as significantly modulated. B, production of IFN-γ and IL-22 by iNKT cells sorted from IAV-infected mice is shown. Lung iNKT (PBS57-loaded CD1d tetramer TCRβ-T) cells were purified from mock-treated or IAV-infected mice (60 h p.i.) and cultured for 2 days without restimulation (1 x 10⁶ cells/well). Cytokines present in the supernatant were quantified by ELISA. A and B, data represent the mean ± S.D. (triplicates) of an experiment of two performed (pool of 10 mice/group).
from IAV-infected mice (60 h p.i.) and cultured without further restimulation. Compared with iNKT cells purified from mock-treated animals, pulmonary iNKT cells from IAV-infected mice produced higher levels of both IFN-γ and IL-22 proteins (Fig. 1B). In contrast, IL-4, IL17A/F, and IL-21 were not detected in the culture supernatant (data not shown). Collectively, upon IAV challenge, iNKT cells selectively produce IFN-γ and more, surprisingly, IL-22, a cytokine known to play a key role in mucosal defense (for reviews, see Refs.38–42). To our knowledge this is the first time that iNKT cells have been shown to produce IL-22 in the context of infection. For the rest of the study we focused on IL-22 production by iNKT cells.

**IAV Entry into iNKT Cells Does Not lead to IL-22 Production**—We next explored the mechanisms underlying the activation of iNKT cells in response to IAV.Invariant NKT cells have recently been shown to be targeted by herpes simplex type 2 virus to produce IL-17 (20). We thus hypothesized that IAV entry in iNKT cells could also result in cytokine release. Before addressing this issue in vitro, we first investigated whether iNKT cells are permissive to IAV. To this end, viral RNA (M2 transcript) was quantified in pulmonary iNKT cells purified from IAV-infected mice. As revealed in Fig. 2A, IAV M2 transcript was detected in iNKT cells 2 and 4 days p.i. Similarly, the transcript of the IAV M2 gene was detected in iNKT cells previously exposed to IAV in vitro (Fig. 2B, left panel). Thus, iNKT cells are susceptible to IAV infection. However, analysis of infectious progeny present in the culture supernatant by plaque assay indicated that IAV does not replicate productively in iNKT cells (Fig. 2B, right panel). Having established viral entry into iNKT cells, we next proceeded to test our hypothesis. For this, iNKT cells were exposed to IAV, and 48 h later, cytokines present in the supernatant were quantified by ELISA. As shown in Fig. 2C, IAV-infected iNKT cells did not produce IL-22 or IFN-γ. Of note, anti-CD3 re-stimulation, used here as a positive control, led to IFN-γ, but not IL-22, release by iNKT cells. To conclude, although permissive to IAV, iNKT cells are not directly activated by IAV to produce IL-22.

**IAV-infected DCs Activate NK1.1neg iNKT Cells to Produce IL-22**—Next, we wanted to determine whether iNKT cells could be indirectly activated by IAV-infected DCs, a cell population known to stimulate iNKT cells during stressful conditions (for reviews, see Refs. 1, 8, and 9). As depicted in Fig. 3A, mouse bone marrow-derived DCs exposed to IAV (m.o.i. = 1) matured in terms of co-stimulatory molecule (CD40, CD86) expression (upper panel) and cytokine (IL-12p40, IL-23, IL-1β, TNF-α, IL-6,
and IFN-β) production (lower panel and not shown). On the other hand, IAV-infected DCs failed to release IL-22 (Fig. 3A).

We next investigated whether DC maturation in response to IAV could lead to IL-22 release by iNKT cells. For this, iNKT cells were purified from the liver of naïve mice to obtain sufficient numbers of cells for in vitro studies. As seen in Fig. 3B, co-culture of IAV-infected DCs with iNKT cells resulted in IL-22 secretion. Dendritic cells have been shown to secrete...
IL-22 under certain circumstances (43, 44), but the use of DCs derived from IL-22−/− mice indicated that iNKT cells are the source of IL-22 in our setting. Invariant NKT cells can be subdivided into two main subsets based on the expression of NK1.1; the NK1.1pos subset being described to preferentially produce IFN-γ and IL-4 and the NK1.1neg subset, more prone to IL-17 release (45–49). As depicted in Fig. 3, NK1.1neg iNKT cells release IL-22 in response to IAV-infected DCs, whereas NK1.1pos iNKT cells failed to do so. Similar data were obtained when iNKT cells were sorted from the lung tissue (Fig. 3). IL-22 release by iNKT cells was also observed during infection (45–49). As depicted in Fig. 3, NK1.1pos iNKT cells release IL-22 in response to IAV, while NK1.1neg iNKT cells did not (Fig. 4). In combination, these data indicate that, among innate sensors recruited by IAV in DCs, the RNA sensors RIG-I and NLRP3 strongly contribute to the production of IL-22 by iNKT cells.

** FIGURE 4. Role of innate sensors in IL-22 release by iNKT cells.** WT, Mydd88−/− and lps-1−/− (panel A), WT and Thi7−/− (panel B), WT, Nod2−/−, and Rig-1−/− (panel C), or WT and Nlrp3−/− (panel D) DCs were co-cultured with purified liver iNKT cells. Cytokine production was quantified by ELISPOT. Data represent the mean ± S.E. of at least three independent experiments performed in triplicate. A–C, a one-way ANOVA has been used to analyze the variance followed by a Bonferroni multiple comparison test to compare all groups. **, p < 0.01; ***, p < 0.001. B–D, differences in mean were analyzed using the two-tailed Student’s t tests. **, p < 0.01.

As mentioned above, the NLRP3 inflammasome was shown to signal DCs upon IAV infection (55, 58, 63). However, Nlrp3 deficiency in DCs had no effect on IL-22 release by iNKT cells (Fig. 4D). The potential role of DC-derived cytokines was next studied, with a special focus on IL-1β, TNF-α, IL-6, and IL-23, which are known to play a role in IL-22 synthesis by T cells (65). As revealed in Fig. 4C, neutralizing Abs against IL-1β, IL-6, and IL-23 reduced the synthesis of IL-22 by iNKT cells (by ~65% and 55%, respectively), whereas the other tested Abs were without effect. Thus, IL-1β and IL-23 secretion by IAV-infected DCs is necessary and sufficient to activate the release of IL-22 by iNKT cells. To further confirm this, purified iNKT cells were treated with IL-1β and/or IL-23 in a DC-free culture system. As shown in Fig. 5D, whereas IL-1β or IL-23 individually failed to promote IL-22 synthesis, their combined addition induced IL-22 production by liver iNKT cells. Importantly, IL-22 production in response to IL-1β/IL-23 was not restricted to liver iNKT cells as pulmonar iNKT cells also produced it, as analyzed by intracellular FACS staining (Fig. 5E). Of note, only the NK1.1neg iNKT subset produced IL-22.

IL-22 Protects Lung Epithelial Cells against Death after In Vitro IAV Infection—We next asked whether IL-1β and IL-23, which are able to promote IL-22 synthesis by iNKT cells in...
*in vitro*, are produced in the lungs during IAV infection. As seen in Fig. 6A, IL-1β, IL-23, and IL-22 proteins were detected in the lungs of infected mice 2 days p.i. Moreover, the transcript level of REG3β, a known target of IL-22 (44), was enhanced during infection (Fig. 6B). This induction was IL-22-dependent as the level of REG3β mRNA remained at the baseline level in IAV-infected Il-22−/− mice. Thus, IL-22 is functional in the lungs of IAV-infected mice.

During infection, IAV initially targets the lung epithelium to replicate, a process leading to epithelial cell death and pathology. Although IL-22 was described to protect epithelial cells in response to a variety of environmental insults (65, 66), its potential role in the context of virus infection has not yet been evaluated so far. Before addressing this question, we first investigated the effect of IL-22 on primary AECs. As seen in Fig. 6C, IL-22 induced an increased transcript level of REG3β, which is known to play a role in epithelium fitness and survival (67). Having validated the biological activity of IL-22 on AEC, we next studied its potential role in the control of cell mortality triggered by IAV infection/replication. As represented in Fig. 6D, left panel, IAV induced cell mortality 24 h and, particularly, 48 h p.i. Strikingly, IL-22 strongly prevented the IAV-triggered cell death. In contrast, although IL-22 shares a structural similarity with the antiviral cytokine IFN-α (68), it failed to reduce IAV replication as assessed by plaque assay (Fig. 6D, right panel). Thus, IL-22 may play a protective role on epithelial cells during IAV infection.

**IL-22 Deficiency Leads to Morphological Alterations of Airway Epithelium during IAV Infection**—We then addressed the *in vivo* role of IL-22 during IAV infection, with a special focus
on airway epithelium. The lack of IL-22 did not significantly modulate the recruitment of neutrophils and mononuclear cells (monocytes/macrophages and lymphocytes) in the BAL fluids 4 days p.i. (Fig. 7A). The IAV-induced production of inflammatory cytokines and chemokines, including IL-6, IL-17A, and CXCL1, was not modulated in the absence of IL-22 (Fig. 7B). Moreover, IL-22 deficiency had no effect on viral replication in the lung tissue (Fig. 7C). Interestingly, the lack of IL-22 led to a more marked morphological alteration of the airway epithelium relative to controls (Fig. 7D). Indeed, compared with WT animals, IAV-infected Il-22−/− mice developed an enhanced bronchial hyperplasia, a consequence of epithelial cell damages. This effect was associated with an augmented loss of the intercellular cohesion of the epithelium. Together, this suggests that IL-22 plays a part in the control of epithelial damages caused by IAV infection.

DISCUSSION

In some, but not all, experimental models, iNKT cells play a key role in antiviral immunity and associated immunopathogenesis (for reviews, see Refs. 11, 69, and 70). However, the mechanisms by which these cells become activated during viral infection as well as their precise functions have not been fully elucidated. Our data show for the first time that iNKT cells produce IL-22 in vivo during infection. We also present detailed mechanisms by which these cells release IL-22 in the context of IAV infection, and we propose that the innate production of IL-22 might protect the pulmonary epithelium against damage caused by viral replication.

The paradoxical role of iNKT cells during viral infection can be attributed to the nature of the cytokines they produce. For instance, production of IFN-γ by iNKT cells enhances the innate and Th1-dependent immune responses of NK cells and CD8+ T cells, ultimately leading to the elimination of virus-infected cells (14, 17, 18). On the other hand, iNKT cells can play a detrimental role in specific models of virus-induced immunopathogenesis by producing IL-17A or IL-13 (19, 20). Others (22, 23) (H1N1) and ourselves (24) (H3N2) have shown that iNKT cells play a positive role in the control of the IAV-
associated pulmonary inflammation. To better understand how iNKT cells function in this system, we first monitored the nature of the cytokines they produce during the early course of IAV infection. Our data show that, relative to controls, iNKT cells from IAV-infected mice produce higher levels of transcripts for IFN-α (but not IL-4) and IL-22. On the other hand, mRNA levels of genes associated with the Th17 lineage, such as IL-17A/F and IL-21, were unchanged. Importantly, the preferential synthesis of IFN-α and IL-22 by iNKT cells in the context of IAV infection was confirmed at the protein level by ELISA.

Interleukin-22 is intensively studied at the moment due to its role in mucosal host defense and immunopathology, and it is of importance to better characterize IL-22-producing cell types as well as mechanisms involved in IL-22 expression. We thus attempted to identify the mechanisms by which iNKT cells release IL-22 in the context of IAV infection. Some viruses, including HIV, lymphocytic choriomeningitis virus, or herpes simplex viruses can selectively target iNKT cells (20, 71, 72). We first investigated whether iNKT cells could be directly activated by IAV to release IL-22. Our data show that H3N2 IAV can infect iNKT cells in vivo and in vitro. Of note, iNKT cells appear to support viral replication in vitro but, in clear contrast to epithelial cells, in a non-productive manner as revealed by plaque assay. In parallel, iNKT cells do not produce IL-22 in response to IAV, indicating that the mechanism of activation is indirect and probably requires APCs, such as DCs. Myeloid DCs can be infected by IAV in vitro and in vivo (73–75) and are particularly well equipped to directly activate iNKT cells. Our data show that IAV-infected DCs can promote IL-22 production by iNKT cells. Thus, our co-culture system mimics the in vivo situation, and we took advantage of this to identify the mechanisms implicated in IL-22 production by iNKT cells.

**FIGURE 7. Effect of IL-22 deficiency on IAV-associated epithelial damage in vivo.** Age-matched WT or Il-22−/− mice were infected with 100 plaque-forming units of IAV Scotland/20/74/H3N2 strain and then sacrificed 4 days p.i. A, total cells, neutrophils, macrophages, and lymphocytes in the BALs were counted. B, IL-6, IL-17A, and CXCL1 concentrations in BAL fluids were quantified by ELISA. C, the viral loads, expressed as plaque forming units (pfu)/mg of lung tissue, were determined by plaque assay. D, representative hematoxylin and eosin-stained tissue sections (magnification × 400) are shown. Sections were scored blindly for levels of epithelial hyperplasia and loss of intercellular cohesion (arrows). Results are representative of three repeated experiments. Data represent the mean ± S.D. (n = 5–11 mice/group). Significant differences are designated by an asterisk (*, p < 0.05 (two-tailed Student’s t test).
iNKT Cells as Producers of IL-22 during IAV Infection

data clearly show that the NK1.1neg subset, but not the NK1.1pos subset, produced IL-22 in response to IAV-exposed DCs.

During infection, the activation of innate sensors in DCs is important to activate iNKT cells (34, 50–53). Recognition of IAV by the host is elicited by various classes of pattern-recognition receptors including members of the TLR (TLR7, TLR3), NLR (NOD2 and NLRP3) and RIG-I-like receptors families (55–64, 76). So far, with the exception of TLR members, no studies have been devoted to investigate the role of cellular sensors expressed by DCs in iNKT cell activation. Our data show that TLR7/MyD88-dependent signaling pathways in DCs are important to promote IL-22 release by iNKT cells, whereas those from TLR3/TRIF are not (data not shown). In parallel, our data indicated a role for IPS-1/RIG-I signaling in IL-22 secretion by iNKT cells. On the other hand, the NLR NOD2 and NLRP3 are dispensable. This latter finding appeared surprising as stimulation of the NLRP3-dependent inflammasome activates caspase 1 to generate mature IL-1β, a cytokine involved in IL-22 production by iNKT cells (Fig. 6). However, the recent demonstration that RIG-I also triggers caspase-1-dependent inflammasome activation and, thus, IL-1β secretion by a mechanism independent of NLRP3 (77) likely explains our results. Together, our data strongly suggest that, in the context of IAV infection, recognition of virus genomic RNA in the endosome by TLR7 as well as recognition of the 5′ triphosphate end of viral RNA in the cytosol by RIG-I is critical to trigger, via DCs, IL-22 release by iNKT cells.

Stressed DCs have been shown to produce and present self lipid(s) to activate iNKT cells with the need for co-factors including IL-12, IL-18, and/or type I IFNs (34, 50–54, 78, 79). Thus, we investigated whether the CD1d molecule is necessary to activate iNKT cells or whether cytokines released by IAV-infected DCs are sufficient to do so. As is the case after murine cytomegalovirus infection (54), our data indicate that CD1d expressed by DCs is dispensable for the activation of iNKT cells. We next hypothesized that activating cytokines produced by IAV-infected DCs may compensate the lack of CD1d function. Using neutralizing Abs, we showed that IL-1β and IL-23 are necessary to induce IL-22 synthesis by iNKT cells. Furthermore, using recombinant cytokines (DC-free system), we showed that the combination of IL-1β and IL-23 induced IL-22 release by iNKT cells. Thus, along with the inflammatory cytokines IL-12 and IL18 (IFN-γ) (52, 54, 79), the combination of IL-1β and IL-23 is sufficient to promote cytokine (IL-22) release by iNKT cells in the absence of a CD1d-restricted agonist. Although the potential role of self lipids presented by iNKT cells themselves cannot be completely ruled out (47, 54), our data suggest that cytokine-, rather than TCR-driven signals are crucial in iNKT cell activation (IL-22 production) during IAV challenge, at least in vitro.

Along with other lymphoid cell types, including NKp46+ NK1.1− mucosal cells (also termed NCR22), mucosa-associated lymphoid tissue inducer cells, conventional NK cells (for reviews, see Refs. 40, 80, and 81), and certain subsets of γδ T lymphocytes (82–84), the present study shows that NK1.1pos iNKT cells also have the potential to produce IL-22. Our finding is in line with that of Doisne and colleagues (47), who recently showed that peripheral lymph node-resident NK1.1pos iNKT cells produce IL-22 in response to stimuli that mimic (bacterial) infection. It is noteworthy that in this setting the transcription factor retinoic acid receptor-related orphan receptor-γt plays a crucial part in IL-22 synthesis (47). Our unpublished results indicate that 40–50% of pulmonary NK1.1pos iNKT cells express receptor-related orphan receptor-γt (15–20% of the total iNKT cell pool) and that this subset is the only producer of IL-22 upon IL-1β and IL-23 stimulation. Attempts are now in progress to determine the precise function of this iNKT cell subset in the context of IAV infection. In parallel, one can wonder whether the early production of IL-22 by iNKT cells is physiologically important in vivo. A recent study has provided the first evidence that this could be the case. Indeed, in response to ConA stimulation, hepatic iNKT cells lacking the herpesvirus entry mediator receptor produced IL-22 to protect against hepatitis (85). IL-22 has both proinflammatory and tissue-protective properties depending on the context in which it is expressed (38). In the lung tissue the beneficial role of IL-22 has recently been demonstrated in experimental models of lung fibrosis induced by repeated exposure to Bacillus subtilis (83), of pneumonia triggered by Klebsiella pneumoniae (86), and of ventilator-induced lung injury (87). On the other hand, IL-22 is deleterious in a model of acute lung inflammation induced by bleomycin (66). The role of IL-22 during (pulmonary) viral infection is still elusive. Interestingly, a recent study demonstrated that during IAV (H1N1) infection, NK also produce IL-22 in the lungs (88). Whether or not the early production of IL-22 by iNKT cells and/or by other cell types is important during IAV infection awaits further studies. Keeping in mind that direct or indirect interactions between iNKT cells and epithelial cells are viewed as important in many physiopathological situations, including infection (for reviews, see Refs. 89 and 90), it is possible that iNKT cell-derived IL-22 has a particular function during IAV infection. Our study shows that IL-22 is biologically active in the lung tissue during infection and that it can protect IAV-infected epithelial cells, the primary target of IAV, against mortality in vitro. Moreover, our finding indicates that IL-22 plays a positive role in the control of epithelial damage early after IAV infection. On the other hand, IL-22 lacks direct antiviral activity against IAV both in vitro and in vivo, as recently shown for hepatitis B and C viruses (91, 92). These data are in contrast with those from Guo and Topham (88), who suggested that IL-22 rather favors viral replication by sustaining epithelial cells. The protective role of IL-22 on epithelial cells is in line with our and other recent observations showing an enhanced damage of the airway epithelium in mice lacking iNKT cells (23, 24). It is possible that the cytoprotective effect of IL-22 is mediated through induction of anti-apoptotic molecules or REG3β (66). Thus, although this remains to be demonstrated in vivo, iNKT cell-derived IL-22 may be important to sustain the respiratory epithelium and preserve the epithelial barrier during IAV infection. In parallel, IL-22 may promote the synthesis of epithelial cytokines/chemokines as well as antimicrobial peptides, some of which are known to neutralize IAV (93–95). Through these mechanisms, IL-22 may be of particular significance during IAV pneumonia, a hypothesis that is currently under active investigation.
To conclude, our results show for the first time that iNKT cells produce IL-22 in the context of an infection. Our data also characterize RIG-I-like receptors as a new class of innate sensors important for iNKT cell activation during viral infection and also reinforce the concept (96) that iNKT cells are uniquely equipped for immediate, cytokine-driven activation during stressful conditions, including infection. Finally, we suggest that IL-22 is important to control epithelial damage caused by viral infection, a finding that may be relevant during IAV-associated pathogenesis.

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