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Potential Role of Invariant NKT Cells in the Control of Pulmonary Inflammation and CD8+ T Cell Response during Acute Influenza A Virus H3N2 Pneumonia

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Influenza virus (IAV) infection results in a highly contagious respiratory illness leading to substantial morbidity and occasionally death. In this report, we assessed the in vivo physiological contribution of invariant NKT (iNKT) lymphocytes, a subset of lipid-reactive αβ T lymphocytes, on the host response and viral pathogenesis using a virulent, mouse-adapted, IAV H3N2 strain. Upon infection with a lethal dose of IAV, iNKT cells become activated in the lungs and bronchoalveolar space to become rapidly anergic to further restimulation. Relative to wild-type animals, C57BL/6 mice deficient in iNKT cells (Jκ18−/− mice) developed a more severe bronchopneumonia and had an accelerated fatal outcome, a phenomenon reversed by the adoptive transfer of iNKT cells prior to infection. The enhanced pathology in Jκ18−/− animals was not associated with either reduced or delayed viral clearance in the lungs or with a defective local NK cell response. In marked contrast, Jκ18−/− mice displayed a dramatically reduced IAV-specific CD8+ T cell response in the lungs and in lung-draining mediastinal lymph nodes. We further show that this defective CD8+ T cell response correlates with an altered accumulation and maturation of pulmonary CD103+ dendritic cells in the mediastinal lymph nodes. Taken together, these findings point to a role for iNKT cells in the control of pneumonia as well as in the development of the CD8+ T cell response during the early stage of acute IAV H3N2 infection. The Journal of Immunology, 2011, 186: 5590–5602.

Influenza infection is one of the most important causes of respiratory tract diseases and is responsible for widespread morbidity and mortality. Annual epidemics typically affect 5–15% of the population and are thought to result in 250,000–500,000 deaths annually (for review, see Ref. 1). Of the three types of influenza viruses, the type A virus is the most virulent to humans and is capable of infecting multiple mammalian and avian species. Human influenza A virus (IAV) can be further divided into different serotypes on the basis of the Ab response to the viral surface glycoproteins hemagglutinin and neuraminidase (1). The most important subtypes for humans are H1N1 and H3N2, the latter being currently associated with more severe disease (1, 2).

The pulmonary inflammation that develops during IAV infection is often due to deleterious host immune responses directed against the pathogen. The initial reactions to IAV are directed by the innate immune system and lead to the containment of viral replication without an excessive inflammatory response. The early response to IAV, which occurs during the first 48 h after the onset of primary infection, has been attributed to stromal cells (epithelial cells, fibroblasts) and immune cells such as neutrophils, macrophages, dendritic cells (DCs), and NK cells (3–6).

Invariant NKT (iNKT) cells represent a population of “innate-like” αβ T lymphocytes expressing markers associated with the NK lineage. These cells have the particularity of recognizing self and exogenous lipid Ags presented by the MHC class I-like molecule CD1d (for reviews, see Refs. 7–11). Upon lipid recognition through their TCR, iNKT cells swiftly secrete cytokines with opposing effects on immune responses. This functional property establishes iNKT cells as innate immune effector cells as well as regulators of adaptive immune responses. Numerous studies have shown that, upon intentional or natural activation, iNKT cells either suppress or enhance immune-mediated
responses during inflammation, cancer, autoimmune diseases and infection (for reviews, see Refs. 8–11). The natural role of iNKT cells in antiviral immunity and in the control of viral replication and pathology has been studied using Jo18Δ−/− mice, which lack iNKT cells. It was reported that the role of iNKT cells during experimental viral infection can vary according to the virus and experimental conditions (for reviews, see Refs. 12, 13). For instance, during HSV type 1 and 2 and lymphocytic choriomeningitis virus infections, iNKT cells play a positive role in the antiviral immune responses and virus-associated pathology (14–17), whereas they rather appear to be deleterious during Sendai virus (18), HSV type 2 (only in aged mice) (19), and Dengue virus serotype 2 (J. Renneses, R. Guabiraba, I. Maillet, R.E. Marques, S. Ivanov, J. Fontaine, C. Paget, V. Quemins, C. Favreau, B. Ryylef, M.M. Teixeira, and F. Trottstein, submitted for publication) infections. Studies using CD1d-deficient mice, which not only lack iNKT cells but also non-iNKT cells, have suggested that NKT cells (either iNKT cells, non-iNKT cells, or both) positively contribute to the immune response to respiratory syncytial virus (20), encephalomyocarditis virus (21), murine CMV (22), and Thelier’s murine encephalomyelitis virus (23). In contrast, non-iNKT cells are rather deleterious during coccasimvirus infection (24). Although suspected (25–27), the role of iNKT cells in human viral infections is not entirely clear.

The consequences of endogenous and exogenous activation of iNKT cells in the control of IAV infection represent an intense area of investigation at the moment. For instance, in the mouse system, in vivo stimulation of iNKT cells by the synthetic glycolipid α-galactosylceramide (α-GalCer) promotes an efficient immune response in the lungs that culminates in the control of viral load and improved disease course (28–32). The natural role of iNKT cells during experimental IAV H1N1 (PR8 strain) infection has been studied using CD1dΔ−/− mice (33–35) and Jo18Δ−/− mice (34). An early study of Benton et al. (33) using CD1dΔ−/−-deficient mice showed that NKT cells may contribute to, but are not required for, cross-protection to influenza viruses of different subtypes. More recently, De Santo et al. (34) and Ishikawa et al. (35), using a sublethal dose of IAV H1N1, showed that CD1dΔ−/−-deficient mice had an increased mortality rate relative to controls and failed to limit viral replication in the lungs, a phenomenon ascribed to a reduced IAV-specific CTL response. Using Jo18Δ−/− mice, De Santo et al. (34) demonstrated that iNKT cells are physiologically important in this setting. In this work, we studied the potential regulatory role of iNKT cells in the development of viral pathogenesis and host responses during acute IAV H3N2 infection. To address this issue, we deliberately chose a highly pathogenic, human-origin IAV strain (Scotland/20/74) that induces severe pneumonia in mice (36). We showed that in this system, endogenous activation of iNKT cells leads to a beneficial effect on the control of airway inflammation and to a delay in the fatal outcome. We also demonstrated that iNKT cells favor the initiation of the IAV-specific CD8+ T cell response, in part by promoting the accumulation and maturation of a particular subset (CD103+) of respiratory DCs in the lung-draining mediastinal lymph nodes (MLNs). These findings suggest a new role for iNKT cells in DC functions and extend the concept that pulmonary iNKT cells might be exploited in the future to trigger immune responses and to avert immune pathology during IAV infection.

Materials and Methods

Mice and viruses

Six- to eight-week-old male wild-type (WT) C57BL/6 (H-2b) mice were purchased from Janvier (Le Genest-St-Isle, France). Jo18Δ−/− mice and CD1Δ−/− mice, back-crossed at least 10 times in C57BL/6, were a gift from Dr. M. Taniguchi (Riken Institute, Yokohama, Japan) (37) and Dr. L. Van Kaer (Vanderbilt University, Nashville, TN) (38), respectively. OT-I TCR-transgenic mice were purchased from Charles River (St Germain sur l’Arbresle, France). For IAV infection, mice were maintained in a biosafety level 2 facility in the Animal Resource Center at the Pasteur Institute, Lille (Lille, France). All animal work conformed to the Pasteur Institute, Lille, Animal Care and Use Committee guidelines (agreement no. AF 16/2009 from the Comite´ d’Ethique en Expe´ rimentation Animale Nord Pas-De- Calais). The IAV strain used in this study (Scotland/20/74, H3N2) was grown in 10-d embryonated hens eggs by standard procedures and titrated on Madin-Darby canine kidney cells as described in Ref. 39.

Reagents and Abs

α-GalCer was from Axxora Life Sciences (Coger S.A., Paris, France). The IAV-derived (SLLFNFRAYV) and OVA-derived (SIINFEKL) peptides were obtained from the Institut de Biologie et Chimie des Prote´ines (Lyon, France). mAbs against mouse CD3 (allophycocyanin-conjugated), NK1.1 (PE- or PerCP-Cy5.5-conjugated), TCR-β (FITC-conjugated), CD69 (PE-conjugated), CD11b (PE- or PerCP-Cy5.5-conjugated), Gr1 (allophycocyanin-conjugated), CD8 (allophycocyanin- or FITC-conjugated), CD19 (allophycocyanin-conjugated), CD103 (PE-conjugated), CD11c (allophycocyanin-conjugated), granzyme B (FITC-conjugated), CD107a (FITC-conjugated), IFN-γ (PE-conjugated), CD86 (biotinylated), CD40 (biotinylated), and isotype controls were purchased from BD Pharmingen (Le Pont de Claix, France). Allophycocyanin-conjugated PBS-57 glycolipid-loaded CD1d tetramer was from the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University, Atlanta, GA), and PE-conjugated Pro5 MHC pentamer (H-2D*, P234-241; SLLFNFRAYV) was from ProImmune (Oxford, UK). Sterile OVA was kindly provided by Dr. G. Randolph (Mount Sinai School of Medicine, New York, NY). OVA was stained using a FluorReporter FITC Protein Labeling Kit from Molecular Probes (Invitrogen, Cergy Pontoise, France). Alamar blue was from A&H Serotec (Düsseldorf, Germany).

IAV infection and assessment of the pathology

For IAV infection, mice were anesthetized and intranasally administered 50 μl PBS containing 600 PFU IAV (Scotland/20/74, H3N2). Mice were then monitored daily for illness and mortality for a period of 16 d. Clinical manifestations appeared around day 8 and included arching of the back, ruffling of the fur, and slowing of activity. Within 9 and 12 d postinfection (p.i.), all mice demonstrated severe sickness and weight loss and death at day 16. Disease was assessed by measuring lung inflammation, viral load in the lungs, and lethality. Mice found to be moribund were euthanized and counted as dead. Mice were euthanized at various intervals for sampling the lung lumen by bronchoalveolar lavage (BAL). The BAL fluid was collected in 3 × 1 ml washes. After light centrifugation, total cell numbers per BAL was determined by manual counting under the microscope. A morphology-based differential cell count was conducted on cytospin preparations from the BAL fluid and stained with Diff-Quik solution (Sigma). For histopathologic examination, lungs were fixed by inflation and immersion in PBS 3.2% paraformaldehyde and embedded in paraffin. To evaluate airway inflammation, we subjected fixed lung slices (5-μm sections) to H&E staining. Evaluators who were blinded to genotype scored lung sections (0 [none] to 5 [extreme]) on the basis of edema, hemorrhages, recruitment of polymorphonuclear (PMN) cells, macrophages, lymphocytes, lesions of alveolitis and bronchitis, area of focal or diffuse consolidation, necrosis and metaplasia of pneumocytes: grade 1, minimal or mild focal lesions combined with the perivascular recruitment of a few inflammatory cells; grade 2, mild to moderate focal lesions within the alveoli and bronchi, a few cells located in the peri-vascular and peribronchial areas; grade 3, mild to moderate focal and diffuse lesions extended to a whole lobule with focal consolidation combined with perivascular and peribronchial recruitment of inflammatory cells; grade 4, severe lesions, focal and diffuse with large areas of lobular pneumopathy including severe lesions of alveolitis, bronchitis, and consolidation (between 20 and 40% of normal lung); grade 5, destruction of the lungs with severe lesions of alveolitis, bronchitis, with necrosis and large areas of consolidation extended to several lobules (<20% of functional lung parenchyma).

Analysis of virus load and of genes associated with viral replication by quantitative RT-PCR

Total RNA from lungs of naive or IAV-infected mice was extracted using RNase free beads for tissue lysis and RNAeasy RNA isolation kit (Qiagen, 5591
Pulmonary iNKT cells become activated during IAV infection

The potential recruitment/expansion of iNKT cells in the lung as well as their activation status have not yet been examined during IAV infection. To investigate this, C57BL/6 mice were infected with IAV Scotland/20/74 (H3N2), and the absolute number of pulmonary PBS-57 glycolipid-loaded CD1d tetramer$^+$ TCR$^+$ (iNKT) cells was first determined during the early course of infection. As depicted in Fig. 1A (upper panel), the number of detectable iNKT cells in the lung tissue remained stable at all time points analyzed (from days 2 to 7 p.i.). This finding was confirmed by quantifying the genomic level of V$\alpha$14J$\delta$18 TCR gene rearrangement (specific to iNKT cells) in the lung tissue using quantitative PCR (data not shown). To investigate the activation status of pulmonary iNKT cells, we monitored the expression of the early activation marker CD69. As revealed in Fig. 1A (lower panel), and relative to mock-treated animals, the level of CD69 expression on lung iNKT cells increased at days 2, 4, and 7 p.i. The potential accumulation and activation status of iNKT cells in the alveolar space were also analyzed. Although iNKT cells were almost undetectable in the BAL fluid of uninfected mice, we observed an influx of iNKT cells into the airways at day 4 p.i., with a peak at day 7 p.i. (Fig. 1B and data not shown). Finally, iNKT cells present in the alveolar space express a high level of...
CD69 (Fig. 1B, right panel). Thus, iNKT cells in lung parenchyma and airways display clear signs of phenotypic activation during the early phase of IAV infection.

It is known that after primary activation, iNKT cells enter into a state of nonresponsiveness and fail to produce cytokines upon subsequent stimulation (43–45). We took advantage of this property to confirm further that pulmonary iNKT cells become activated during IAV infection. To this end, lung cells from mock- or IAV-infected mice were stimulated with the iNKT cell superagonist α-GalCer, and cytokine release was quantified. As expected, lung cells from uninfected mice produced copious amounts of IFN-γ (Fig. 1C) and IL-4 (data not shown) in response to α-GalCer. In marked contrast, lung cells from mice infected 4 and 7 d earlier produced much lower amounts of cytokines upon α-GalCer stimulation. To verify that the observed effect was not dependent on decreased APC functions in the mixed lung cell culture, lung cells from naive or infected mice were cocultured with α-GalCer–pulsed bone marrow DCs. This procedure did not restore cytokine release by lung cells isolated from infected animals (data not shown), indicating that the effect is intrinsic to iNKT cells. Overall, IAV infection is accompanied by an activation of iNKT cells in the lungs followed by a state of nonresponsiveness.

Mice deficient in iNKT cells show significantly increased mortality but not reduced or delayed viral clearance

Excessive inflammation in the lung is detrimental after a variety of respiratory challenges. Infection of mice with IAV H3N2 (Scotland/20/74) causes acute bronchiolitis and pneumonia that eventually leads to death. It is known, in the mouse system, that iNKT cells are versatile controllers of inflammatory responses. To address the potential role of iNKT cells in the development and/or control of pulmonary immunopathology, WT and Jα18−/− (iNKT cell-deficient) mice were first used in survival studies with a lethal dose (600 PFU) of virus. In WT animals, the first clinical manifestations appeared on day 7 p.i. and within 8 and 12 d p.i., all animals demonstrated severe sickness ending in death (85% mortality at day 16) (Fig. 2A, left panel). Notably, Jα18−/− mice developed accelerated clinical manifestations and succumbed more rapidly to infection relative to WT animals. No survival was observed in infected Jα18−/− mice past day 11 after viral challenge (p < 0.05). The role of iNKT cell in disease regulation was confirmed using CD1d−/− animals, which lack iNKT cells as well as non-iNKT cells (Supplemental Fig. 1). These data are in line with those from De Santo et al. and Ishikawa et al. who reported enhanced mortality in IAV (H1N1)-infected Jα18−/− (34) and CD1d−/− (34, 35) mice relative to controls and strongly suggest...
Figure 2. Survival rates and viral load in WT and iNKT cell-deficient mice infected with a lethal dose of IAV. A. Age-matched WT or Jo18<sup>−/−</sup> mice were infected with 600 PFU IAV Scotland/20/74/H3N2 strain and then followed for mortality. In the left panel, the survival of Jo18<sup>−/−</sup> mice was monitored and compared with that of WT mice (n = at least 15 mice/group). Log-rank test for comparisons of Kaplan–Meier survival curves indicated a significant increase in the mortality of Jo18<sup>−/−</sup> mice compared with that of WT animals. *p < 0.05. In the right panel, Jo18<sup>−/−</sup> mice were i.v. injected with 1 x 10<sup>6</sup> cell-sorted NKT cells or with PBS 24 h before IAV challenge. The survival of reconstituted Jo18<sup>−/−</sup> mice was monitored and compared with that of Jo18<sup>−/−</sup> mice injected with PBS alone (n = 10 to 15/group). *p < 0.05. B. Analysis of the viral load in the lung of IAV-infected WT or Jo18<sup>−/−</sup> mice. In the left panel, on the stated days p.i., IAV M2 mRNA levels were measured by quantitative RT-PCR. Data are normalized to expression of hprt. Shown are IAV M2/hprt mRNA expression ratios. Data represent the mean ± SEM of three independent experiments performed in triplicate (n = 12/group/day). In the right panel, the viral load, expressed as PFU/mg of lung tissue, was determined by plaque assay 4 d p.i. Shown is the mean ± SEM of one representative experiment of two (n = 5). C. mx1 and ifnb mRNA copy numbers were determined by quantitative real-time PCR. Data are normalized to expression of gapdh and are expressed as fold increase over average gene expression in naive mice. Data represent the mean ± SEM of three independent experiments performed in triplicate (n = 12/group/day).

To determine whether the accelerated clinical signs and mortality in infected Jo18<sup>−/−</sup> mice are due to the impairment of viral clearance after influenza administration, the viral load was monitored at day 4 (the peak of viral replication) and day 7 p.i. by quantitative RT-PCR and plaque assay. At day 4 p.i., the viral load in the lungs in WT and Jo18<sup>−/−</sup> mice was identical (Fig. 2B). At day 7 p.i., both WT and Jo18<sup>−/−</sup> mice had a lower and similar viral load in their lungs. In agreement with this, the transcript levels of genes associated with viral replication (ifnb, mx1) were not significantly different between infected WT and Jo18<sup>−/−</sup> mice (Fig. 2C). Thus, in our experimental system, iNKT cell deficiency does not lead to an impaired containment and clearance of IAV in the lungs during infection. This suggests that, in this infectious system, inflammatory injury, rather than uncontrolled viral replication, is an important determinant of the more rapid mortality observed in Jo18<sup>−/−</sup> mice.

Mice deficient in iNKT cells develop increased pneumopathy

To compare the extent of pulmonary inflammation, lungs from WT and Jo18<sup>−/−</sup> mice were harvested for histology during the acute-phase response. Relative to infected WT animals, infected Jo18<sup>−/−</sup> mice developed a marked increase in lung inflammation (Fig. 3). This was characterized by a more marked infiltration of neutrophils and macrophages, but not lymphocytes, in the lung tissue (Fig. 3, bottom panel) and in the BALs (data not shown). Histological scoring of H&E-stained lung sections confirmed the significant enhancement of airway inflammation in Jo18<sup>−/−</sup> mice (Fig. 3, bottom panel). In WT mice, mild to moderate lesions of acute pneumopathy were observed including small and confluent areas of alveolitis, bronchiolitis, and one small focus of limited consolidation in ∼40% of mice. No necrosis of alveoli nor of bronchus epithelia was observed in WT infected mice. By contrast, lesions in Jo18<sup>−/−</sup> mice were more severe showing a larger spectrum of inflammatory lesions. In particular, severe lesions of
and alveolar macrophages between WT or Jα18−/− mice revealed no differences in the infection rate of bronchial epithelial cells 

crosis are indicated. Results of a representative experiment of three re-

type and consolidation. In the 

levels of immunopathology. The percentages of bronchial epithelium ne-

A

B

C

D

bronchiolitis with necrosis of epithelia were observed. Finally, the 

lungs of ~80% of Jα18−/− mice showed large areas of lobular consolidation. iNKT cell deficiency thus leads to a significant in-

crease in IAV-associated airway inflammation.

Mice deficient in iNKT cells show normal NK cell activation 

but an altered IAV-specific CD8+ T cell response in the lungs

In vivo activation of iNKT cells triggers downstream stimulation of various immune cells including NK cells and CD8+ T cells, known to be important during IAV infection. For instance, activated iNKT cells can stimulate NK cells to produce IFN-γ. To study the 

role of iNKT cells on the transactivation of NK cells in the context of IAV infection, we first compared the frequency of IFN-γ+ NK cells in the lungs of IAV-infected WT and Jα18−/− animals. As shown in Fig. 4A (left panel), pulmonary NK cells from WT 

infected mice labeled positively for IFN-γ (but not IL-4, data not shown) at days 4 and 7 p.i., as judged by intracellular FACS 

staining. Notably, iNKT cell deficiency did not reduce the frequency of IFN-γ+ NK cells and even significantly increased it at 

day 4 p.i. Similarly, the expression of CD107a (Fig. 4A, right panel) and granzyme B (not shown), two molecules known to 

participate in cellular cytotoxicity, was also enhanced on NK cells 

from Jα18−/− mice relative to that of WT mice at day 4 p.i. Thus, iNKT cells are not necessary to activate NK cells in our experimental IAV infection model.

IAV infection leads to a rapid recruitment, expansion, and activation of CD8+ T lymphocytes in pneumatic lungs. The virus-

specific CD8+ T cell response was thus compared in terms of cell 

number and IFN-γ production. For this, cells specific for an 
immunodominant Db-restricted CD8+ T epitope derived from the viral polymerase 2 protein (PA224–233) (46) were analyzed in the 
lungs of infected WT and Jα18−/− mice. As measured by MHC class I pentamer staining, CD8+ DPA224–233+ cells accumulated in 

the lungs of infected WT animals as early as 4 d p.i. and their 

absolute number was higher at day 7 p.i. (Fig. 4B). Notably, re-

lative to WT controls, Jα18−/− mice had a dramatically decreased 

number of IAV-specific CD8+ T cells in the lungs at days 4 and 7 

p.i. This phenomenon was also observed in the BAL (Supple-

mental Fig. 2). As depicted in Fig. 4C, the absolute number of 

IAV-specific CD8+ T cells was partially (~40%), but significantly, 

restored after adoptive transfer of NKT cells into Jα18−/− mice 

(shown is day 4 p.i.). Finally, upon restimulation with PA224–233, 

the release of IFN-γ by lung cells isolated from infected Jα18−/− 

mice was dramatically reduced compared with that from WT 

animals (Fig. 4D). These results demonstrate that iNKT cell de-


iNKT cells do not control the recruitment and the suppressive activity of CD11b+ Gr1+ cells in the lung during the early step of IAV infection

During H1N1 IAV infection, it appears that the defective IAV-

specific CD8+ T cell response observed in Jα18−/− mice is due to 

an enhanced recruitment and an increased suppressive activity of a population of myeloid cells expressing Gr1 (34). We thus 

investigated whether this is also the case in our experimental system using a pathogenic H3N2 IAV strain. To do this, we 

first quantified the frequency and the number of CD11b+ Gr1+ cells, 

defined as myeloid-derived suppressor cells (MDSCs) in mice 

(47). As shown in Fig. 5A, and relative to noninfected animals, 

CD11b+ Gr1+ cells accumulated in the lungs of infected mice at 

days 4 and 7 p.i. (by ~3.6- and 7.9-fold in terms of absolute 

numbers, respectively). However, no difference was observed, in 
terms of frequency and cell number, between IAV-infected WT 

and Jα18−/− mice (Fig. 5A). To analyze potential differences in 

their suppressive activity, CD11b+ Gr1+ cells were sorted and 
tested for their ability to reduce the proliferation of OT-I cells 

upon OVA peptide stimulation. As seen in Fig. 5B, CD11b+ Gr1+ 
cells from naive mice significantly inhibited OVA-specific T cell 

proliferation (by ~35%), but this effect was not enhanced with 
cells sorted from IAV-infected mice. Furthermore, the suppressive 
activity of CD11b+ Gr1+ purified from IAV-infected WT and 

Jα18−/− animals was not different. These data suggest that the 
defective IAV-specific CD8+ T cell response in the lungs of 
inNKT cell-deficient mice is not due to a heightened suppressive 
activity of CD11b+ Gr1+ cells in this organ.

Jα18−/− mice show impaired IAV-specific CD8+ T cell priming in MLNs

These findings prompted us to investigate the possibility that the 
diminished CD8+ T cell response in the lung could be caused by 
an altered primary stimulation in the MLNs. As shown in Fig. 6A, 

CD8+ DPA224–233+ cells accumulate in the MLNs of infected WT 

animals as early as 3 d p.i. In marked contrast, the absolute 

number of pentamer-positive CD8+ T cells was dramatically lower
Finally, the IAV-specific CD8+ T cell response was fully restored in Jα18-/- mice reconstituted with NKT cells (Fig. 6B). Together, these data strongly suggest that the altered pulmonary CD8+ T cell response observed in iNKT cell-deficient animals is due to a defected priming in the lung-draining lymph nodes (LNs).

**FIGURE 4.** Characterization of the NK cell and CD8+ T cell responses in IAV-infected WT and Jα18-/- mice. Four and seven days p.i., lung cells from infected mice were recovered and analyzed for NK cell and CD8+ T cell responses. A, In the upper panel, gated CD3+ NK1.1+ cells from mock and infected (4 d p.i.) WT or Jα18-/- mice were analyzed for intracellular IFN-γ (and granzyme, not shown) production and for cell surface CD107a expression. The average percentages ± SEM of pulmonary CD3+ NK1.1+ cells positive for IFN-γ (left panel) and CD107a (right panel) are represented (n = 9 mice/group). Of note, the total number of pulmonary NK cells is identical in influenza-infected WT and Jα18-/- mice (data not shown). B, Representative dot plots of gated PA224-233-specific CD8+ T cells from mock and IAV-infected (7 d p.i.) mice are shown. C, In the left panel, the number of PA224-233-specific CD8+ T cells represents the average (±SEM) of results obtained in three independent experiments performed (n = 10 mice/group). In the right panel, Jα18-/- mice were reconstituted or not with NKT cells and 4 d p.i., the number of PA224-233-specific CD8+ T cells in the lung tissue was determined by flow cytometry (n = 6/group). D, Lung cells were restimulated with PA224-233 peptide (10 μg/ml) for 3 d. IFN-γ production was measured by ELISA (n = 12). Of note, lung cells from naive WT and Jα18-/- mice produce equal amounts of cytokines after anti-CD3 stimulation (not shown). Although increased compared with noninfected animals, the number of cells, including total CD8+ T cells, in the lungs was not different between IAV-infected WT and Jα18-/- mice (data not shown). A and B, Differences in mean were analyzed using the two-tailed Student t test. C, A one-way ANOVA was used to analyze the variance followed by a Bonferroni multiple comparison test to compare all groups. *p < 0.05, **p < 0.01, ***p < 0.001.

in infected Jα18-/- animals. Finally, the IAV-specific CD8+ T response was fully restored in Jα18-/- mice reconstituted with NKT cells (Fig. 6B). Together, these data strongly suggest that the altered pulmonary CD8+ T cell response observed in iNKT cell-deficient animals is due to a defected priming in the lung-draining lymph nodes (LNs).
One-way ANOVA was used to analyze the variance followed by a Bonferroni multiple comparison test to compare all groups. The numbers of pulmonary CD11b+ Gr1+ within lung MNCs of IAV-infected mice. The lungs were harvested 4 and 7 d p.i., and the relative proportions (left panel) and cell numbers (right panel) of CD11b+ Gr1+ cells were calculated by FACS staining. Control refers to mock-inoculated mice. Data represent the mean percentage ± SEM of four independent experiments (n = 12). B, Analysis of in vitro suppressive activity of CD11b+ Gr1+ cells. OT-I splenocytes were cultured in the presence (or not) of the OVA peptide SIINFEKL (2 μg/ml) with or without pulmonary CD11b+ Gr1+ cells purified from mock or IAV-infected mice (day 4). Data represent the average ± SD of n = 5 mice/group. A, Differences in mean were analyzed using the two-tailed Student t test. B, A one-way ANOVA was used to analyze the variance followed by a Bonferroni multiple comparison test to compare all groups. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 5.** Analysis of CD11b+ Gr1+ frequency/number and suppressive activity in infected WT and Jα18−/− mice. A, Percentages and total cell numbers of pulmonary CD11b+ Gr1+ within lung MNCs of IAV-infected mice. The lungs were harvested 4 and 7 d p.i., and the relative proportions (left panel) and cell numbers (right panel) of CD11b+ Gr1+ cells were calculated by FACS staining. Control refers to mock-inoculated mice. Data represent the mean percentage ± SEM of four independent experiments (n = 12). B, Analysis of in vitro suppressive activity of CD11b+ Gr1+ cells. OT-I splenocytes were cultured in the presence (or not) of the OVA peptide SIINFEKL (2 μg/ml) with or without pulmonary CD11b+ Gr1+ cells purified from mock or IAV-infected mice (day 4). Data represent the average ± SD of n = 5 mice/group. A, Differences in mean were analyzed using the two-tailed Student t test. B, A one-way ANOVA was used to analyze the variance followed by a Bonferroni multiple comparison test to compare all groups. *p < 0.05, **p < 0.01, ***p < 0.001.

The accumulation of CD103+, but not CD11bhigh, pulmonary DCs to the MLNs is reduced in IAV-infected Jα18−/− mice

The migration of virus Ag-loaded pulmonary DCs to the MLNs plays a key role in the induction of the CD8+ T cell response early after IAV infection (42, 48–50). We thus hypothesized that the defective CD8+ T cell response observed in Jα18−/− mice could be due to an altered accumulation of respiratory DCs in the MLNs. To investigate this, endotoxin-free FITC-conjugated OVA was inoculated 2 d after IAV infection, and 24 h later the number of DCs positive for FITC was quantified in MLNs from WT and Jα18−/− mice. Because two predominant populations of respiratory DCs, which are mostly CD8α+ (51), have been described (52, 53), FITC+ DCs were discriminated on the basis of CD11b and CD103 expression. Respiratory DCs undergo spontaneous migration to secondary lymphoid tissues via afferent lymphatics (50, 54). In agreement with this, inoculation of FITC-conjugated OVA in noninfected animals resulted in the accumulation of CD11bhighCD103− (here referred to as CD11bhigh) and CD11b+CD103+ (here referred to as CD11b+CD103+) DCs in the MLNs (Fig. 7). In this setting, resident LN DCs (mostly CD103+CD11b−) were not FITC+. Relative to uninfected animals, the number of FITC+CD11bhigh and FITC+CD103+ DCs strongly augmented in the MLNs of IAV-infected WT animals (∼5.5- and 6.5-fold increase over controls, respectively). Notably, the increased number of emigrating FITC+CD103+ DCs in infected Jα18−/− mice was less substantial (∼3.4-fold increase compared with mock-treated Jα18−/− mice), whereas the fold increase of FITC+CD11bhigh DC number was of equal amplitude relative to that observed in infected WT animals (∼4.8-fold increase). Thus, the lack of iNKT cells results in a diminished accumulation of pulmonary CD103+ DCs in the MLNs during IAV infection.

iNKT cell deficiency leads to a reduced maturation of respiratory DCs in the MLNs

The maturation of DCs is a crucial step in the induction of T cell activation and polarization. In this setting, iNKT cells may serve as important components (55, 56). We thus compared the maturation status of DCs in the MLNs of infected WT and Jα18−/− mice (4 d p.i.). Before this, the number of lung-derived DCs, as well as MLN resident DCs, was quantified. Of note, relative to infected WT animals, the IAV-associated enhancement of CD103+ DC number in the MLNs of Jα18−/− mice was of lower amplitude, whereas that of CD11b+ and CD103−CD11b− (LN resident) DCs was not significantly affected (Fig. 8A). Thus, the increased accumulation of CD103+ DCs observed in Jα18−/− mice was partially (∼60%) overcome by NKT cell transfer (Fig. 8B). Infection with IAV induced an increased synthesis of CD86 and CD40 on CD103+ DCs from WT mice. In contrast, CD11b+ and CD103−CD11b− (LN resident) DCs was not significantly affected (Fig. 8A). Of interest, the reduced accumulation of CD103+ DCs observed in Jα18−/− mice was partially (∼60%) overcome by NKT cell transfer (Fig. 8B). Infection with IAV induced an increased synthesis of CD86 and CD40 on CD103+ DCs from WT mice. In contrast, CD11b+ and CD103−CD11b− (LN resident) DCs was not significantly affected (Fig. 8A). Thus, iNKT cell deficiency not only affects the accumulation of CD103+ DCs but also their maturation in the MLNs. It is likely that these phenomena are responsible, at least in part, for the reduced priming of CD8+ T cells in iNKT cell-deficient animals.

**Discussion**

In some, but not all, experimental models of viral infections, iNKT cells have been shown to exert positive or negative responses,
Viral infection of the respiratory tract can lead to the activation of iNKT cells, which is important in the development of the local IA V-immunopathology and susceptibility to mortality. We also suggest that iNKT cells play a part in the control of infection, endotoxin-free FITC–OVA was inoculated intranasally, and the number of FITC+ DCs was determined in WT or Jo18−/− mice (24 h post FITC–OVA inoculation). FITC+ DCs were discriminated on the basis of CD11bhiCD103neg (referred to as CD11bhigh), CD11bintCD103+ (referred to as CD103+), and CD103− CD11b+ (resident LN) total and FITC+ DCs in the MLNs of mock-treated or IA V-infected WT mice (one representative animal/group). Data in the bottom panel represent the mean percentage ± SD (n = 5). One representative experiment of three is shown. Of note, the total number of cells in the MLNs was not significantly different between infected WT and Jo18−/− mice (4.6 ± 0.93 × 10^6 and 3.9 ± 0.79 × 10^6, respectively, at day 4 p.i.). *p < 0.05, **p < 0.01.  

CD69 was strongly increased on pulmonary (tissue and BAL) iNKT cells. Finally, upon in vitro stimulation with the superagonist α-GalCer, lung iNKT cells from IA V-infected mice failed to produce cytokines suggesting that they acquired a hyporesponsive phenotype, a phenomenon that occurs after primo-activation (43–45). The mechanisms by which lung iNKT cells become activated during H3N2 infection are still unknown. In this setting, activation of iNKT cells might be triggered by cytokines produced from APC, by TCR ligation with self-lipids, or both (for review, see Ref. 57). Several viruses, including human CMV and Kaposi sarcoma-associated herpesvirus, vesicular stomatitis virus, vaccinia virus, HSV type 1 (low dose), have been shown to upregulate CD1d surface expression on APC (58, 59), whereas others, including Kaposi sarcoma-associated herpesvirus, vesicular stomatitis virus, vaccinia virus, HSV type 1 (high dose), and HIV 1, reduce its surface expression on APC (58, 59), whereas others, including Kaposi sarcoma-associated herpesvirus, vesicular stomatitis virus, vaccinia virus, HSV type 1 (low dose), have been shown to upregulate CD1d expression to escape iNKT activation (59–65). In the case of IA V, this evasion mechanism does not appear to occur, at least in vitro (Ref. 34, and our unpublished data). The mechanisms (role of the CD1d molecule and/or inflammatory cytokines) by which iNKT cells become activated during H3N2 IA V infection are currently being studied.
The clinical response to IAV infection ranges from mild disease to severe pneumonia, according to IAV strains and inoculation doses. In our experimental system, mice died at times after the peak of virus replication in the lung suggesting that inflammatory injury, rather than uncontrolled viral replication, is an important determinant of the fatal outcome. Physiologically, iNKT cells can augment or inhibit inflammatory responses through a variety of mechanisms depending on the context (i.e., sterile or nonsterile inflammation) and the targeted organ. Several experimental models have highlighted the detrimental role of iNKT cells in lung inflammation including allergic reaction, airway hyperreactivity, and (virus-induced) chronic obstructive pulmonary disease (18, 66, 67). In this study, we evaluated their potential regulatory function in IAV-induced pathology. In multiple repeated experiments, we consistently observed that mice lacking iNKT cells, both Jα18−/− and CD1d−/− mice, died more rapidly compared with infected WT animals, a phenomenon fully reversed by the adoptive transfer of FACS-purified NKT cells into Jα18−/− mice before IAV infection (data not shown). It is also possible that the reduced number of IAV-specific CD8+ T cells in Jα18−/− mice, or to the contrary, the enhanced infiltration of neutrophils and/or macrophages play a part in this phenomenon. Finally, iNKT cells might also influence lung repair processes for instance to counteract epithelial damage (i.e., necrosis). Attempts are now under way to investigate these issues and to identify more subtle functions of iNKT cells in IAV-associated pneumonia.

In contrast with Ref. 34 (H1N1 strain, sublethal dose), iNKT cell deficiency did not lead to an impaired containment and clearance of IAV in the lungs in our experimental system (H3N2 strain, lethal dose). This finding is not without precedent as iNKT cells have been shown to be dispensable to the control of the viral load in some experimental systems (22, 68, 69). In line with this observation, the lack of iNKT cells did not reduce the NK cell response. First, NK cell accumulation in the lung was almost identical in WT and Jα18−/− mice (data not shown). It is also possible that the reduced number of IAV-specific CD8+ T cells in Jα18−/− mice, or to the contrary, the enhanced infiltration of neutrophils and/or macrophages play a part in this phenomenon. Finally, iNKT cells might also influence lung repair processes for instance to counteract epithelial damage (i.e., necrosis). Attempts are now under way to investigate these issues and to identify more subtle functions of iNKT cells in IAV-associated pneumonia.

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cells and/or the local IL-12 production are sufficient to activate NK cells (70, 71). Of note, it is possible that the enhanced NK cell response in Jo18<sup>−/−</sup> mice at day 4 p.i. may exacerbate the disease by damaging the affected tissues through direct cytotoxicity or by the release of inflammatory cytokines and chemokines (72). We next examined the magnitude of the early IAV-specific CD8<sup>+</sup> T cell response in the lungs of infected Jo18<sup>−/−</sup> mice. In agreement with Ref. 34, which focused on a distinct immunodominant epitope (NP<sub>366-374</sub>), we observed that Jo18<sup>−/−</sup> mice exhibited a much less robust pulmonary CD8<sup>+</sup> T cell response than that of WT animals as assessed by PA<sub>224-233</sub> epitope-specific CD8<sup>+</sup> T cell number and IFN-γ production (Fig. 4B). Moreover, the frequency of granzyme B and CD107a-expressing CD8<sup>+</sup> T cells was also reduced by 30–50% in Jo18<sup>−/−</sup> mice (data not shown). Together, these data indicate that iNKT cell deficiency leads to a dramatically reduced CD8<sup>+</sup> T cell response in the lung, an effect that is not associated with a lack of control of viral replication as in Ref. 34 (H1N1 strain, sublethal dose). This difference can be explained by the fact that IAV-specific CD8<sup>+</sup> T lymphocytes confer protection against low (sublethal) dose, but not high dose (our study), viral challenge (73). In our system, the increased NK cell response in concert with the remaining CD8<sup>+</sup> T cell response may be sufficient to control the lung viral load in infected Jo18<sup>−/−</sup> mice. It is also possible that other cells of the immune system cooperatively control IAV replication.

We next attempted to better understand how iNKT cells cripple the magnitude of the lung CD8<sup>+</sup> T cell response during IAV infection. A recent report has suggested that iNKT cells not only control the recruitment of MDSCs in the lungs but also their suppressive function on the CD8<sup>+</sup> T cell response (34). The role of iNKT cells on the functions of MDSCs has recently been confirmed in the context of antitumor immunity (74). In our experimental system, the relatively low suppressive activity of CD11b<sup>+</sup> Gr1<sup>+</sup> cells isolated from noninfected mice was not enhanced p.i., whatever the mouse genotype (iNKT cell competent or deficient animals). Thus, unlike in Ref. 34, our data do not support a role for iNKT cells in the trafficking (and/or expansion) of CD11b<sup>+</sup> Gr1<sup>+</sup> cells into the lungs or in their immunosuppressive functions. Although the precise reasons for this discrepancy remain unclear, it may be due to experimental differences. For instance, the previous study used a sublethal dose of H1N1 IAV (34), whereas we used a lethal dose of H3N2 IAV. The time points when the cells were sorted (day 6 p.i. versus day 4 p.i.) as well as the basis of the sorting (Gr1<sup>+</sup> versus CD11b<sup>+</sup> Gr1<sup>+</sup> cells) may also explain this discrepancy. Our data also suggest that iNKT cell deficiency does not modulate the frequency/number of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cells in the lungs during infection (data not shown). These findings prompted us to investigate the possibility that the diminished CD8<sup>+</sup> T cell response in the lung could be caused by altered primary stimulation in the lung-draining MLNs rather than inhibition of T cell expansion in the lung tissue. Indeed, it is known that secondary restimulation of IAV-specific CD8<sup>+</sup> T response takes place in the lungs (as early as 4 d p.i.) and that it is dependent on the recruitment of TNF and inducible NO synthase-producing DCs (so-called TipDCs) (75–77). However, we did not observe differences in the frequency and number of TipDCs between infected WT and Jo18<sup>−/−</sup> mice (data not shown). Our data show that the IAV-specific CD8<sup>+</sup> T response that normally develops in the MLNs of WT animals is strongly reduced in Jo18<sup>−/−</sup> mice. This supports the hypothesis that iNKT cells act on the primary stimulation of CD8<sup>+</sup> T cells in the MLNs during IAV infection. It is known that DCs and iNKT cells can potentially influence their mutual stimulation/maturation, including during infection (55, 56). DCs act during the priming phase of iNKT cell activation, and iNKT cells promote DC maturation and cytokine production, in particular through IFN-γ production. Migration of respiratory DCs from the lungs to the regional MLNs is a key step in the initiation of virus-specific CD8<sup>+</sup> T cell responses after infection (48, 49). Our data show that the defective CD8<sup>+</sup> T cell response observed in infected Jo18<sup>−/−</sup> mice is associated with a reduced accumulation of respiratory CD103<sup>+</sup> DCs, but not CD11b<sup>+</sup> DCs, in the MLNs. This phenomenon may be due to a lack of (iNKT cell-derived) factors able to promote the selective emigration of this DC subset. The reduced number of CD103<sup>+</sup> DCs in the lung tissue of naive Jo18<sup>−/−</sup> mice (30–50% reduction compared with that in WT animals, data not shown) may also, in part, explain our observation. Recent findings have revealed that the CD103<sup>+</sup> DC subset is of crucial importance in the promotion of the CD8<sup>+</sup> T cell response during respiratory viral infection, including vaccinia virus and IAV (42, 78), although other DC subsets also play a role in the latter system (49). In parallel, our data show that CD103<sup>+</sup> DCs present in the MLNs express less CD86 and CD40 in infected Jo18<sup>−/−</sup> mice compared with that in WT animals. Thus, the lack of iNKT cells not only negatively affects the selective accumulation of respiratory CD103<sup>+</sup> DCs to the MLNs but also their maturation process. This latter finding is in line with a recent study reporting the preferential modulating effect of iNKT cells on DCs (in this case the CD8<sup>+</sup> DC subset) during infection (56). Thus, along with other recently described mechanisms (55, 56, 79, 80), our data suggest a new mechanism (promotion of DC emigration) by which iNKT cells may control the development of CD8<sup>+</sup> T cells in vivo. This observation may be relevant in many pathological situations.

Overall, our data suggest a key and early role for iNKT cells in an acute model of pneumonia triggered by an H3N2 IAV strain. During IAV infection, both endogenous and exogenous (α-GalCer) iNKT cell activation appear beneficial to promote immune responses and to control pulmonary inflammation. It is possible that a better understanding of the mode of in vivo iNKT cell activation and the precise functions they exert during IAV infection will be beneficial to better harness these cells for prophylactic (vaccine) and/or therapeutic purposes in the future.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Corrections


The tenth author's name was published incorrectly. The correct name is Giovanna Barba-Spaeth.

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Supplementary figure legends

Supplementary Figure 1: Survival rate in WT and CD1d<sup>−/−</sup> mice infected with a lethal dose of IAV. Age- and sex-matched WT or CD1d<sup>−/−</sup> mice were infected with 600 pfu IAV Scotland/20/74/H3N2 strain and then followed for mortality. The survival of CD1d<sup>−/−</sup> mice was monitored and compared to WT mice (n = at least 10 mice/group). Log-rank test for comparisons of Kaplan-Meier survival curves indicated a significant increase in the mortality of CD1d<sup>−/−</sup> mice compared to WT animals. **p < 0.01.

Supplementary Figure 2: Analysis of specific-CD8<sup>+</sup> D<sup>b</sup>PA<sub>224-233</sub><sup>+</sup> T cells in the BAL of IAV-infected WT and J<sub>α</sub>18<sup>−/−</sup> C57Bl/6 mice (600 pfu i.n., 4 days p.i). A, Representative flow cytometry plots defining the percentage of CD8<sup>+</sup> D<sup>b</sup>PA<sub>224-233</sub><sup>+</sup> T cells in the BAL of mock- and IAV-infected WT mice. B, The absolute number of CD8<sup>+</sup> D<sup>b</sup>PA<sub>224-233</sub><sup>+</sup> T cells in the BAL (pool of 4 mice, one experiment out of three is shown) of IAV-infected WT and J<sub>α</sub>18<sup>−/−</sup> mice are represented.
Supplementary Fig. 2

A

MHC I Pentamer (SSLENFRAYV)

CD8

0%

12.2%

B

Total number of Pmel-17-specific CD8+ T cells

WT mice  Jα18−/− mice