of pro- and anti-inflammatory cytokines upon stimulation with α-GC, a highly potent ligand for *i*NKT cells (Kawano et al. 1997). In contrast, cytokine production by *i*NKT cells is much more finely regulated under physiological environment, which could result in production of a set of Th2 cytokines (Sakuishi et al. 2007).

*i*NKT cells are segregated into $CD4^+CD8^-$ and $CD4^-CD8^-$ double negative (DN) subsets. It has been shown that each subset differs remarkably in their functional properties. In humans, about 40–60% of *i*NKT cells are $CD4^+$, and a large majority of the remaining cells are DN cells. Some *i*NKT cells express CD8α, but only very few cells co-express CD8β. The $CD4^+$ subset potently produces both Th1 and Th2 cytokines, whereas the DN population selectively produces the Th1 cytokines (IFN-γ and TNF-α) and preferentially up-regulates perforin in response to IL-2 or IL-12 (Gumperz et al. 2002; Lee et al. 2002). It is also known that the $CD4^+$ and DN *i*NKT cells differentially express chemokine receptors: CCR4 on $CD4^+$ cells and CCR1, CCR6, and CXCR6 on DN cells (Kim et al. 2002). These results suggest the presence of a functional dichotomy in *i*NKT cells.

3.1.2 iNKT Cells and Their Ligands

To evaluate the potential of *i*NKT cells to regulate autoimmune diseases, it is particularly important to understand how they recognize a glycolipid antigen bound to CD1d. The CD1d molecule, highly conserved among mammalian species (Exley et al. 2000), is primarily expressed on the cells of hematopoietic origin, including thymocytes, B cells, macrophages, and DCs, and could also be induced on T cells upon activation. The binding cleft of the CD1d molecule consists of two nonpolar lined grooves, which makes it ideal for the presentation of hydrophobic antigens such as glycolipids. In 1997, a marine sponge-derived glycosphingolipid, α-GC, was identified as a potent ligand for mouse *i*NKT cells (Kawano et al. 1997). It was subsequently found that α-GC is stimulatory for human *i*NKT cells as well (Brossay et al. 1998). Thereafter, a synthetic α-GC has been used extensively for research (Fig. 3). A widely supported view on the topology of TCR/ligand/CD1d is that the two lipid chains of α-GC would be inserted into the CD1d hydrophobic grooves and α-linked sugar moiety becomes accessible for the TCR of *i*NKT cells (McCarthy et al. 2007). More recently, crystal structure analysis has demonstrated that the invariant α-chain of the *i*NKT cells would selectively recognize the α-linked sugar of α-GC (Borg et al. 2007). It is of note that glycolipids with a-linked sugars such as α-GC could not be found in mammalian tissues, but are rather ubiquitously present in the environment. After LPS-negative α-proteobacteria extracts were found to contain glycosphingolipids stimulatory for *i*NKT cells, a growing number of bacterial lipid antigens has been shown to stimulate *i*NKT cells (Bendelac et al. 2007), including diacylglycerol glycolipid extracted from Borrelia burgdorferi (Kinjo et al. 2005). Given that the TCRs of *i*NKT cells recognize such pathogen-derived antigens, the lipid antigens may be an important initiator for triggering the immune response in bacterial and parasite infection. However, it has recently been demonstrated that *i*NKT cells are activated during infection without recognizing a bacteria component

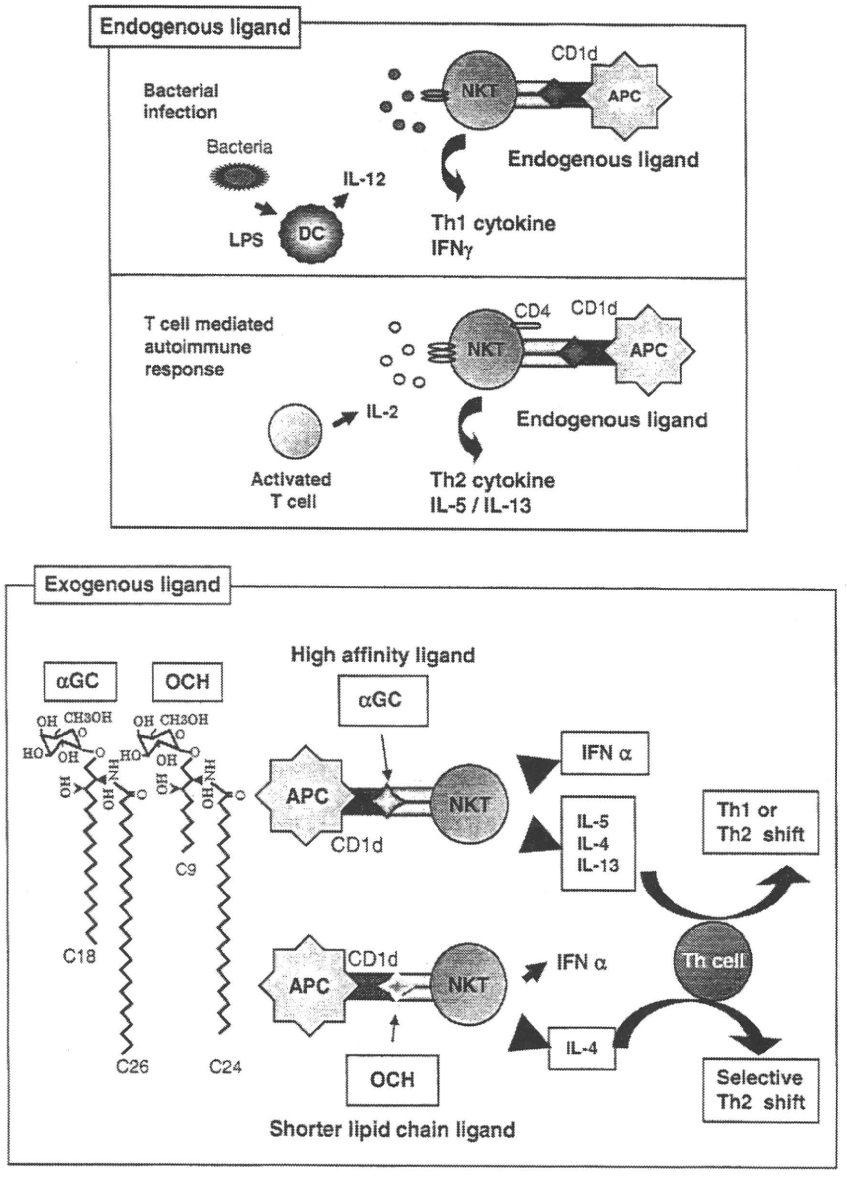

Fig. 3 Effects of lipid chain lengths in alpha-galactosylceramides on cyotokine release by natural killer T cells

via TCR (Brigl and Brenner 2004; Mattner et al. 2005). The antigen recognized by the TCR of *i*NKT cells is thought to be an endogenous ligand bound with CD1d, but not an exogenous microbial ligand. These studies also showed that the role for the bacterial LPS is to trigger production of IL-12 from DCs. Although *i*NKT cells

exhibit little response to the endogenous ligand/CD1d *i*NKT cells expressed by DCs, the presence of excessive amount of IL-12 would remarkably augment the *i*NKT cell response to endogenous ligand, which leads to production of a large amount of IFNγ from *i*NKT cells. Thus, *i*NKT cells may act as crucial amplifiers of Th1 cells in the initial inflammatory response to the pathogens.

Of note, not only Th1 but Th2 cytokine response could also be amplified through a similar mechanism. We have recently revealed that in the presence of excessive IL-2, TCR recognition of putative endogenous ligand would trigger production of IL-5 and IL-13 from human $CD4^+$ *i*NKT cells (Sakuishi et al. 2007). These findings indicate that under physiological conditions, cytokine milieu would be decisive in directing *i*NKT cell responses towards Th1 or Th2, and are relevant for understanding the mechanism of how *i*NKT cells would regulate the adaptive immune response in vivo (Fig. 3).

Since α-anomeric glycolipids do not exist in mammalian tissues, a number of β-anomeric glycolipids have been evaluated for their possible role as an endogenous ligand for *i*NKT cells. The search has led to the identification of lysosomal glycolipid isoglobotrihexosylceramide (iGb3) as a putative endogenous ligand (Zhou et al. 2004; Mattner et al. 2005). However, it has recently been demonstrated that *i*NKT cells are normal in number and function in iGb3 synthetase deficient mice, despite of lacking endogenous iGb3 (Porubsky et al. 2007). Moreover, a highly sensitive HPLC assay has failed to detect the presence of iGb3 in various mouse tissues except for the dorsal root ganglion. Nor was iGb3 detected in any human tissue (Speak et al. 2007). Therefore, the search for endogenous ligand is still not over. Regarding the pathogenesis of MS, it is of key interest whether any myelin-derived lipid antigen may stimulate *i*NKT cells.

Another subject of growing interest is to use *i*NKT cell ligands as therapeutic agents for autoimmune diseases. The prototypical ligand α-GC showed some efficacy for autoimmune diseases (Hong et al. 2001). However, as it provokes production of a wide range of cytokines including proinflammatory ones, it may worsen some disease conditions. To overcome this problem, structurally altered analogs of α-GC were synthesized and their ability to inhibit the development of autoimmune disease has been examined. A work from our laboratory has demonstrated that an α-GC analog bearing a shorter sphingosine chain compared with α-GC (named as OCH) would selectively stimulate IL-4 production from *i*NKT cells, whereas α-GC stimulation induces both IL-4 and IFNγ (Miyamoto et al. 2001; Oki et al. 2004). Accordingly, OCH stimulation of *i*NKT cells favors a Th2 bias of immune response in vivo as compared with α-GC stimulation and showed better efficacy for treatment of various autoimmune disease models (Fig. 3) (see Sect. 3.3 as well).

3.2 Studies of iNKT Cells in MS

Using single-strand conformation polymorphism (SSCP), a method for examining the TCR repertoire, we have previously analyzed blood samples from subjects with MS as well as other neurological diseases (Illes et al. 2000). Expression of the

invariant Vα24-Jα18 rearrangement, the invariant TCR α-chain expressed by human *i*NKT cells, was greatly reduced in the blood lymphocytes of the patients with MS, compared with those from healthy subjects. The reduction was not observed in the patients with other autoimmune/inflammatory neurological diseases. Interestingly, the Vα24-Jα18 TCR was only rarely found in the CNS lesions of MS but was often detected in the biopsy samples from chronic inflammatory demyelinating polyneuropathy (CIDP).

More recently, we have reanalyzed the frequency of *i*NKT cells in the peripheral blood of MS by using flow cytometry. A striking reduction of the total number of *i*NKT cells was confirmed in the peripheral blood of the patients with MS in a drug-free remission state (Araki et al. 2003). Interestingly, when $CD4^+$ and DN *i*NKT cells were analyzed separately, a remarkable *i*NKT cell reduction was found to reflect a great reduction of DN *i*NKT cells, that are known to preferentially produce proinflammatory cytokines (Gumperz et al. 2002; Lee et al. 2002). Moreover, we found that the $CD4^+$ *i*NKT cell lines from MS patients were significantly biased for Th2: they produced much more IL-4 than those from healthy subjects, although the production of IFN-γ was not altered significantly (Araki et al. 2003). Collectively, the changes found in *i*NKT cells (a reduction of DN and Th2 bias of $CD4^+$ *i*NKT cells) are thought to be beneficial for maintaining the remission state of MS.

It is also worthwhile to mention that the currently available drugs may exert their actions through targeting *i*NKT cells. Although the drug-free remission state of MS was associated with a great reduction of *i*NKT cells in the peripheral blood (Araki et al. 2003), patients who were continuously given a low dose oral corticosteroid showed a normal frequency of *i*NKT cells in the blood, indicating that oral corticosteroid treatment may restore the frequency of *i*NKT cells (Araki et al. 2004). Interestingly, the cytokine profile of DN NKT cells from the corticosteroid-treated MS showed a trend for Th2 bias. This may represent one of the mechanisms of the corticosteroid effects in MS and other autoimmune diseases.

In a recent longitudinal study, IFN-β treatment significantly increased the number of *i*NKT cells in the peripheral blood mononuclear cell within same patients (Gigli et al. 2007). Furthermore, *i*NKT cells of IFN-β treated individuals showed a dramatically improved secretion of INF-γ, IL-4, and IL-5 in response to α-GC stimulation compared with those isolated from the same individuals before IFN-β treatment. The study also showed up-regulation of key costimulatory molecules expressed by DCs in the IFN-β treated patients. Thus, immune regulatory effect of IFN-β therapy in MS may possibly mediate *i*NKT cells.

3.3 iNKT Cells as a Therapeutic Target in MS/EAE

Results of EAE studies give us clues to understanding the role of *i*NKT cells in the pathogenesis of MS. It is well known that SJL/J mice are very susceptible to induction of EAE and other autoimmune diseases. In this strain of mice, *i*NKT cells are reduced in number and defective in IL-4 production (Yoshimoto et al. 1995),

allowing us to speculate that the *i*NKT cell defects may account for the autoimmune susceptible nature. On the contrary, transgenic overexpression of the invariant TCR of *i*NKT cells was found to protect NOD strain of mice from development of EAE. This EAE protection was associated with an inhibition of antigen-specific IFN-γ production but was independent of IL-4 (Mars et al. 2002). These results indicate an inverse correlation of *i*NKT cell numbers/functions with the susceptibility to EAE, raising a simple idea that expanding *i*NKT cells may be beneficial for treating patients with MS.

After α-GC was identified as a potent ligand for *i*NKT cells, several laboratories have examined whether in vivo injection of α-GC may modify the clinical course of EAE by stimulating *i*NKT cells. A study by Singh et al. showed that α-GC is capable of down-modulating EAE, by inducing Th2 bias of *i*NKT cells (Singh et al. 2001). Furlan et al. also showed an efficacy of α-GC in EAE, but they did not reveal a Th2 bias but rather showed an enhanced IFNγ production by the liver *i*NKT cells (Furlan et al. 2003). In an independent study by Jahng et al., injection of α-GC with aim to suppress EAE resulted in diverse outcome, which depends on the administration route, timing of injection, and dose of this glycolipid (Jahng et al. 2001). Although the reason for these discrepancies remain unclear, it is possible that source of the mice, quality of the animal facilities, or even gut flora might have influenced the results.

It was subsequently found that CD28-B7 costimulatory signals play a critical role in stimulating *i*NKT cells with α-GC. When *i*NKT cells were stimulated with α-GC in the presence of anti-B7 (CD80) antibody *in vitro*, they selectively produced Th2 cytokines (Pal et al. 2001). *In vivo* stimulation of *i*NKT cells along with blocking CD28-B7 interactions was found to suppress the onset of EAE (Pal et al. 2001). These results collectively indicated that proper stimulation of *i*NKT cells might lead to suppression of pathogenic Th1 responses. We have then explored whether a Th2 polarizing ligand could be identified among α-GC analogs. As discussed briefly in Sect. 3.1.2, we have found that an analog of α-GC, called OCH, bearing a shorter sphingosine chain could selectively induce production of IL-4 but not of IFN-γ and could modulate disease process of EAE when injected *in vivo* (Miyamoto et al. 2001). This protective effect against the development of EAE was abrogated by a simultaneous injection of anti-IL-4 antibody. Moreover, the protective effect of OCH could not be seen in IL-4 knockout mice, indicating that IL-4 produced from *i*NKT cells is involved in the disease suppression.

The molecular mechanism for the selective IL-4 production by OCH has been intensively studied in our laboratory. Owing to the truncation of sphingosine chain, OCH binds to CD1d molecule less stably compared to α-GC. We are proposing that the unstable OCH-CD1d interaction, which does not allow continuous TCR stimulation, is a key to understanding the Th2 polarizing character of OCH (Oki et al. 2004). When *i*NKT cells are stimulated by a-GC, IL-4 is produced within a few hours, which is then followed by production of a large quantity of IFN-γ (Pal et al. 2001). Of note is that de novo protein synthesis is required for the *i*NKT cell production of IFN-γ but not of IL-4 (Oki et al. 2004). Subsequent analysis has revealed that c-Rel protein is selectively induced, when *i*NKT cells are simulated by α-GC. Inhibiting c-Rel expression in *i*NKT cells has led to a selective IL-4 induction as a result of

suppressed production of IFN-γ, as seen with OCH stimulation. Taken together, it can be postulated that unstable binding of OCH with CD1d leads to disrupted TCR signaling, which does not induce expression of c-Rel and of its down-stream molecule IFN-γ. Compared with α-GC, which is capable of fully inducing c-Rel and IFN-γ, OCH would exhibit a unique Th2 polarizing effect on *i*NKT cells *in vitro* and *in vivo*. Intriguingly, in vivo injection of OCH induces defective IFN-γ production not only by NKT cells but also by NK cells (Oki et al. 2005). Mechanistic analysis has revealed that an injection of OCH induces an insufficient induction of CD40L in addition to lower primary IFN-γ production by the NKT cells, leading to a marginal IL-12 production by DCs. A combination of these differences between OCH and α-GC stimulation would account for the lower secondary IFN-γ production by NKT and NK cells by OCH. Of note, McCarthy et al. have recently confirmed that shortening of the phytosphingosine chain increased the rate of lipid dissociation from CD1d molecule and induced less sustained TCR signals (McCarthy et al. 2007). In this study, they have also demonstrated the decreased affinity of TCR to OCH bound-CD1d.

Other lipid chain truncated analogs of α-GC have been reported to display a similar skewing of cytokine profile towards Th2 but the mechanism seems to differ from that found in OCH (Goff et al. 2004; Yu et al. 2005). Taken together, altered glycolipid provides attractive means for *i*NKT cells mediated intervention of inflammatory autoimmune disease such as EAE and human MS.

4 MR1- Restricted Invariant T Cells in MS

Another novel invariant NK cell receptor-positive T cell population besides *i*NKT cells has been described in mice and humans. They are preferentially located in the gut lamina propria and are generally termed mucosal-associated invariant T (MAIT) cells (Treiner et al. 2003). Of interest, they are absent in germ-free mice, which indicates the role of gut flora for generation and maintenance of this lymphocyte. The discovery of this population is dated back to 1993, when DN T cell population expressing an invariant TCR α-chain was described along with the identification of Vα24 *i*NKT cells (Porcelli et al. 1993). It is now established that the new invariant T cells are distinct from *i*NKT cells in the expression of another conserved CDR3α sequence (Vα7.2-Jα33 in humans and Vα19-Jα33 in mice) and restricted use of Vβ2 and Vβ13 in mice and humans. Unlike *i*NKT cells selected by CD1d, they are selected by another MHC class Ib molecule, MR1, that is also highly conserved among species (Treiner et al. 2003). The mouse MAIT cells were isolated from $NK1.1^+$ T cells in the liver of CD1d deficient mice lacking "conventional" *i*NKT cells, allowing us to call the cells "Vα19-Jα33 NKT cells." As seen with "conventional" NKT cells, human MAIT cells constitutively express memory phenotype and some NK cell markers other than CD57 (Treiner et al. 2005) (Fig. 1). Several lines of evidence suggest that MR1 presents lipid ligands such as α-mannocylceramide (Shimamura et al. 2007). Although the function of MAIT cells is unclear at the moment, their cardinal features such as the semiinvariant repertoire, restriction by

monomorphic class I-like molecule and the natural memory phenotype suggest that *i*NKT cells and MAIT cells may exhibit similar and/or complementary functions.

When expression of Vα7.2 invariant TCR for human MAIT cells was investigated in MS patient samples, there was a striking difference between the MAIT and *i*NKT cell invariant TCR in their expression. Expression of the invariant TCR chain for NKT cells was clearly reduced in the peripheral blood of MS patients (Illes et al. 2000), whereas invariant TCR for MAIT cells was clearly detected in the great majority of the patients (Illes et al. 2004). Parallel analysis of CNS lesions from MS patients showed that MAIT cells would infiltrate the majority of the lesions, whereas *i*NKT cells do not (Illes et al. 2000, 2004). The differential expression of the two invariant chains in samples from MS suggests that MAIT cells and NKT cells may complement each other and MAIT cells may substitute deficiency of *i*NKT cells in MS.

The protective role of MAIT cells is further delineated by the study of mouse EAE. We found that overexpression of the invariant Vα19-Jα33 TCR in B6 mice is protective against EAE induction and progression (Croxford et al. 2006). Consistently, EAE was exacerbated in MR1 deficient mice, which lack Vα19-Jα33 invariant T cells. The protective effect was found to accompany a reduced production of inflammatory mediators as well as an increased secretion of IL-10. We have also demonstrated that IL-10 production occurred in part through interactions between B cells and Vα19 MAIT cells involving ICOS costimulatory molecule.

5 Concluding Remarks

NK cells and *i*NKT cells are groups of innate lymphocytes with multi potential qualities. Recent advances in cell biology of these cells have brought our attention to their ability in regulating autoimmune inflammatory responses. Selective induction of their regulatory properties could be an effective means for modification of autoimmune disease affecting the CNS. It is also notable that NK cells and *i*NKT cells change their phenotypes, number, and gene expression profile during disease course of MS. They could be good targets also for those who attempt to identify useful biomarkers for MS.

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Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity

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Infection with influenza A virus represents a major public health threat worldwide, particularly in patients with asthma. However, immunity induced by influenza A virus may have beneficial effects, particularly in young children, that might protect against the later development of asthma, as suggested by the hygiene hypothesis. Herein, we show that infection of suckling mice with influenza A virus protected the mice as adults against allergen-induced airway hyperreactivity (AHR), a cardinal feature of asthma. The protective effect was associated with the preferential expansion of $CD4^-CD8^-$, but not $CD4^+$, NKT cells and required T-bet and TLR7. Adoptive transfer of this cell population into allergen-sensitized adult mice suppressed the development of allergen-induced AHR, an effect associated with expansion of the allergen-specific forkhead box $p3^+$ ($Foxp3^+$) Treg cell population. Influenza-induced protection was mimicked by treating suckling mice with a glycolipid derived from *Helicobacter pylori* (a bacterium associated with protection against asthma) that activated NKT cells in a CD1d-restricted fashion. These findings suggest what we believe to be a novel pathway that can regulate AHR, and a new therapeutic strategy (treatment with glycolipid activators of this NKT cell population) for asthma.

Introduction

Bronchial asthma, a complex and heterogeneous trait, is a major public health problem, affecting nearly 10% of the general population and disproportionately affecting children. Moreover, the prevalence of asthma has increased dramatically over the past 3 decades, an increase thought to be due to changes in our environment. These environmental changes include reductions in the incidence of infectious diseases that may exert protective effects against asthma, as suggested by the hygiene hypothesis (1). While the infectious agents responsible for this relationship, and the precise mechanisms by which infectious microorganisms might protect against asthma, are very poorly understood, epidemiological studies suggest that infection with bacteria (e.g., *Helicobacter pylori* [refs. 2, 3], endotoxin [ref. 4], or *Acinetobacter lwoffii* [ref. 5]) or viruses (e.g., hepatitis A virus [refs. 6, 7]) might reduce the likelihood of developing asthma.

The role of viral infection in modulating the development of asthma is particularly complex because many different viruses affect the respiratory tract, some appearing to enhance and some to protect against the development of asthma. For example, infection with human rhinovirus in children before 3 years of age increases the later risk of developing asthma (8), while other respiratory viral infections appear to protect against the later development of asthma (9–14). However, in older individuals with established asthma, respiratory viral infection, particularly with influenza A virus, almost always triggers acute symptoms of asthma (15–17). These discrepancies may be due to the timing of the infection, since infection in very young children may profoundly alter the developing innate immune system in such a way as to protect against the later development of asthma, or to the specific immunological cell types activated by a given infectious agent.

To improve our understanding of the role of respiratory viral infection in children in the development of asthma, we studied a mouse model of asthma in which suckling mice were infected with the influenza A virus (H3N1), and were subsequently studied as adults for susceptibility to allergen-induced airway hyperreactivity (AHR), a cardinal feature of asthma. We found that H3N1 infection in suckling mice protected the mice as adults against allergen-induced AHR. The protective effect was associated with the preferential expansion of a subpopulation of suppressive double-negative (DN) NKT cells and was mimicked by treatment of suckling mice with several specific glycolipids, including one derived from *H. pylori*.

Results

Infection of suckling mice with H3N1 protects against AHR. We infected suckling pups (2 weeks old) or adult mice (8 weeks old) with the influenza A/Mem71 (H3N1) virus, and 6 weeks later the mice were examined for susceptibility to OVA-induced AHR (Figure 1A). H3N1 infection

Authorship note: Michio Shimamura, Petr Illarionov, and Dale T. Umetsu contributed equally to this work.

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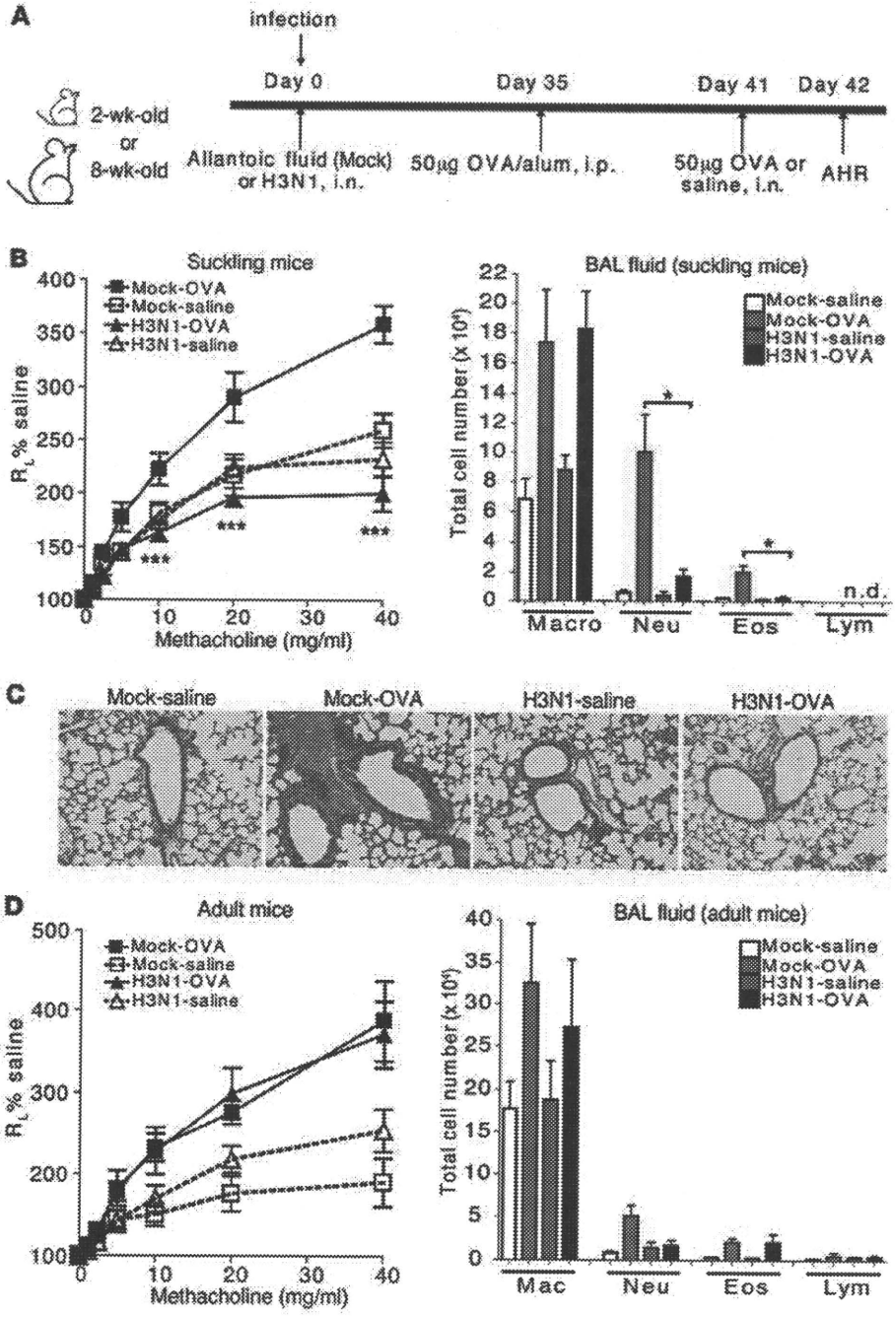

Figure 1
Infection of suckling mice with H3N1 protects the mice against AHR when adults. (**A**) Schematic showing the protocol for OVA-induced AHR. Two-week-old (suckling) or 8 week-old (adult) mice were treated with influenza A virus (H3N1) or control AF (mock infection) and assessed 6 weeks later as adults for AHR. (**B**) BALB/c mice ($n = 8$ per group) treated with H3N1 or AF at 2 weeks of age were assessed 42 days after infection for OVA-induced AHR. Changes in lung resistance (R_L) were measured in anesthetized, tracheotomized, intubated, and mechanically ventilated mice (left panel). ***$P < 0.001$ compared with mock-infected group. Cells in BAL were collected and analyzed 24 hours after the final OVA challenge (right panel). *$P < 0.05$ compared with mock-infected group. (**C**) Representative lung sections stained with H&E (original magnification, ×10) from mock- or H3N1-infected mice treated with saline or challenged with OVA. (**D**) Eight-week-old BALB/c mice ($n = 5$ per group) were infected with H3N1 or AF. Six weeks after infection, the mice were assessed for OVA-induced AHR by measuring lung resistance (left panel). Cells in BAL were collected and analyzed 24 hours after the final OVA challenge (right panel). Data are representative of 3 independent experiments.

in 2-week-old mice protected the mice as adults (at 8 weeks of age) against OVA-induced AHR (Figure 1B) and airway inflammation (Figure 1, B and C). In contrast, severe OVA-induced AHR and airway inflammation developed in the mock-infected mice at 8 weeks of age. Whereas infection in 2-week-old suckling mice conferred protection, infection in 8-week-old adult mice with H3N1 did not protect against subsequent OVA-induced AHR or airway inflammation (Figure 1D).

Adoptive transfer of NKT cells cannot reconstitute OVA-induced AHR in Jα18$^{-/-}$ mice. Infection with a different influenza virus strain (H3N2) enhanced the ability of respiratory tolerance to prevent OVA-induced AHR (11), consistent with the idea that influenza infection is complex and can affect multiple compartments of the immune system. Because infection with the influenza A virus has been shown to directly activate NKT cells (18), which play a very important role in asthma (19), we asked whether infection with the H3N1 virus affected the function of NKT cells. We therefore purified NKT cells from mice infected with H3N1 as sucklings (42 days after infection) and adoptively transferred these cells (92%–97% purity; Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI44845DS1) into adult OVA-sensitized, NKT cell–deficient recipients (Jα18$^{-/-}$ mice) (Figure 2A). After receiving the H3N1-exposed NKT cells, the Jα18$^{-/-}$ mice, which have CD1d-restricted non-invariant (but not invariant) TCR NKT cells, and which cannot develop

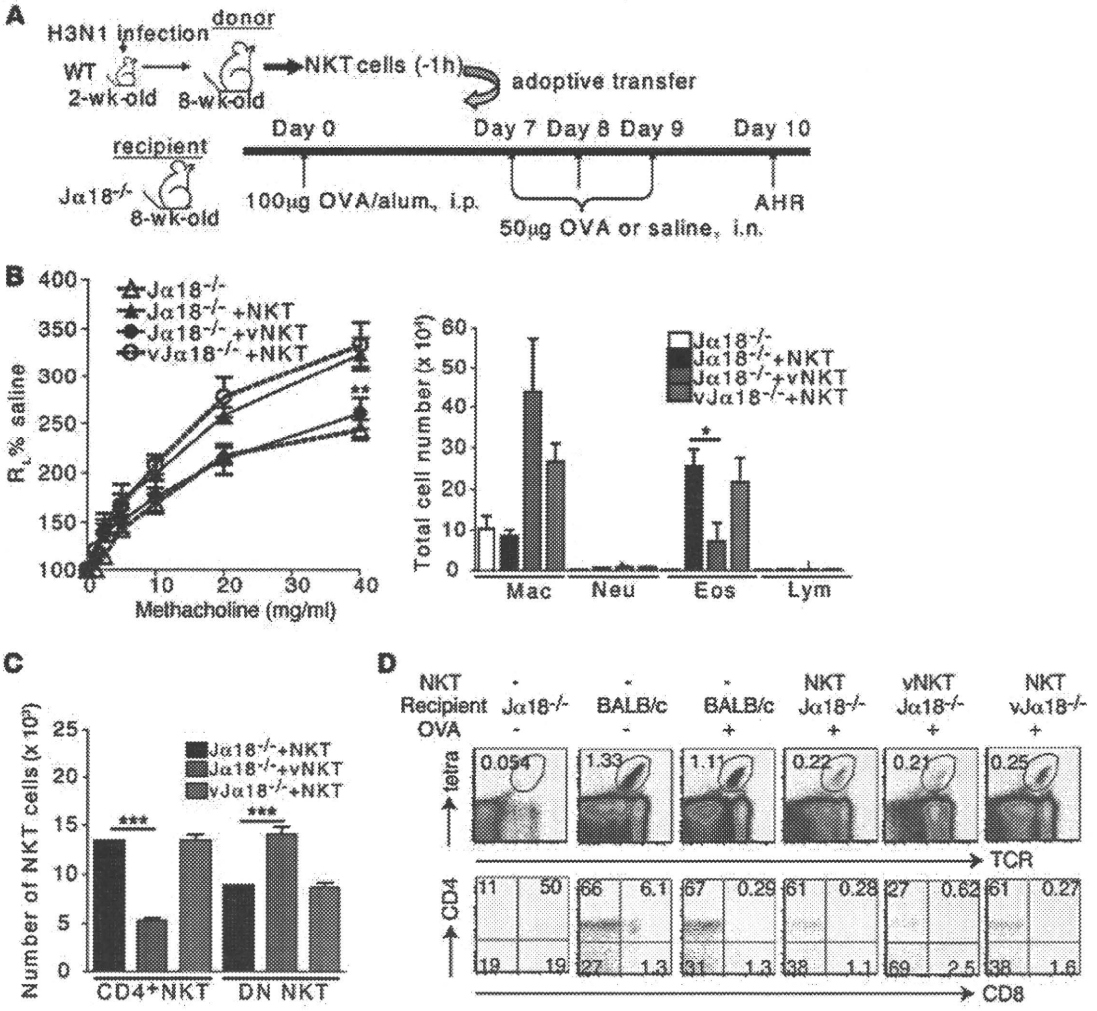

Figure 2

Adoptive transfer of H3N1-exposed NKT cells fails to reconstitute OVA-induced AHR. (**A**) Schematic showing the protocol for adoptive transfer of NKT cells to OVA-immunized *Jα18*$^{-/-}$ recipients. The donor mice were infected with H3N1 or mock infected at 2 weeks of age. Six weeks after infection, NKT cells were purified and adoptively transferred into OVA-sensitized *Jα18*$^{-/-}$ mice, which were then challenged with OVA and assessed for AHR. (**B**) Adoptive transfer of H3N1-exposed NKT cells (vNKT) to *Jα18*$^{-/-}$ mice failed to reconstitute OVA-induced AHR (measured as lung resistance in response to methacholine challenge) (left panel). Adoptive transfer of NKT cells from mock-infected mice (NKT) fully reconstituted AHR. H3N1 infection at 2 weeks of age of *Jα18*$^{-/-}$ mice (v*Jα18*$^{-/-}$) and reconstitution at 8 weeks of age with NKT cells from mock-infected mice did not protect against AHR (n = 8–10 per group). BAL fluid was collected and analyzed (right panel). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with *Jα18*$^{-/-}$ + NKT group. (**C** and **D**) Lung cells were isolated from the recipients after measurement of AHR, and the absolute numbers (**C**) and percentages (**D**) of lung CD4^{+} or CD4^{-}CD8^{-} (DN) NKT subsets were assessed by FACS. Upper panels show dot plots for NKT cells in lung leukocytes. After gating on the NKT cells, the cells were analyzed for CD4 and CD8 (lower panels). $^{***}P < 0.001$ compared with WT NKT group. Data are representative of 3 independent experiments.

allergen-induced AHR unless reconstituted with functional invariant TCR NKT cells (20–22), failed to develop OVA-induced AHR (Figure 2B). In contrast, transfer of NKT cells from mock-infected mice to *Jα18*$^{-/-}$ mice fully reconstituted AHR. Moreover, H3N1 infection in 2-week-old *Jα18*$^{-/-}$ suckling mice (*vJα18*$^{-/-}$ mice) and later reconstitution (at 8 weeks of age) with NKT cells from mock-infected mice did not prevent OVA-induced AHR (Figure 2B), indicating that early exposure of all of the non-NKT cells in *Jα18*$^{-/-}$ mice (e.g., conventional CD4^{+} and CD8^{+} T cells) to H3N1 was not effective in preventing AHR. Finally, in the lungs of mice receiving the H3N1 virus–exposed NKT cells (42 days after infection), significantly more CD4^{-}CD8^{-} (DN) NKT cells and significantly fewer CD4^{+} NKT cells were present (Figure 2, C and D), suggesting that H3N1 infection of 2-week-old suckling mice reduced the inflammatory function of the NKT cells, possibly by altering the CD4^{+} versus DN NKT cell subset proportions.

H3N1 infection accelerates the expansion of pulmonary NKT cells in suckling mice. In 2-week-old naive suckling mice, few NKT cells were present in the lungs, although this number increased normally to adult levels over a 6-week period (Figure 3A). Importantly, H3N1 infection but not mock infection in suckling mice greatly accelerated the expansion of the pulmonary NKT cell numbers (Figure 3B).

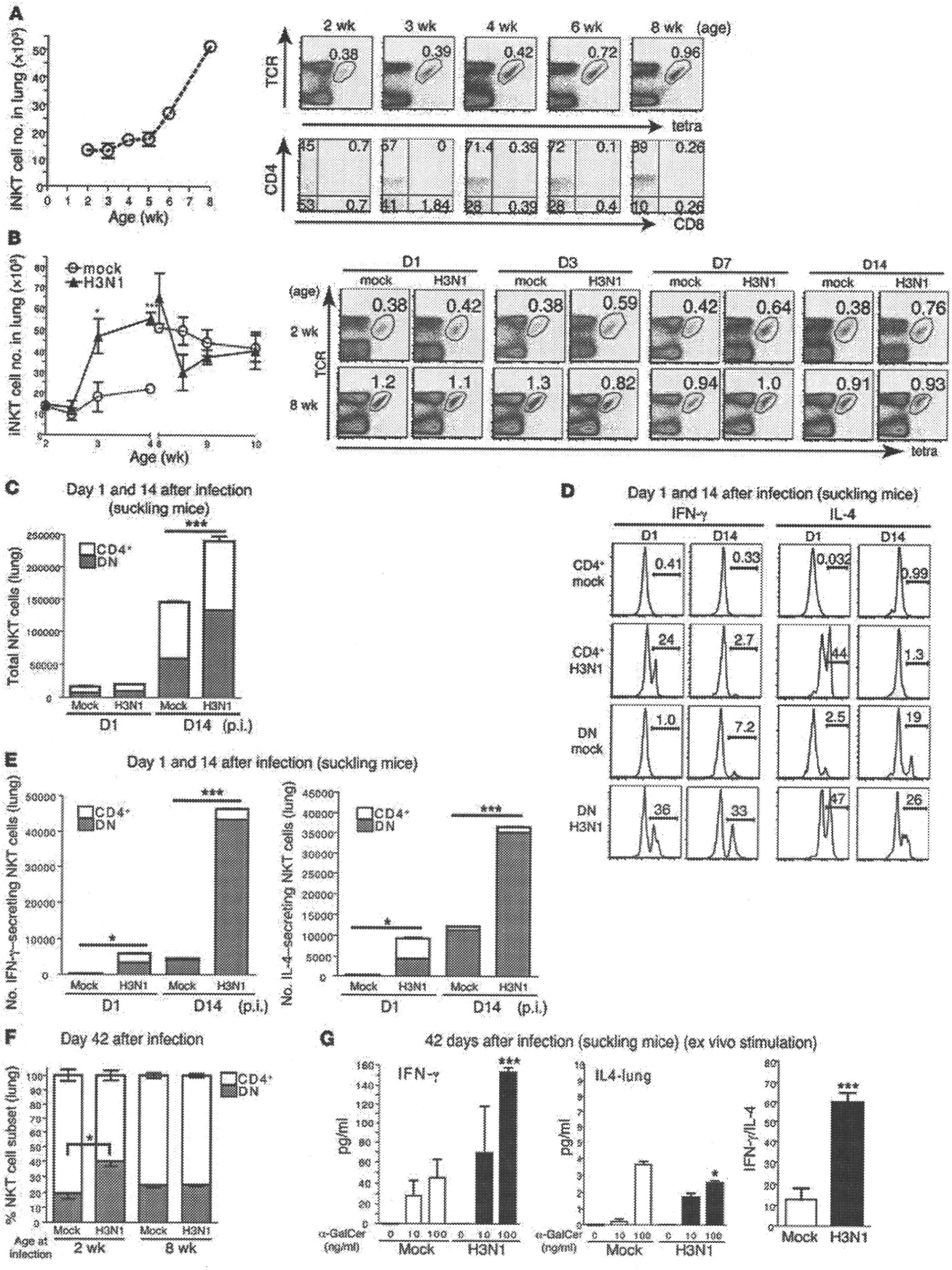
A
iNKT cell no. in lung (×10³)
Age (wk)
2 wk
3 wk
4 wk
6 wk
8 wk (age)
TCR
tetra
CD4
CD8
B
mock
H3N1
iNKT cell no. in lung (×10³)
Age (wk)
D1
D3
D7
D14
mock
H3N1
(age)
2 wk
8 wk
TCR
tetra
C
Day 1 and 14 after infection (suckling mice)
Total NKT cells (lung)
CD4⁺
DN
Mock
H3N1
D1
D14 (p.i.)
D
Day 1 and 14 after infection (suckling mice)
IFN-γ
IL-4
D1
D14
CD4⁺ mock
CD4⁺ H3N1
DN mock
DN H3N1
E
Day 1 and 14 after infection (suckling mice)
No. IFN-γ–secreting NKT cells (lung)
No. IL-4–secreting NKT cells (lung)
CD4⁺
DN
D1
D14 (p.i.)
F
Day 42 after infection
% NKT cell subset (lung)
CD4⁺
DN
Age at infection
2 wk
8 wk
G
42 days after infection (suckling mice) (ex vivo stimulation)
IFN-γ
IL4-lung
IFN-γ/IL-4
pg/ml
α-GalCer (ng/ml)
Mock
H3N1

Figure 3
H3N1 infection in 2-week-old mice alters the phenotype of the NKT cells. (**A**) Lung cells were isolated over a 6-week period and analyzed for NKT cells. Left: Absolute numbers of lung NKT cells. Right: Percentage of NKTs (top) in lung leukocytes. NKT cells were analyzed for CD4 and CD8 (bottom). (**B**) Left: BALB/c mice (n = 3/group) were infected with H3N1 or AF at 2 or 8 weeks of age, and lung NKT cells were assessed over 2 weeks. Right: Percentage of NKT cells in lungs of 2-week-old and 8-week-old mice. (**C**) Two-week-old BALB/c mice were mock infected or infected with H3N1, and pulmonary CD4$^+$ NKT and DN NKT cell numbers were assessed on days 1 and 14 after infection. (**D** and **E**) NKT cells from **C** were assessed for CD4, IFN-γ, and IL-4 expression (**D**) and absolute numbers quantified (**E**). (**F**) BALB/c mice (n = 4–5/group) were infected with H3N1 or mock infected at 2 or 8 weeks of age, and lung samples were taken 42 days later to assess NKT cell subsets. One of 2 independent experiments is shown. (**G**) Two-week-old BALB/c mice were infected with H3N1 or mock infected. After 42 days, lung cells were harvested and stimulated ex vivo with vehicle or α-GalCer for 96 hours. IFN-γ and IL-4 in supernatants from triplicate wells were determined by ELISA and the IFN-γ/IL-4 ratio calculated. $^*P < 0.05$, $^{***}P < 0.001$ compared with mock infection.

In contrast, H3N1 infection in adult mice had little effect on pulmonary NKT cell numbers. In fact, H3N1 infection in the adult mice transiently reduced the number of NKT cells, possibly due to activation-induced TCR downregulation (Figure 3B). In 2-week-old suckling naive mice, approximately 50% of the pulmonary NKT cells were CD4$^+$, and over time this fraction increased such that in 8-week-old adult naive mice, 89% of the pulmonary NKT cells were CD4$^+$ (dot plots in Figure 3A). However, H3N1 infection of suckling mice preferentially increased the number of DN NKT cells by day 14 after infection (Figure 3C). Both CD4$^+$ and DN NKT cells from the suckling mice secreted IFN-γ on day 1 of infection, but 14 days after infection only DN but not CD4$^+$ pulmonary NKT cells continued to secrete IFN-γ (and IL-4), as assessed with intracellular staining without in vitro restimulation (Figure 3D). Thus, 14 days after infection the great majority of cytokine-secreting cells in the lungs were DN NKT cells (Figure 3E).

Analysis of the mice 42 days after H3N1 infection showed that the proportion of DN versus CD4$^+$ NKT cells in the lungs doubled, whereas 42 days after H3N1 infection in 8-week-old mice, there was no effect on the proportion of DN NKT cells in the lungs (Figure 3F). Assessment of the cytokine profile of NKT cells 42 days following infection after ex vivo stimulation with α-galactosylceramide (α-GalCer, which specifically activates NKT cells) demonstrated increased IFN-γ but not IL-4 production by the H3N1-exposed NKT cells (Figure 3G), resulting in a greatly increased IFN-γ/IL-4 ratio (Figure 3G). These results suggested that H3N1 infection in suckling mice preferentially expanded a unique NKT cell population in the lungs that, by day 42, preferentially produced IFN-γ but not IL-4 and was associated with a reduced expression of CD4.

Adoptive transfer of H3N1-exposed NKT cells suppresses AHR and induces Treg cells. While the H3N1-exposed NKT cells (vNKT) could not induce AHR when transferred into *Jα18*$^{-/-}$ mice (Figure 2), they were not anergic, but instead potently suppressed OVA-induced AHR (Figure 4, A and B) and inflammation (Figure 4C), as assessed by adoptive transfer 42 days after infection into adult WT OVA-sensitized mice. In contrast, NKT cells from mock-infected mice (WT NKT) (Figure 4, B and C) or from adult mice infected with H3N1 (data not shown) did not suppress OVA-induced AHR. The proportion of DN NKT cells in the lungs of mice receiving the H3N1 virus–exposed NKT cells was increased (Figure 4D), consistent with the idea that H3N1 infection in suckling mice preferentially expands a subpopulation of DN NKT cells.

To more clearly demonstrate that the DN NKT cell subpopulation was responsible for the suppression of AHR, we purified CD4$^+$ and DN NKT cell subpopulations from the spleens of mice (purity 96%–99%) (Supplemental Figure 1C), which had been infected with H3N1, and adoptively transferred these cells into OVA-sensitized mice. Figure 4E shows that the DN but not the CD4$^+$ NKT cell population suppressed AHR that developed on challenge of the mice with OVA, confirming that the H3N1-exposed DN NKT cell population was responsible for this effect.

The suppression of AHR by the transferred H3N1-exposed NKT cells was associated with a 50% increase in the number of natural Foxp3$^+$ Treg cells and with a 300% increase in the number of adaptive OVA-specific Foxp3$^+$ Treg cells in the lungs (assessed by transferring DO11.10 Tg OVA-specific Foxp3$^-$ T cells from DO11.10 Tg × *Rag*$^{-/-}$ mice), compared with when NKT cells from mock-infected mice were transferred (Figure 4F). Furthermore, the inhibitory effect of the NKT cells exposed to H3N1 was reversed by treatment of the recipient mice with an anti-CD25 mAb (Figure 4G). These results together indicated that H3N1-exposed NKT cells could suppress the development of experimental asthma, and that natural and adaptive Treg cells might mediate the suppressive effects of the NKT cell population.

We found a similar suppressive NKT cell population in Vα14 TCR Tg mice. Adult Vα14 TCR transgenic mice have a 5- to 10-fold increase in the number of NKT cells in the spleen, of which the majority (53%) are DN NKT cells (Supplemental Figure 1B), whereas in WT BALB/c mice, only 11% of the splenic NKT cells are DN (Supplemental Figure 1B). Adoptive transfer of NKT cells purified from Vα14 TCR Tg mice into adult WT OVA-sensitized BALB/c mice greatly suppressed the development of OVA-induced AHR and airway inflammation (Figure 4, H–J). Transfer of Vα14 TCR Tg NKT cells was also associated with a 50% increase in the number of natural Foxp3$^+$ Treg cells and in a 300% increase in the number of adaptive OVA-specific Foxp3$^+$ Treg cells (assessed by transfer of DO11.10 Tg OVA-specific cells), compared with transfer of naive (WT) NKT cells (Figure 4F). These results suggest that NKT cells in Vα14 Tg mice were similar to NKT cells from suckling mice exposed to H3N1, in that they had suppressive activity for allergen-induced AHR.

The protective effect of H3N1 infection depends on TLR7 and T-bet. Since influenza A virus is a single-stranded RNA (ssRNA) virus, and since T-bet participates in IFN-γ production and in NKT cell maturation (23), we infected 2-week-old *Tlr7*$^{-/-}$*Tbet*$^{-/-}$ mice and control WT BALB/c mice with the H3N1 virus. Six weeks later, the mice were examined for OVA-induced AHR (protocol shown in Figure 5A). Whereas H3N1 infection in suckling WT mice protected against subsequent OVA-induced AHR and airway inflammation (Figure 5, B and C), H3N1 infection in suckling *Tlr7*$^{-/-}$ or suckling *Tbet*$^{-/-}$ mice failed to protect against, and even exacerbated, OVA-induced AHR and airway inflammation. Furthermore, the ratio of IFN-γ production to IL-4 production in NKT cells from *Tlr7*$^{-/-}$ mice was reduced (Supplemental Figure 2D), while IFN-γ was reduced and IL-13 and IL-17 production increased in NKT cells in *Tbet*$^{-/-}$ mice compared with WT mice (Supplemental Figure 2, A and E). (Note that *Tbet*$^{-/-}$ mice have reduced numbers of NKT cells, particularly in the liver [ref. 23] but have significant numbers of pulmonary NKT cells compared with WT mice [ref. 24]).

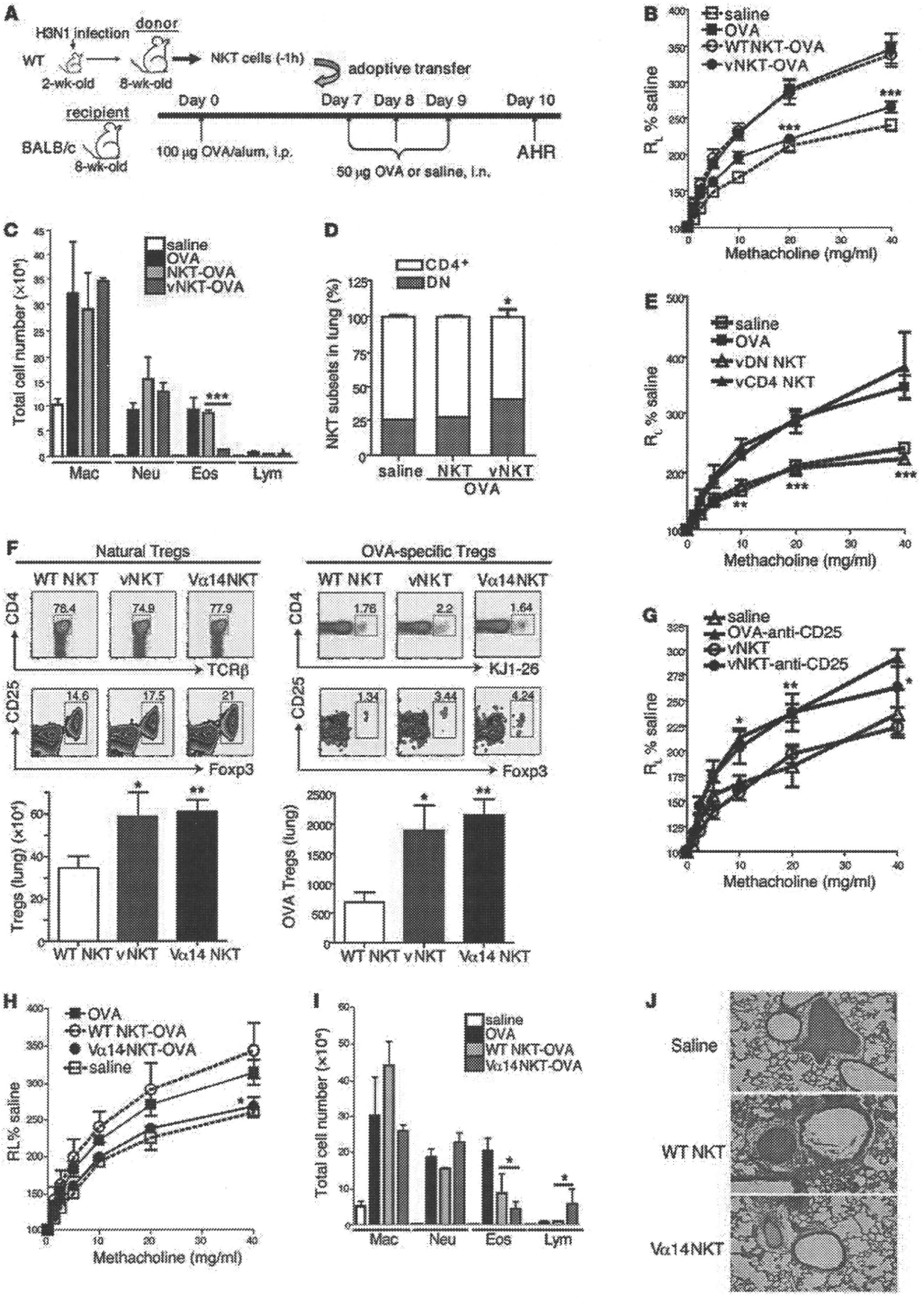
A
H3N1 infection
donor
WT
2-wk-old
8-wk-old
NKT cells (-1h)
adoptive transfer
recipient
BALB/c
8-wk-old
Day 0
Day 7
Day 8
Day 9
Day 10
100 μg OVA/alum, i.p.
50 μg OVA or saline, i.n.
AHR
B
saline
OVA
WT NKT-OVA
vNKT-OVA
RL % saline
Methacholine (mg/ml)
C
saline
OVA
NKT-OVA
vNKT-OVA
Total cell number (×10⁴)
Mac
Neu
Eos
Lym
D
CD4+
DN
NKT subsets in lung (%)
saline
NKT
vNKT
OVA
E
saline
OVA
vDN NKT
vCD4 NKT
RL % saline
Methacholine (mg/ml)
F
Natural Tregs
OVA-specific Tregs
WT NKT
vNKT
Vα14NKT
CD4
TCRβ
CD25
Foxp3
KJ1-26
Tregs (lung) (×10⁴)
OVA Tregs (lung)
G
saline
OVA-anti-CD25
vNKT
vNKT-anti-CD25
RL % saline
Methacholine (mg/ml)
H
OVA
WT NKT-OVA
Vα14NKT-OVA
saline
RL % saline
Methacholine (mg/ml)
I
saline
OVA
WT NKT-OVA
Vα14NKT-OVA
Total cell number (×10⁴)
Mac
Neu
Eos
Lym
J
Saline
WT NKT
Vα14NKT

Figure 4
H3N1-exposed NKT cells suppress AHR and increase OVA-specific Tregs. (**A**) Protocol for adoptive transfer of NKT cells. (**B** and **C**) Lung resistance was measured in recipient mice (**B**; n = 15/group) and BAL cells collected (**C**). (**D**) Relative numbers of CD4$^+$ versus DN NKT cells in recipients' lungs were assessed (**E**) H3N1-exposed CD4$^-$CD8$^-$NKT (vDN NKT) or CD4$^+$NKT (vCD4 NKT) cells were purified and transferred as in **A**. Lung resistance was measured in recipient mice (n = 5/group). (**F**) Eight-week-old WT BALB/c mice received 5 × 10^4 DO11.10 *Rag*$^{-/-}$ T cells and were sensitized with OVA/alum. Seven days later, NKT cells from WT BALB/c, Vα14tg, or H3N1-infected mice were adoptively transferred into OVA-sensitized mice. After OVA challenge, the numbers of natural Tregs (CD4$^+$C25$^+$Foxp3$^+$) and adaptive OVA antigen–specific Tregs (CD4$^+$ CD25$^+$ Foxp3$^+$KJ1-26$^+$) were determined. Absolute cell numbers were calculated (n = 5/group). (**G**) Eight-week-old WT BALB/c recipients were depleted of Tregs through injections of anti-CD25 mAb (clone PC61; 0.5 mg) and assessed as in **A** (n = 5/group). (**H** and **I**) NKT cells from WT or Vα14 Tg were transferred to OVA-sensitized BALB/c mice (n = 4–6/group), which were assessed as in **A** (**H**), and BAL cells were analyzed (**I**). (**J**) Representative lung sections from recipients described in **H** were H&E stained (original magnification, ×10). Data represent 2–3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT NKT-OVA (**B**–**D**), OVA (**E**), WT NKT (**F**, **H**, and **I**), and OVA-vNKT (**G**).

As noted above (Figure 3F), protection against AHR was associated with an increase in the number of DN NKT cells following H3N1 infection in WT mice, which did not occur in *Tlr7*$^{-/-}$ or *Tbet*$^{-/-}$ mice (Figure 5D). Moreover, adoptive transfer of NKT cells purified 6 weeks after H3N1 infection of WT, but not *Tlr7*$^{-/-}$ or *Tbet*$^{-/-}$ mice, into OVA-sensitized WT BALB/c mice suppressed OVA-induced AHR and airway inflammation (Figure 5, E and F). Taken together, these results indicate that protection by H3N1-exposed NKT cells against AHR depends on TLR7 and T-bet.

Induction of protection with α-C-GalCer and a glycolipid from H. pylori. Since NKT cells appeared to mediate the effects of H3N1 infection, we examined a panel of glycolipids that specifically activate NKT cells for the capacity to replicate the beneficial effects of H3N1 infection. We first examined the effects of α-C-GalCer, a synthetic C-glycoside analog of α-GalCer that preferentially induces IFN-γ but not IL-4 synthesis (25–27). Treatment of suckling mice with α-C-GalCer (5 μg), but not α-GalCer, which induces production of both IFN-γ and IL-4, protected the mice as adults (42 days later) from the development of OVA-induced AHR (Figure 6A). The protective effect was dependent on T-bet, since *Tbet*$^{-/-}$ mice were not protected by treatment with α-C-GalCer (Figure 6B). Moreover, adoptive transfer of NKT cells exposed to α-C-GalCer protected recipients against the development of AHR and airway inflammation (Figure 6C).

We also found a second glycolipid, PI57, a cholesterol-derived lipid from *H. pylori* (28), that could protect against the development of AHR (Figure 6D). *H. pylori*, a bacteria that colonizes the stomach (29) and is associated with protection against asthma (2, 3), produces cholesteryl α-glucosides (30), including cholesteryl 6-O-acyl α-glucoside (AGlc-Chol) (Supplemental Figure 4), which was chemically synthesized (PI57) (Figure 6D). PI57, when administered i.p. to 2-week-old mice, increased the total number of NKT cells, particularly the number of DN NKT cells, found in the lung 2 weeks later (Figure 6, E and F). In contrast, treatment with α-GalCer increased both CD4$^+$ and DN NKT cells in the lungs. Importantly, treatment of 2-week-old mice with PI57 (50 or 100 μg) (Figure 6G) protected the mice from the development of OVA-induced AHR, induced 6 weeks after the glycolipid treatment. On the other hand, treatment of 2-week-old mice with PBS30, a lipid present in the cell walls of *Sphingomonas* bacteria (31, 32), failed to protect the mice from OVA-induced AHR (Figure 6H). Moreover, adoptive transfer of NKT cells from PI57-treated, but not vehicle-treated, 2-week-old mice (harvested 6 weeks after treatment) into OVA-sensitized WT mice, suppressed AHR and airway inflammation (Figure 6, I and J). Transfer of NKT cells from α-GalCer–treated mice reduced AHR slightly, but this was not statistically significant (Supplemental Figure 3A). The production of IFN-γ by the NKT cells was important, since the protective effect of PI57, like that of H3N1 and α-C-GalCer, was dependent on T-bet, since PI57 treatment of 2-week-old *Tbet*$^{-/-}$ mice did not protect against subsequent OVA-induced AHR (Supplemental Figure 3B). These results together suggest that a subset of NKT cells that can be specifically activated by some but not all glycolipid antigens, and that preferentially produces IFN-γ, mediates the protective effects of H3N1 infection.

PI57 is a CD1d-dependent NKT cell antigen. To demonstrate that PI57, like α-C-GalCer, can directly activate NKT cells, we showed that PI57, when added to cultures of NKT cell lines plus DCs, induced the production of IFN-γ in a CD1d-restricted manner, since cytokine production was blocked by anti-CD1d mAb (Figure 7A). In addition, PI57 induced higher levels of IFN-γ and less IL-4 in NKT cell lines compared with PBS30 (from *Sphingomonas*) or α-GalCer, and did so in a CD1d-restricted manner, since DCs from *Cd1d*$^{-/-}$ mice failed to support PI57-induced cytokine production (Figure 7B). Furthermore, the PI57 response occurred by direct activation of NKT cells, since PI57 induced cytokine production in NKT cell lines with DCs from *Myd88*$^{-/-}$ or *Trif*$^{-/-}$ mice (Figure 7B), and since 3 different NKT cell hybridomas derived from Vα14 NKT cells but not from Vα14$^-$ T cells produced IL-2 in response to immobilized recombinant CD1d previously loaded with PI57 but not with PI56, a control glycolipid (Figure 7C). Moreover, CD1d tetramers loaded with PI57 stained 10%–23% of NKT cells in an NKT cell line (Figure 7D). Of the PI57-CD1d tetramer$^+$ cells, 92% were CD4$^-$ (DN) (data not shown). This strongly suggests that PI57 bound to CD1d was directly recognized by the TCR of a population of NKT cells. Finally, human NKT cells were also activated by PI57, since NKT cells lines (Figure 7E) as well as a Vα24$^+$ NKT cell clone (BM2a.3) (Figure 7F) responded to this glycolipid. The response was also directly induced, since plate-bound CD1d loaded with PI57 induced IFN-γ in BM2a.3 cells (Figure 7G). Taken together, these results indicated that both mouse and human NKT cells were directly activated by PI57, an *H. pylori* glycolipid, in a CD1d-restricted manner.

Discussion

Herein, we showed that infection of 2-week-old pups with influenza A virus H3N1 protected against the subsequent development of allergen-induced AHR, whereas infection of adult (8-week-old) mice with H3N1 did not protect against the subsequent development of AHR. The protective effect H3N1 in suckling mice was associated with the maturation and expansion of a specific subset of NKT cells, which suppressed the development of allergen-induced AHR, demonstrated by adoptive transfer of these NKT cells into normal allergen-sensitized adult mice. The protective NKT cell subset required T-bet, as the NKT cells had to be derived from T-bet$^+$ mice; this subset also produced IFN-γ and was present in NKT cell populations enriched for DN (CD4$^-$) NKT cells. Adoptive transfer of the protective NKT cell population was associated

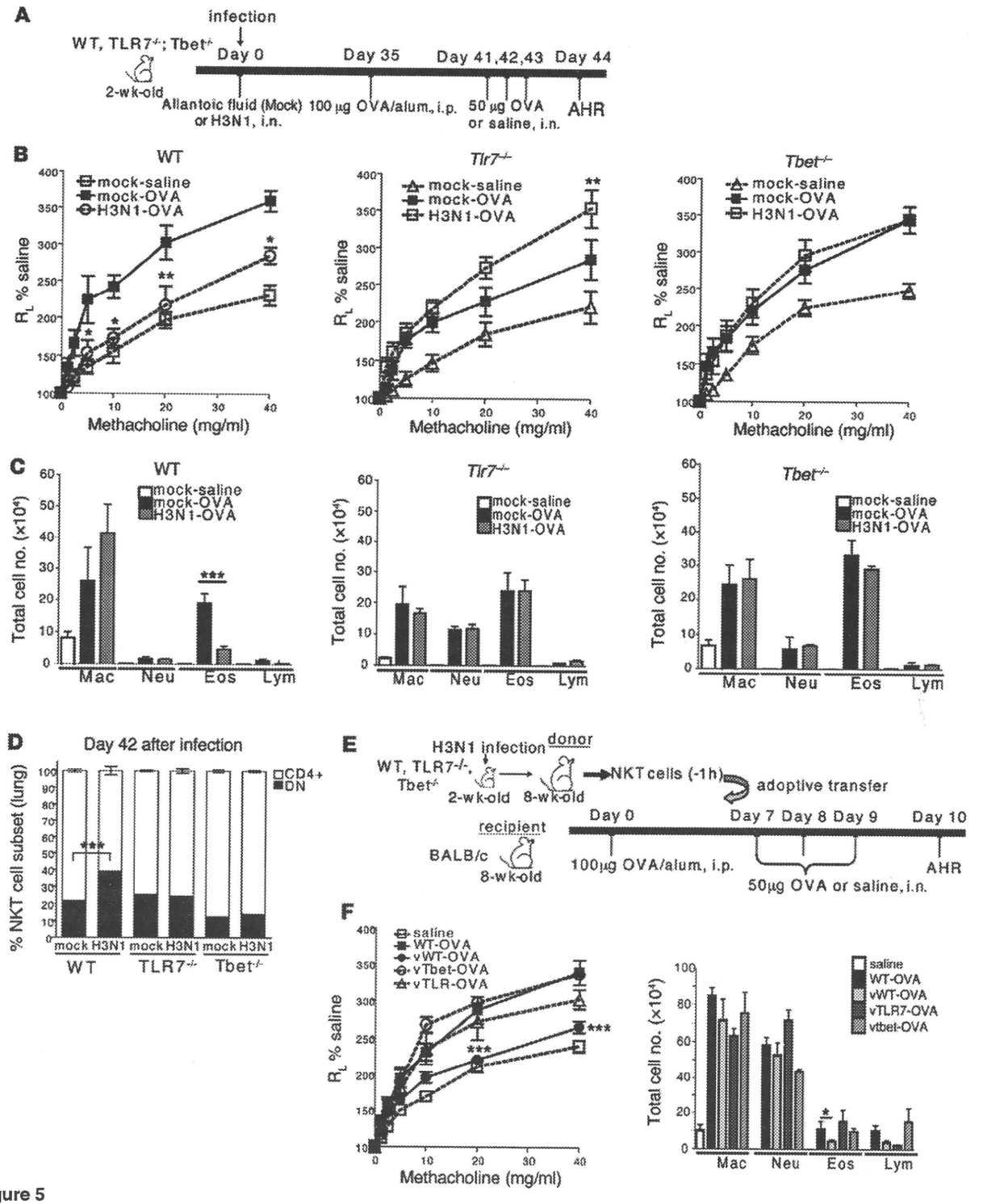

Figure 5

The protective effect of H3N1 infection depends on TLR7 and T-bet. (**A**) Schematic showing the protocol for WT, *Tlr7*$^{-/-}$, or *Tbet*$^{-/-}$ mice infected at 2 weeks of age with H3N1 virus or mock infected and examined for OVA-induced AHR at 8 weeks of age (n = 4–6 per group). (**B**) Lung resistance was measured. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the mock-OVA group. (**C**) BAL cells from **B** were collected. (**D**) WT, *Tlr7*$^{-/-}$, or *Tbet*$^{-/-}$ mice were infected with H3N1 or mock at 2 weeks of age, and lung samples were taken 42 days later to asses for NKT cell subsets. ***P < 0.001 compared with the mock group. (**E**) Schematic showing the adoptive transfer of NKT from virus-infected WT, *Tlr7*$^{-/-}$, or *Tbet*$^{-/-}$ mice to OVA-sensitized BALB/c recipients (n = 4–6 per group). The donor mice were infected with H3N1 or mock-infected at 2 weeks of age. NKT cells were purified from these mice 42 days after infection and transferred to OVA-sensitized BALB/c mice, which were then challenged with OVA to induce AHR. (**F**) Left: After OVA challenge, AHR was measured as described in **D**. Right: Cells in BAL were assessed. ***P < 0.001 compared with the WT-OVA group. Data are representative of 2 independent experiments.