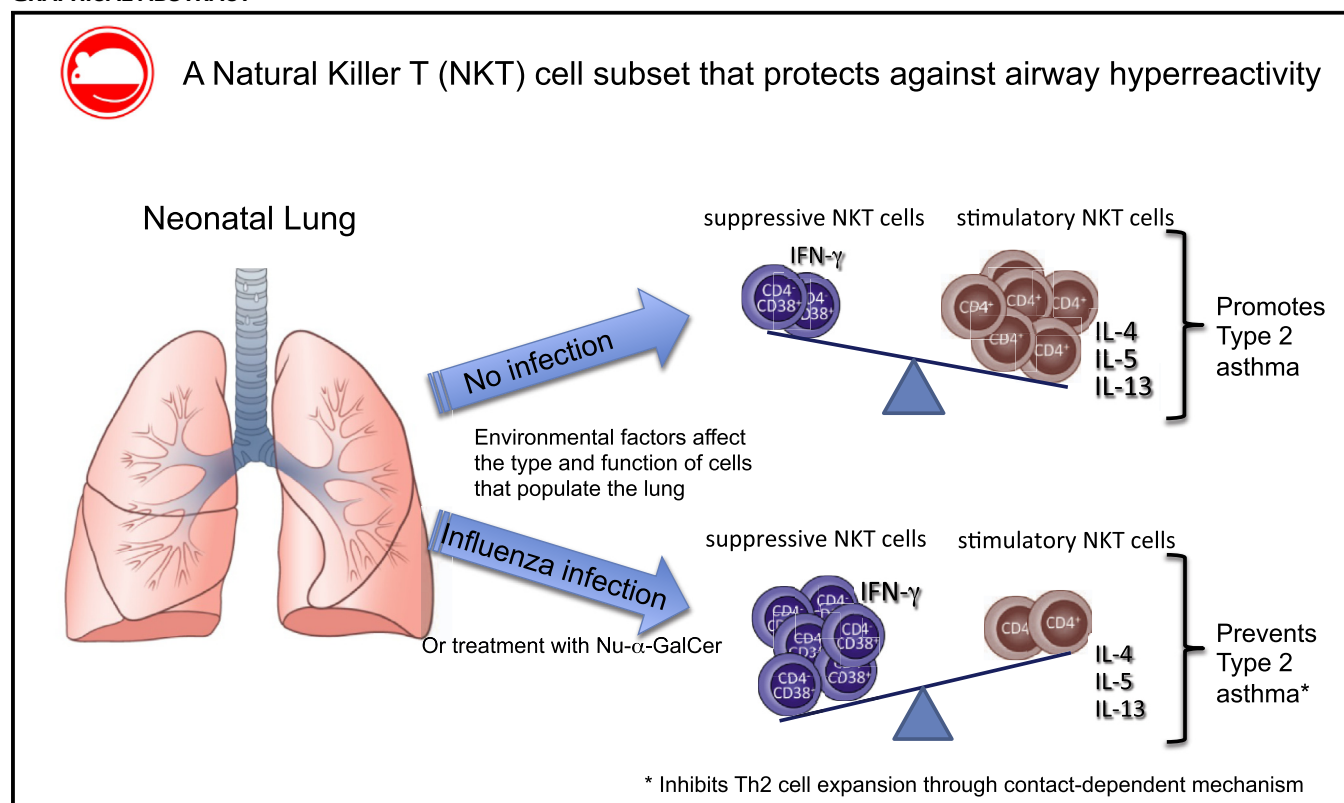


A natural killer T-cell subset that protects against airway hyperreactivity



Ya-Ting Chuang, PhD,^{a,b} Krystle Leung, MS,^{a,c} Ya-Jen Chang, PhD,^{a,d} Rosemarie H. DeKruyff, PhD,^{a,e} Paul B. Savage, PhD,^f Richard Cruse, PhD,^g Christophe Benoit, PhD,^g Dirk Elewaut, PhD,^{h,i} Nicole Baumgarth, PhD,^j and Dale T. Umetsu, MD, PhD^{a,*}
Boston, Mass; Taipei, Taiwan; Stanford, Davis, and San Francisco, Calif; Provo, Utah; and Ghent, Belgium

GRAPHICAL ABSTRACT



Background: Infection of suckling mice with influenza virus expands a CD4⁻CD8⁻ double-negative (DN) natural killer T (NKT) cell subpopulation that protects the mice as adults

against allergen-induced airway hyperreactivity (AHR). However, this NKT cell subset has not been characterized, and the underlying mechanisms of protection remain unknown.

From ^athe Department of Pediatrics, Boston Children's Hospital, Harvard Medical School, Boston; ^bthe Department of Medical Research, National Taiwan University Hospital, Taipei; ^cthe Department of Medicine, Harvard Medical School, Boston; ^dthe Institute of Biomedical Sciences, Academia Sinica, Taipei; ^ethe Department of Medicine, Stanford University; ^fthe Department of Chemistry and Biochemistry, Brigham Young University, Provo; ^gthe Division of Immunology, Department of Microbiology and Immunobiology, Harvard Medical School, Boston; ^hthe Department of Rheumatology, Faculty of Medicine and Health Sciences, and ⁱthe Unit of Molecular Immunology and Inflammation, VIB Inflammation Research Center, Ghent University; and ^jthe Department of Pathology, Microbiology & Immunology, School of Veterinary Medicine, University of California, Davis.

*Dr Umetsu is currently affiliated with Genentech, South San Francisco, California.

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Corresponding author: Dale T. Umetsu, MD, PhD, 1 DNA Way, MS 453b, South San Francisco, CA 94080. E-mail: dale.umetsu@gmail.com.

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Objective: We characterized this specific NKT cell subpopulation that developed during influenza infection in neonatal mice and that suppressed the subsequent development of AHR.

Methods: A cell-surface marker was identified by comparing the mRNA expression profile of wild-type CD4⁺ NKT cells with that of suppressive V α 14 DN NKT cells. The marker-enriched NKT cell subset was then analyzed for its cytokine profile and its suppressive *in vitro* and *in vivo* abilities.

Results: We showed that DN NKT cells with high CD38 expression produced IFN- γ , but not IL-17, IL-4, or IL-13, and inhibited development of AHR through contact-dependent suppression of helper CD4 T-cell proliferation. The NKT subset expanded in the lungs of neonatal mice after infection with influenza and also after treatment of neonatal mice with Nu- α -GalCer, which effectively increased DN CD38^{hi} NKT cell numbers.

Conclusion: These results suggest that early/neonatal exposure to infection or antigen challenge affects subsequent lung immunity by altering the cellular composition of cells in the lung and that some subsets of NKT cells suppress AHR. These results provide a possible mechanism by which prior infections can protect against the development of allergic asthma and might be further explored as a protective measure for young children. (J Allergy Clin Immunol 2019;143:565-76.)

Key words: Natural killer T subset, asthma, airway hyperreactivity, hygiene hypothesis, influenza, CD38

Asthma, a major public health concern in industrialized countries that affects 330 million persons worldwide,¹ is characterized by symptoms, including wheezing, shortness of breath, and coughing, as well as by airway inflammation, which, when chronic, results in airway remodeling. In addition, although asthma is heterogeneous and includes allergic and nonallergic phenotypes,² airway hyperreactivity (AHR) is a cardinal feature of asthma that correlates with disease severity.^{3,4} The most common form of asthma, allergic asthma, is orchestrated by T_H2 cells and type 2 innate lymphoid cells secreting IL-4, IL-5, IL-13, and IL-9, which increase IgE production; enhance the growth and differentiation of airway eosinophils, basophils, and mast cells; and directly induce the development of AHR.^{5,6} However, the mechanisms that regulate and control allergic asthma beyond allergic sensitization are not fully understood.

The public health concerns regarding asthma are magnified by the dramatic increase in the prevalence of asthma and other atopic conditions, including allergic rhinitis, atopic dermatitis, and food allergy, that has occurred over past decades.^{1,7} This increase appears to be due to changes in the environment, such as reductions in the incidence of certain infections or alterations in the gastrointestinal microbiome.^{7,8} This relationship between the increasing prevalence of asthma and allergy and changes in the microbial environment is poorly understood but has been referred to often as the hygiene hypothesis.⁹ The mechanisms by which these environmental changes (eg, specific infections or microbiota) affect the development of asthma and allergy and the development of immunity in young children are poorly defined.

Our previous study investigated the effect of viral respiratory tract infection by using a mouse model of asthma.¹⁰ We showed that influenza A virus infection of suckling mice protected against the subsequent development of allergen-induced AHR as adults.¹⁰ This protection was associated with preferential expansion of

Abbreviations used

AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
BALT:	Bronchus-associated mucosal tissue
ConA:	Concanavalin A
CT:	Cycle threshold
DC:	Dendritic cell
DN:	Double negative
α -GalCer:	α -Galactosylceramide
NK:	Natural killer
NKT:	Nature killer T
NU:	Naphthylurea
OVA:	Ovalbumin
Tg:	Transgenic
TLR:	Toll-like receptor
WT:	Wild-type

CD4⁺CD8[−] natural killer T (NKT) cells in the lungs of suckling mice infected with influenza, and adoptive transfer of these double-negative (DN) NKTs suppressed the development of allergen-induced AHR. In the current report we explore the precise characteristics of DN NKT cells and the protective mechanisms involved, which might help explain the hygiene hypothesis and immune mechanisms that protect against asthma and allergic diseases.

METHODS

Mice

Adult wild-type (WT) BALB/c ByJ mice were purchased from the Jackson Laboratory (Bar Harbor, Me) and bred at Boston Children's Hospital. V α 14 transgenic (Tg) mice were provided by Albert Bendelac (University of Chicago, Chicago, Ill) and backcrossed to a BALB/c background for more than 10 generations. For studies in suckling mice, BALB/c mice were bred, and offspring were infected with influenza A virus at 2 weeks of age and then weaned at 3 weeks of age. The Animal Care and Use Committee at Boston Children's Hospital approved all animal protocols.

Reagents

α -Galactosylceramide (α -GalCer) was synthesized by P. B. Savage (Brigham Young University, Provo, Utah). Naphthylurea (NU)-modified α -GalCer (NU- α -GalCer) glycolipids were described earlier¹¹ and used *in vivo* and for *in vitro* NKT cell cultures.

Culture and *in vitro* stimulation of NKT cells

WT BALB/c and V α 14 Tg splenocytes were stimulated with α -GalCer (100 ng/mL) to expand the NKT cell population. After 72 hours of stimulation, splenocytes were washed and replaced in fresh medium for another 24 hours and then stained with phycoerythrin-conjugated CD1d tetramer and fluorescein isothiocyanate-conjugated anti-T-cell receptor β mAbs and sorted by using BD FACSaria II cell sorter (BD Biosciences, San Jose, Calif). The purity of the sorted cells was greater than 98%. The sorted NKT cells were cultured in complete Dulbecco modified Eagle medium supplied with murine IL-15 (PeproTech, Rocky Hill, NJ) in the presence of γ -irradiated (2000 rad) bone marrow-derived dendritic cells (DCs; DC/NKT cell ratio = 1:20). Cells were subcultured and restimulated with fresh irradiated DCs once a week. Before stimulation, NKT cells were washed, rested for 24 hours after removal of IL-15, and then stimulated in 96-well plates (10⁵ cells/well) with bone marrow-derived DCs (5 $\times$ 10³ cells/well) in the presence of α -GalCer. After 48 hours, supernatants of NKT cells were analyzed for IL-4, IL-5, IL-10, IL-13, IL-17, and IFN- γ by using ELISA with anti-cytokine antibodies.

Induction of AHR and measurement of airway responsiveness in the ovalbumin model

BALB/c mice were sensitized with 100 μ g of ovalbumin (OVA; Sigma-Aldrich, St Louis, Mo) in alum administered intraperitoneally (on day 0) to induce AHR. After sensitization, mice were exposed to intranasal antigen (50 μ g/d OVA) or normal saline for 3 consecutive days (days 7–9). AHR was assessed on the day after the last OVA challenge. Airway compliance (Volume change of lung vs airway – Intrapleural pressure change, reflecting elastic resistance of the lung and chest wall) was measured in some experiments. Control mice received intraperitoneal injection of PBS and intranasal administration of normal saline.

Immunization with NU- α -GalCer *in vivo*

Offspring were immunized at 2 weeks of age with NU- α -GalCer and weaned at 3 weeks. Two-week-old pups (suckling mice) were immunized (intraperitoneally) with 1 μ g of NU- α -GalCer in 100 μ L of PBS.

Further information on the materials and methods used in this study is included in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS

DN NKT cells from V α 14 Tg but not WT mice show a direct suppressive effect on CD4⁺ T-cell proliferation

Our previous study demonstrated that a specific suppressive DN NKT subset expanded in the lungs of WT suckling mice exposed to influenza virus.¹⁰ To further analyze these cells, which are quite rare, we used V α 14 Tg mice, which have 25 to 50 times more DN NKT cells in the spleen than do WT mice. Adoptive transfer of NKT cells purified from V α 14 Tg mice into adult WT BALB/c mice greatly suppressed the development of OVA-induced AHR, which was measured as change in airway resistance on challenge with increasing concentrations of methacholine (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org) and eosinophilic airway inflammation (see [Fig E1, B](#)). Therefore we hypothesized that the DN NKT cells present in V α 14 Tg mice might be similar to those we found in H3N1-exposed mice that suppressed asthma. By comparing the cytokine profiles of NKT cells from WT BALB/c versus V α 14 Tg mice, we found that on activation, more V α 14 Tg NKT cells stained for IL-10 and fewer stained for IL-13, which might explain their ability to suppress allergen-induced AHR ([Fig 1, A](#)).

We further established an *in vitro* suppressive assay system to directly study the suppressive effect of DN V α 14 Tg NKT cells on the CD4⁺ T-cell response. First, we stimulated splenocytes from WT and V α 14 Tg mice *in vitro* with α -GalCer to expand the number of NKT cells and then purified the NKT cells using flow cytometric sorting. WT CD4⁺, WT DN, V α 14 CD4⁺, and V α 14 DN NKT cells (see [Fig E2](#) in this article's Online Repository at www.jacionline.org) were cocultured for 5 days with carboxyfluorescein succinimidyl ester–labeled WT CD4⁺ T cells stimulated with concanavalin A (ConA). We observed that only DN V α 14 NKT cells suppressed the ConA-stimulated proliferative response of CD4⁺ T cells in a dose-dependent manner (NKT/T-cell ratio, 1:1 to 1:4). In contrast, the other 3 NKT subpopulations, WT CD4⁺, WT DN, and V α 14 CD4⁺ NKT cells, all had a stimulatory effect on CD4⁺ T-cell proliferation ([Fig 1, B](#)). Because the WT DN NKT cells were nonsuppressive and DN NKT cells from WT suckling mice exposed to influenza

were suppressive, we next investigated whether a specific cell-surface marker could distinguish suppressive DN NKT cells.

Microarray analysis of DN V α 14 versus CD4⁺ WT NKT cells

To identify surface markers that might distinguish the suppressive NKT subset in WT mice, we compared the gene expression pattern of DN V α 14 NKT cells with that of WT CD4⁺ NKT cells. Gene expression profiling revealed that most genes in the perforin/granzyme pathway were greatly upregulated in DN V α 14 NKT cells, which is known to be a main difference between DN and CD4⁺ NKT populations.^{12–14} We also found several genes related to regulatory T-cell function, such as *Ctla4*, *Klag1*, *CD38*, and *Havcr2* (Tim-3), which were also upregulated in V α 14DN NKT cells ([Fig 2, A and B](#)). These microarray results were confirmed by using real-time RT-PCR ([Fig 2, C](#)). Moreover, we found that cell-surface expression of CD38 was consistently greater in DN V α 14 NKT cells compared with that in WT CD4⁺, WT DN, and V α 14 CD4⁺ NKT cells ([Fig 2, D](#)), suggesting that CD38 might be a useful marker to distinguish the suppressive NKT cell subset.

CD38 expression enriches for the NKT cells that suppress T-cell proliferation

CD38 has been studied previously as an activation marker^{15,16} but also as part of the regulatory T-cell transcriptional signature,^{17,18} making it a rational candidate marker for the suppressive NKT cell subset in WT mice.

To test this hypothesis, we sorted WT splenic NKT cells into 4 populations, CD4⁺CD38^{hi}, CD4⁺CD38^{lo}, DN CD38^{hi}, and DN CD38^{lo} cells ([Fig 3, A](#), and see [Fig E3](#) in this article's Online Repository at www.jacionline.org), and then performed an *in vitro* suppressive assay by coculturing with carboxyfluorescein succinimidyl ester–labeled CD4⁺ T cells stimulated with ConA. We observed that only DN CD38^{hi} NKT cells suppressed the ConA-stimulated proliferative response of CD4⁺ T cells in a dose-dependent manner (all other subpopulations did not suppress), supporting the hypothesis that CD38 could be used to enrich for the suppressive NKT subset ([Fig 3, B](#)).

We next studied the underlying mechanism of DN CD38^{hi} NKT cell–mediated suppression in a transwell system by adding DN CD38^{hi} NKT cells to the upper chamber of the transwell, with CD4⁺ T cells in the lower chamber. We observed that the 0.4 μ mol/L pore size transwell membrane blocked the suppressive effect of these cells on CD4⁺ T-cell proliferation ([Fig 3, C](#)). In contrast, mixing of the cells directly together resulted in significant suppression of the CD4⁺ T-cell proliferation, suggesting that the suppressive activity of DN CD38^{hi} NKT cells was dependent on cell-cell contact. This suppression might be in part due to programmed cell death protein 1–programmed death ligand 1 mechanisms because anti-PD-L1 mAbs partially blocked the *in vitro* activity of the NKT cells (see [Fig E4](#) in this article's Online Repository at www.jacionline.org) but not dependent on NKG2D (encoded by *Klrk*, see below), cytotoxic T lymphocyte–associated antigen 4, or TGF- β because adding anti-NKG2D, anti-cytotoxic T lymphocyte–associated antigen 4, or anti-TGF- β mAbs did not inhibit the suppressive activity in this assay (see [Fig E5](#) in this article's Online Repository at www.jacionline.org).

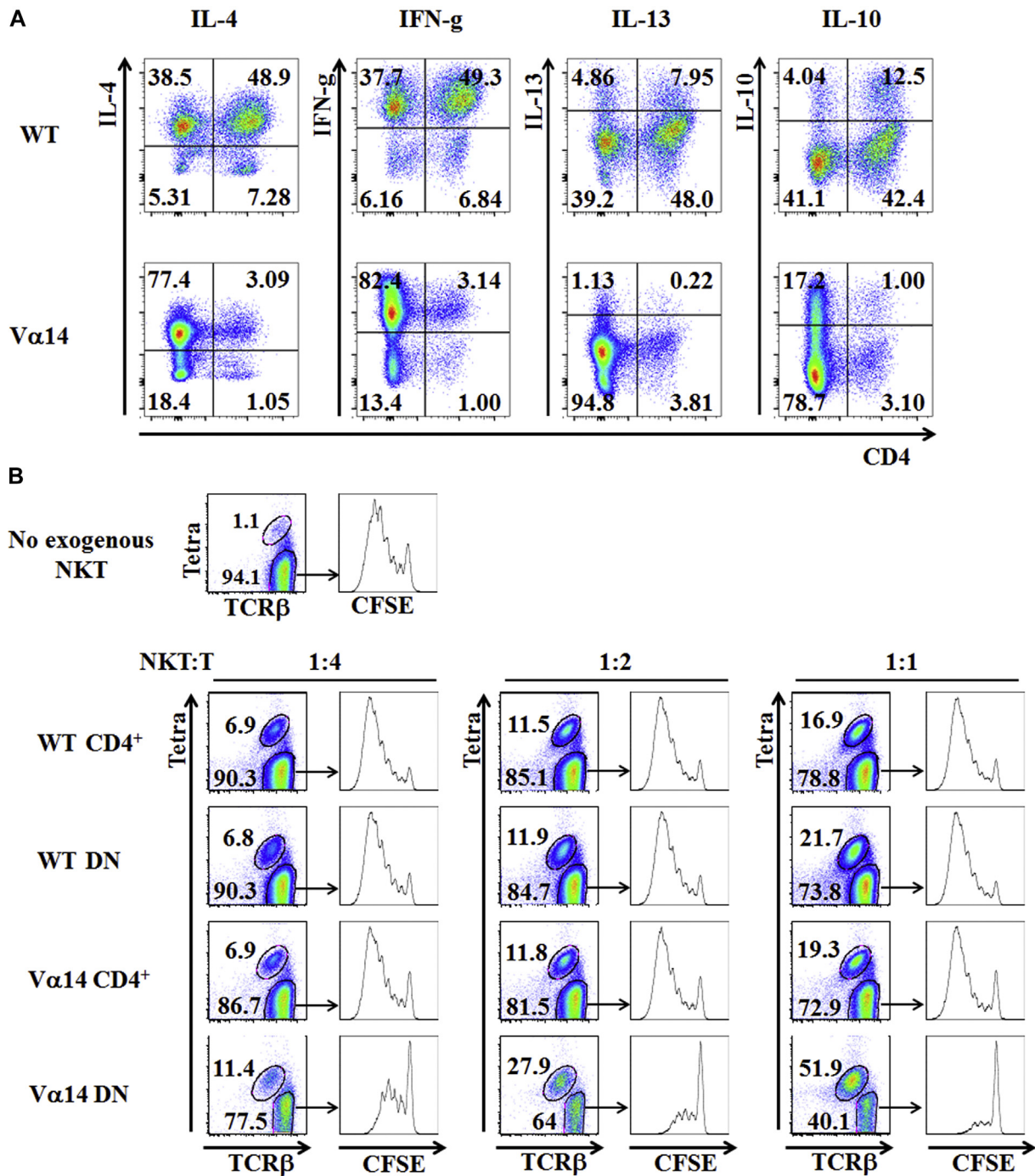


FIG 1. DN V α 14 Tg NKT cells showed a direct suppressive effect on CD4⁺ T-cell proliferation. **A**, WT or V α 14 Tg splenocytes were stimulated with α -GalCer for 96 hours and then reactivated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours. Cytokine production profiles were analyzed by means of intracellular cytokine staining (gated on TCR β ⁺ CD1d tetramer-positive NKT cells). **B**, Carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ T cells isolated from WT mice were used as responders and stimulated with ConA (0.45 μ g/mL). CD4⁺ and DN subpopulations of NKT cells from WT and V α 14 Tg mice were sorted and added to the culture at the indicated NKT/T-cell ratios. Histograms show the CFSE dilution of CD4⁺ T cells after 5 days of culture (gated on TCR β ⁺ CD1d tetramer-negative T cells). TCR, T-cell receptor.

DN CD38^{hi} NKT cells can suppress OVA-induced AHR *in vivo*

We next examined the *in vivo* suppressive activity of the DN CD38^{hi} NKT cells derived from WT mice by transferring the cells intratracheally (Fig 4, A). This resulted in a significant reduction in

the severity of OVA-induced AHR in recipient mice compared with mice that received the DN CD38^{lo} NKT cells (Fig 4, B and C). Furthermore, DN CD38^{hi} NKT cells significantly reduced eosinophilic airway inflammation (Fig 4, D), demonstrating the inhibitory activity of the DN CD38^{hi} NKT subset on airways disease *in vivo*.

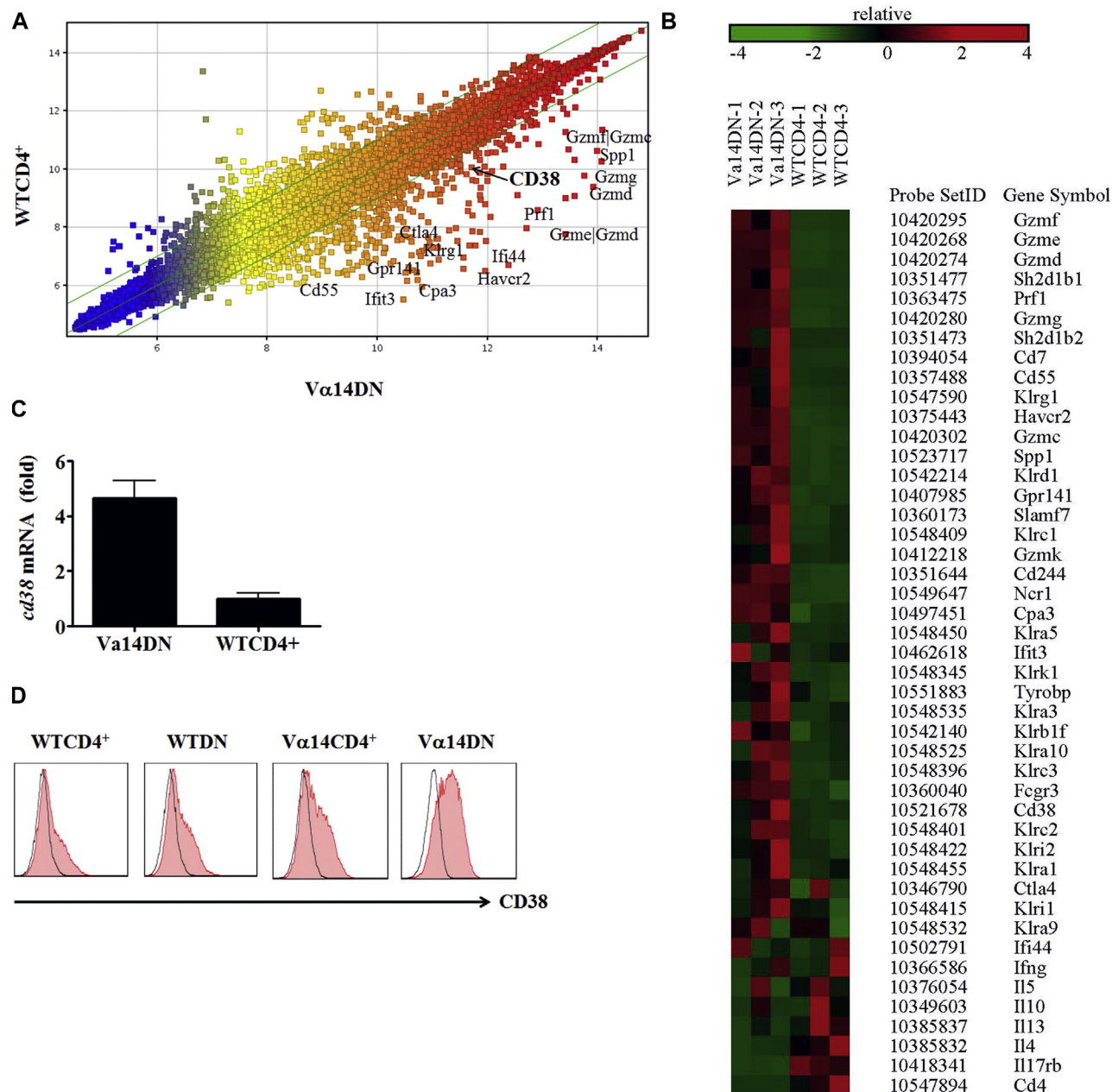
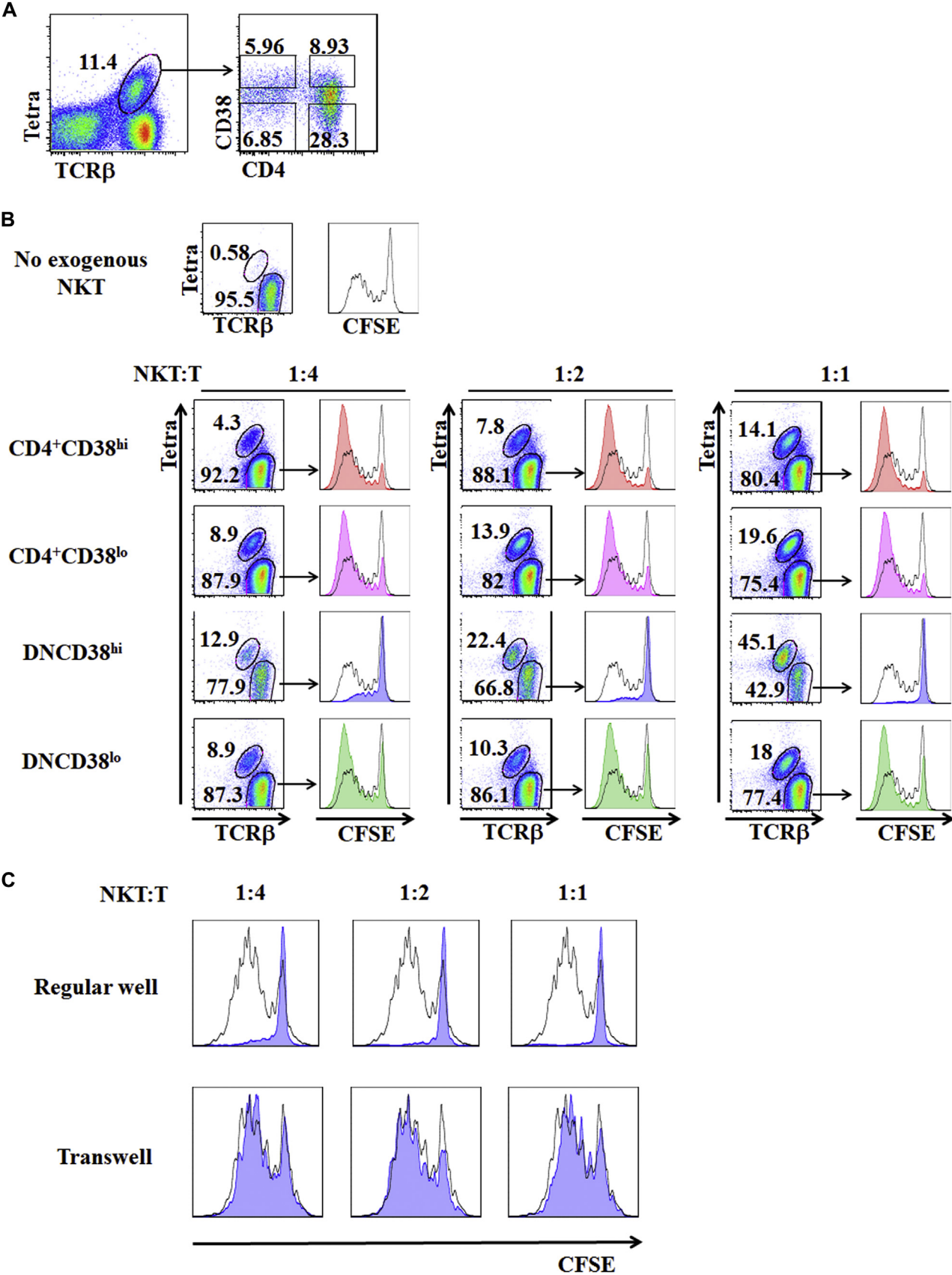


FIG 2. Microarray gene analysis of the suppressive Vα14 DN NKT cell population versus the stimulatory WT CD4⁺ NKT cell population. **A**, Scatter plot of Affymetrix microarray analysis displays the log of raw intensity values for the genes of interest between WT CD4⁺ and Vα14 DN NKT cell populations. Genes with a fold-change expression of greater than 2 are shown on the left and right of the outer diagonals, whereas the center diagonal indicates a less than 2-fold change. **B**, Heat maps of differentially expressed genes from microarrays of Vα14 DN and WT CD4⁺ NKT cell populations. **C** and **D**, CD38 expression on different subpopulations of NKT cells by using PCR (Fig 2, C) or flow cytometry (Fig 2, D). Black line histograms represent staining with isotype control for anti-CD38 (Fig 2, D).

DN CD38^{hi} suppressive NKT cells differentially express killer cell-related C-type lectins and T_H1 cytokines

We next examined additional characteristics of this novel suppressive NKT cell subset. Gene expression analysis of the 4 populations (DN CD38^{hi}, DN CD38^{lo}, CD4⁺CD38^{hi}, and CD4⁺CD38^{lo} NKT cells) revealed differential expression of genes encoding killer cell-related C-type lectin receptors, such

as *Klra*, *Klrc*, *Klrd*, *Klrj*, and *Klrk* (Fig 5, A). Real-time quantitative RT-PCR of selected differentially expressed genes revealed a high degree of correlation with the Affymetrix GeneChip (Affymetrix, Santa Clara, Calif) analysis data (Fig 5, B), providing further verification that the expression of *Klra*, *Klrc*, *Klrd*, and *Klrk* was increased in the DN CD38^{hi} suppressive NKT cell subset. In addition to these genes, we also examined cytokine expression profiles in the 4 WT BALB/c NKT subsets. Expression levels



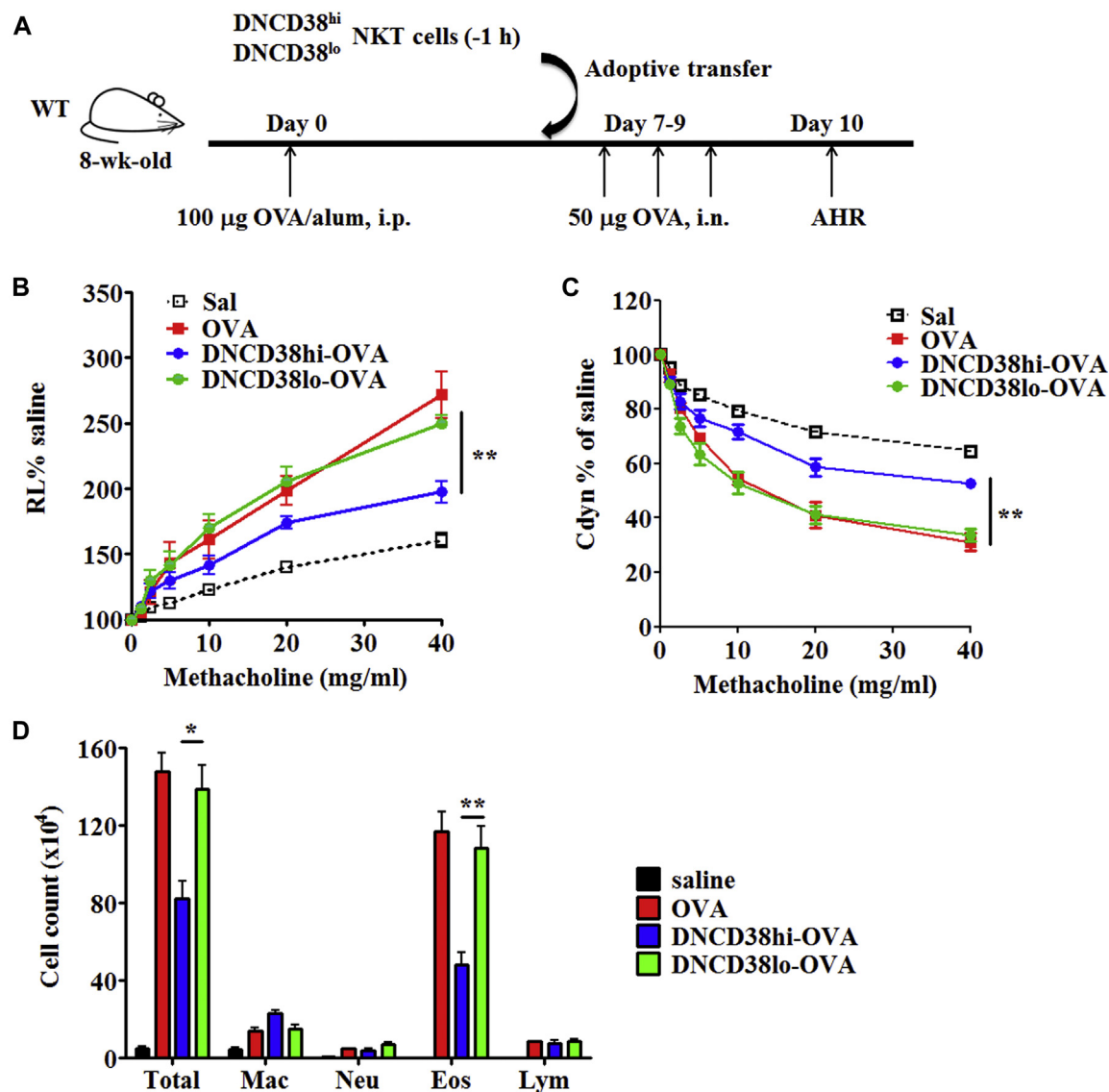


FIG 4. Adoptive transfer of DN CD38^{hi} NKT cells suppresses OVA-induced AHR. WT splenocytes were stimulated with α -GalCer to expand the NKT population. After 96 hours of stimulation, the DN CD38^{hi} and DN CD38^{lo} NKT subpopulations were sorted for adoptive transfer experiments. **A**, Schematic diagram of adoptive transfer of DN CD38^{hi} or DN CD38^{lo} NKT cells. *i.n.*, Intranasal; *i.p.*, intraperitoneal. **B-D**, DN CD38^{hi} or DN CD38^{lo} NKT cells were transferred (3×10^5 cells per mouse) to OVA-sensitized BALB/c mice ($n = 4-6$ mice per group). Changes in lung resistance (RL; Fig 4, B) and compliance (Fig 4, C) were measured in recipient mice. Fig 4, D, Cells in bronchoalveolar lavage (BAL) fluid were collected and analyzed. *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. * $P \leq .05$ and ** $P \leq .01$, 2-way ANOVA (Fig 4, B and C) or the Student 2-tailed *t* test (Fig 4, D).

FIG 3. WT DN CD38^{hi} NKT cell subset showed a direct suppressive effect on CD4⁺ T-cell proliferation. **A**, WT BALB/c splenocytes were stimulated with α -GalCer (100 ng/mL) to expand the NKT cell population. After 96 hours of stimulation, the splenic TCR β^+ CD1d tetramer-positive NKT cell population was gated and then 4-way sorted into CD4⁺CD38^{hi}, CD4⁺CD38^{lo}, DN CD38^{hi}, and DN CD38^{lo} subsets by using the BD FACSAria II cell sorter. **B**, Carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ T cells were used as responders and stimulated with ConA (0.45 µg/mL). Different subsets of NKT cells were added to culture at the indicated NKT/T-cell ratios. Color-filled histograms show CFSE dilution of CD4⁺ T cells after 5 days of coculture (gated on TCR β^+ CD1d tetramer-negative T cells), and black histograms represent the control (no NKT cell coculture). **C**, CFSE-labeled CD4⁺ T cells were placed in the lower chamber of the transwell, and DN CD38^{hi} NKT cells were administered into the upper chamber of the transwell. CD4⁺ T-cell proliferation was measured by using CFSE dilution after 5 days of incubation. *TCR*, T-cell receptor.

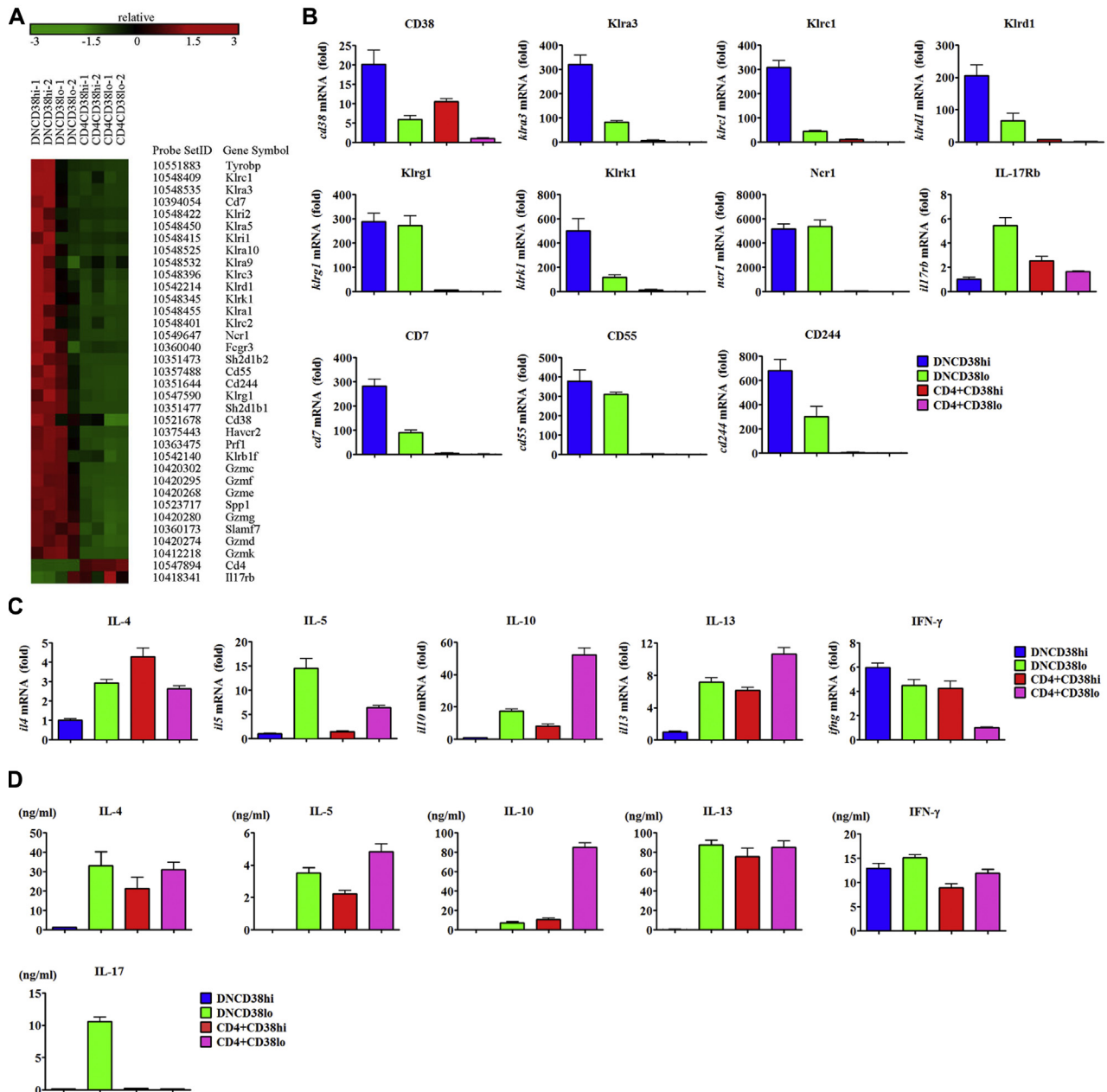


FIG 5. Microarray gene analysis and cytokine profiles of NKT cell subsets. **A**, Heat map of microarray data showing the selected differentially expressed genes among CD4⁺CD38^{hi}, CD4⁺CD38^{lo}, DN CD38^{hi}, and DN CD38^{lo} NKT cell subsets of WT BALB/c mice. **B**, Quantitative PCR analysis of *CD38*, *Klra3*, *Klrc1*, *Klrd1*, *Klrg1*, *Klrk1*, *Ncr1*, *IL17Rb*, *CD7*, *CD55*, and *CD244* mRNA expression by CD4⁺CD38^{hi}, CD4⁺CD38^{lo}, DN CD38^{hi}, and DN CD38^{lo} NKT cell subsets of WT BALB/c mice. Each PCR reaction was normalized to the housekeeping gene *Gapdh* and related to CD4⁺CD38^{lo} NKT cell mRNA levels, except IL-17RB, which was normalized to DN CD38^{hi} cells. **C**, Quantitative PCR analysis of *Il4*, *Il5*, *Il10*, *Il13*, and *Ifng* mRNA expression by CD4⁺CD38^{hi}, CD4⁺CD38^{lo}, DN CD38^{hi}, and DN CD38^{lo} NKT cell subsets of WT BALB/c mice. Each PCR reaction was normalized to the housekeeping gene *Gapdh* and related to the NKT cell subset with the lowest mRNA level (DN CD38^{hi} cells, except for IFN- γ , normalized to CD4⁺CD38^{lo}). **D**, Sorted CD4⁺CD38^{hi}, CD4⁺CD38^{lo}, DN CD38^{hi}, and DN CD38^{lo} NKT cell subsets from WT BALB/c mice were cocultured with bone marrow-derived dendritic cells (BMDC) for 48 hours in the presence of α -GalCer (100 ng/mL). Cytokine production by 4 NKT cell subsets were analyzed by means of ELISA.

of type 2-related cytokine transcripts, such as *Il4*, *Il5*, *Il10*, and *Il13*, were much lower in DN CD38^{hi} suppressive NKT subset compared with the other 3 subsets (Fig 5, C). Activation of sorted NKT cell subsets cocultured with bone marrow-derived DCs and

α -GalCer (Fig 5, D) induced large amounts of IFN- γ but minimal amounts of type 2- and type 17-related cytokines. On the other hand, IL-4, IL-5, and IL-13 were produced exclusively by the DN CD38^{lo}, CD4⁺CD38^{hi}, and CD4⁺CD38^{lo} NKT cell

populations, whereas IL-17 was mainly produced by the DN CD38^{lo} NKT cell population and IL-10 was produced predominantly by the CD4⁺CD38^{lo} NKT cell population.

Influenza infection in 2-week-old mice increases the DN CD38^{hi} suppressive NKT cell subset

Because our previous studies showed that influenza infection in suckling mice protected against subsequent development of allergen-induced AHR in adulthood by expanding DN NKT cells in the lungs, we next examined whether these cells that expanded with influenza infection expressed markers, such as CD38. Indeed, analysis of the mice 2 weeks after influenza A infection (age 4 weeks) showed that the suppressive DN CD38^{hi} NKT cell subpopulation increased in both lungs and spleens and persisted at least 5 weeks after infection (age, 7 weeks; Fig 6, A and B).

Protection by a 6'-derivatized α -GalCer analog is associated with expansion of DN CD38^{hi} NKT cells

To replicate the beneficial effects of influenza virus infection, we examined a panel of 6'-derivatized α -GalCer analogues to find one that might expand DN CD38^{hi} NKT cells in suckling mice. Among the glycolipid compounds tested, Nu- α -GalCer was the most promising. Nu- α -GalCer, featuring an NU substituent, can induce serum levels of IFN- γ comparable with α -GalCer combined with markedly reduced IL-4 levels, thereby inducing a significant increase in T_H1 bias.¹¹ Treatment of suckling mice with Nu- α -GalCer preferentially increased the number of suppressive DN CD38^{hi} NKT cell numbers in the lung at 4 weeks of age, and this increase lasted to at least 7 weeks of age or 5 weeks after Nu- α -GalCer administration (Fig 6, C and D). Although Toll-like receptor (TLR) 7 might be required for Nu- α -GalCer to limit AHR because *Tlr7*^{-/-} mice were not protected against OVA-induced AHR,¹⁰ TLR7 stimulation alone (with resiquimod) was not sufficient to expand suppressive DN CD38^{hi} NKT cells *in vivo* (see Fig E6 in this article's Online Repository at www.jacionline.org). Importantly, treatment of 2-week-old mice with Nu- α -GalCer protected the mice from development of allergen (OVA)-induced AHR and airway inflammation when sensitized and challenged at least 5 weeks after glycolipid treatment (Fig 6, E-G). Together, these results suggest that the DN CD38^{hi} suppressive NKT cell subset can be activated and expanded by Nu- α -GalCer and can mediate protective effects on allergic asthma similar to the effect observed with influenza virus infection.

DISCUSSION

In this study we describe a novel subset of murine DN NKT cells enriched by high expression of CD38. These CD38^{hi} DN NKTs suppressed CD4⁺ T-cell proliferation through direct cell-cell contact *in vitro* and prevented development of allergen-induced AHR *in vivo*. NKT cells constitute a small population of lymphocytes that express characteristics of both natural killer (NK) cells and conventional T cells. On activation, NKT cells rapidly secrete a large amount of type 1 and type 2 cytokines and chemokines; these then activate DCs, macrophages, NK cells, T cells, and B cells and ultimately drive the development of adaptive immunity.⁶ Although a number of heterogeneous subsets of NKT cells have been described previously that express distinct cytokines and functions,¹⁹ for the first time, we show that a novel

NKT cell subset can inhibit allergen-induced AHR, presumably by suppressing CD4⁺ T-cell activity through a contact-dependent mechanism. Moreover, this subset of NKT cells expanded in the lungs of neonatal mice after infection with influenza A virus or after treatment with a T_H1-biasing α -GalCer analog.

Although suppressive/regulatory NKT cells have been described previously, such as in the context of transplantation, graft tolerance, and prevention of type 1 diabetes mellitus,²⁰⁻²⁹ none have been shown previously to function in suppressing allergen-induced AHR by having direct inhibitory activity on CD4⁺ T cells. In previous studies regulatory NKT cells produced IL-4 or IL-10^{26,30-32} or expressed forkhead box protein 3.³³ A recently described NKT cell subset residing in adipose tissue expressed the transcription factor E4 promoter-binding protein 4, which controlled IL-10 production, induced an anti-inflammatory phenotype in macrophages, and enhanced the function of regulatory T cells in adipose tissue.²⁹ In contrast, the suppressive NKT cells we describe arose in the lungs of young mice after neonatal infection, were DN, produced IFN- γ , and, in particular, expressed CD38 and genes for cytotoxicity molecules.

The mechanism through which the NKT cells that we describe suppressed CD4 T-cell activity might involve cytotoxic activity because this DN CD38^{hi} suppressive NKT cell subset expressed many cytotoxicity-related molecules, including perforin and granzyme. Alternatively, these NKT cells might suppress CD4⁺ T-cell activity in part through programmed cell death protein 1-programmed death ligand 1 mechanisms because anti-PD-L1 mAbs partially blocked the *in vitro* activity of the NKT cells (see Fig E4). Inhibition was not likely mediated by IL-10 because the suppressive CD38^{hi} NKT cell subset did not produce IL-10; rather, CD38^{lo} NKT cells produced IL-10 in our system (Fig 5, D).

In our current report we describe NKT cells that prevent AHR, whereas NKT cells that augmented AHR had been described previously.^{34,35} Thus in allergen-induced models of asthma in mice, AHR did not develop in NKT cell-deficient mice, although some degree of eosinophilic airway inflammation remained in the absence of NKT cells.³⁴ Furthermore, in patients with ozone-induced AHR, a model of air pollution-associated asthma, as well as in Sendai virus-induced AHR, a model of virus-induced asthma, AHR did not develop in NKT cell-deficient mice.^{36,37} In each of these models, a distinct NKT cell subset was required for AHR: CD4⁺IL-17RB⁺ IL-4/IL-13-producing NKT cells were required in allergen-induced AHR, CD4⁻NK1.1⁻ IL-17-producing NKT cells were required in patients with ozone-induced AHR, and CD4⁺ NKT cells interacting with alveolar macrophages were required in Sendai virus-induced AHR. These results in mouse models support the idea that asthma is heterogeneous, with several distinct mechanistic or pathogenic pathways,^{38,39} and that multiple subsets of NKT cells are involved in asthmatic patients, some augmenting and some preventing asthma development.⁴⁰ Moreover, this heterogeneity in NKT cell subsets might also explain the diverse functions of NKT cells in multiple distinct diseases.

Although some subsets of NKT cells might be pathogenic in asthmatic patients, several groups have also proposed a role for NKT cells in inhibiting asthma. For example, in mice a single intraperitoneal administration of α -GalCer to activate NKT cells before allergen challenge inhibited the development of AHR.⁴¹⁻⁴³ However, this inhibition might have been due to induction of NKT cell anergy by α -GalCer treatment and not necessarily

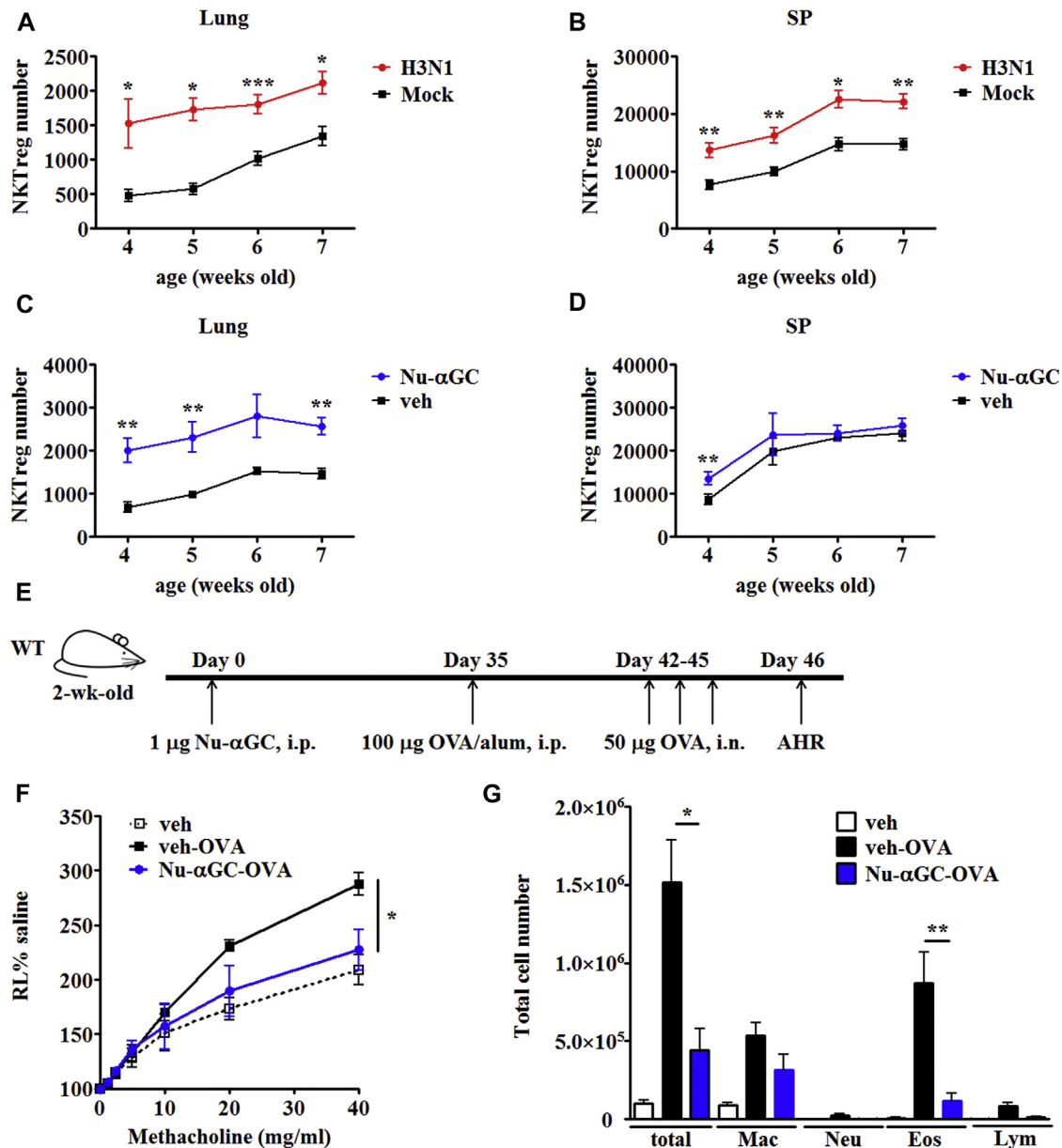


FIG 6. Induction of protection is associated with an increased number of DN CD38^{hi} NKT cells by H3N1 infection and Nu-α-GalCer treatment in suckling mice. **A** and **B**, WT BALB/c mice were infected with H3N1-infected or mock-infected cells at 2 weeks of age. Lung and spleen cells were isolated from H3N1-infected or mock-infected mice over a 5-week period. NKT cells were analyzed for CD4 and CD38 expression. Absolute numbers of DN CD38^{hi} NKT cells (*NKTreg*) were calculated in the lung (Fig 6, **A**) and spleen (Fig 6, **B**). * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$, Student 2-tailed *t* test. **C** and **D**, WT BALB/c mice were treated with Nu-α-GalCer (1 μg) administered intraperitoneally at 2 weeks of age. Lung and spleen cells were isolated from Nu-α-GalCer-treated or mock-treated mice weekly up to 5 weeks after treatment. NKT cells from the lung or spleen were then analyzed for CD4 and CD38 expression, and absolute numbers of DN CD38^{hi} NKT cells in the lung (Fig 6, **C**) and spleen (Fig 6, **D**) were calculated. ** $P \leq .01$, Student 2-tailed *t* test. **E**, Schematic showing the protocol for WT BALB/c mice treated at 2 weeks of age with Nu-α-GalCer (or vehicle/mock). Mice were sensitized and challenged with OVA as indicated and examined for OVA-induced AHR at 8 weeks of age ($n = 4-6$ mice per group). *i.n.*, Intranasal; *i.p.*, intraperitoneal. **F**, AHR was determined by measurement of lung resistance. **G**, Bronchoalveolar lavage fluid cells from treated mice were collected and analyzed. * $P \leq .05$ and ** $P \leq .01$, 2-way ANOVA (Fig 6, **F**) or the Student 2-tailed *t*-test (Fig 6, **G**).

due to activation of regulatory NKT cell activity. In contrast, we described a situation in which infection of suckling mice with influenza A virus protected the mice as adults against the

development of allergen-induced AHR, and this was associated with preferential expansion of a DN CD38^{hi} population of NKT cells in the lungs.

Our current studies describing the effects of infection or exposure of neonatal mice to an analog of α -GalCer that protects the mice by altering a component of the innate immune system in the lungs, such as NKT cells, are reminiscent of the hygiene hypothesis, which states that early exposure to infectious agents or microbiota protects against later development of asthma and allergy.⁹ However, neither the specific mechanism by which protection develops nor the specific microbes or communities of microbes that might protect are fully known or understood. Studies demonstrating that germ-free mice have a greater propensity for developing type 2 responses and have increased IL-4-producing NKT cells in lungs⁴⁴ are consistent with this idea, and *Helicobacter pylori*, hepatitis A virus, *Lactobacillus johnsonii*, and others have been suggested as important microbes that might alter innate immune compartments and confer protection.^{45,46} More recently, studies have shown that the diversity of microbiota in the lung/gut correlates with protection against asthma and allergy⁴⁷⁻⁴⁹ and that microbial exposures might provoke a form of innate “memory” and protection by increasing and expanding the cellular/lymphoid composition in organs, including the lungs.^{50,51} Infection with influenza in mice is known to induce development of bronchus-associated mucosal tissue (BALT), which is important for rapid initiation of subsequent pulmonary responses to unrelated airway challenges,⁵² suggesting a role for BALT, once established, in shaping and managing later pulmonary immunity as another form of “immunologic memory.” Finally, KLRG⁺, IFN- γ -producing, effector memory-like NKT cells in the lung have also been described,⁵³ with similarities to the NKT cells that we describe that might persist for long periods of time in the lung, although their specific anatomic location in the lung (eg, in BALT) has not been determined.

An important finding in this report is that the protective effects of neonatal influenza infection could be replicated by administration of a glycolipid that activated IFN- γ -producing NKT cells. We showed a similar effect previously using a glycolipid from *H pylori* or with α -C-GalCer,¹⁰ suggesting that certain microbes might inhibit the development of asthma in young children by having direct effects on NKT cells. However, the glycolipid Nu- α -GalCer was much more efficient in inducing the inhibitory effect of the *H pylori* glycolipid. Several synthetic α -GalCer analogues, such as α -C-GalCer or OCH, can induce preferential production of T_H1- or T_H2-biasing response by NKT cells.⁵⁴ Although the effects of α -C-GalCer are similar to those of Nu- α -GalCer, Nu- α -GalCer and its related aromatic 6'-derivatives have much greater potency because of a stronger interaction with CD1d caused by induction of significant conformational changes in CD1d leading to sustained interactions with NKT cells, which might explain its potency in preferentially inducing a T_H1-biased response. Accordingly, Nu- α -GalCer showed marked T_H1 biasing when administered intraperitoneally to C57BL/6 mice,^{11,54} which is consistent with its ability to expand a protective DN, IFN- γ -producing, CD38^{hi} NKT cell population. Among these 6'-derivatives, Nu- α -GalCer, which features an NU substituent, showed the most significant T_H1 bias in both C57BL/6 and BALB/c mice; Nu- α -GalCer also showed enhanced tumor suppression relative to α -GalCer in the B16 murine melanoma model, suggesting high antigenic potency.¹¹ Notably, Nu- α -GalCer induced an analogous T_H1 bias in human PBMCs *in vitro*, and these PBMCs induced increased IL-2 secretion and human NKT cell proliferation *in vitro*.¹¹ The conservation of T_H1 bias and NKT cell expansion in mice and human subjects,

in association with an ability to potentially expand a suppressive NKT cell population in neonatal mice, suggests that Nu- α -GalCer might be particularly promising as a therapeutic intervention in human subjects to prevent asthma. However, additional studies will be required to determine whether human CD38^{hi} NKT cells behave in a manner similar to these murine CD38^{hi} NKT cells.

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Key messages

- Influenza infection in young mice expands a novel regulatory NKT cell subset in the lungs.
- These regulatory NKTs inhibit helper T cells and suppress the subsequent development of allergic asthma.
- This effect can be mimicked by treatment of young mice with a glycolipid that preferentially activates regulatory NKT cells.

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METHODS

Microarray analysis and real-time PCR

Total RNA was isolated and purified from sorted NKT cells by using the RNeasy kit (Qiagen, Hilden, Germany). RNA samples were sent to the Microarray Core Facility at the Dana Farber Cancer Institute (Boston, Mass) for amplification and hybridization on the Mouse Gene 1.0 ST array (Affymetrix) to obtain genome-wide expression profiles. The resulting data were subsequently analyzed in the Multiplot suite^{E1} in GenePattern by first filtering out genes with low expression values (<100 in all subsets) and noisy genes (coefficient of variation > 0.8) and then selecting for genes upregulated or downregulated 2-fold (fold change, ≤ 0.5 or ≥ 2) in CD4⁺CD38^{hi} NKT cells relative to the other 3 subsets. Because many of the significantly upregulated/downregulated genes were related to the NK cell-mediated cytotoxicity pathway (KEGG), additional relevant genes not fitting the fold-change criteria are shown in the heat map; the heat map was generated with the GENE-E module (<http://www.broadinstitute.org/cancer/software/GENE-E/>) in GenePattern.

cDNA was synthesized from purified total RNA samples by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif) and amplified by means of real-time PCR on an ABI7300 with TaqMan primers and probes (Applied Biosystems, Foster City, Calif) for the indicated genes in triplicate wells to confirm and compare the relative expression values of specific genes among the 4 NKT cell subsets. Gene expression was normalized by using *Gapdh*. For each gene of interest, the Δ cycle threshold (CT) was calculated by subtracting the CT of *Gapdh* from the CT of the gene of interest. $\Delta\Delta$ CT was calculated by subtracting out the Δ CT of the NKT cell subset with the lowest mRNA level to calculate fold change as $2^{-\Delta\Delta$ CT} relative to that of the NKT cell subset. The SD for $\Delta\Delta$ CT was calculated by using the standard square root of squares method. Error bars in the figures were calculated with an upper bound of $2^{-\Delta\Delta$ CT + SD($\Delta\Delta$ CT).

Collection and analysis of bronchoalveolar lavage fluid

Immediately after AHR measurement, mice were killed, lungs were lavaged twice with 0.5 mL of PBS, and fluid was pooled. Cells in bronchoalveolar lavage fluid were counted and analyzed, as previously described.^{E2} The relative number of different types of leukocytes was determined from slide preparations of bronchoalveolar lavage fluid stained with Diff-Quik solution (Dade Behring, Deerfield, Ill).

Influenza infection

Two-week-old suckling mice were anesthetized and inoculated intranasally with influenza A virus (strain Mem/71[H3N1], which carries hemagglutinin A/Memphis/1/71 [H3] and neuraminidase A/Bellamy/42 [N1]). The virus was

grown and harvested from 10-day embryonated chicken eggs. The dose of virus used (1.2×10^4 plaque-forming units per mouse) causes nonlethal pneumonia of both suckling and adult mice, with complete viral clearance around day 7 after infection. Control (mock-infected) mice were treated with allantoic fluid diluted 1:500 in PBS.

Lung and spleen cell isolation for fluorescence-activated cell sorting analysis

Mice immunized with either Nu- α -GalCer or PBS were killed at weekly time points from week 2 to week 7 (early adulthood) with an overdose of pentobarbital. Whole lungs and spleens were removed and surface rinsed in PBS. Lungs were diced and incubated on an orbital shaker for 1 hour at 37°C in 3 mL of Dulbecco modified Eagle medium with 0.16 mg/mL collagenase (CS14; Worthington Biochemical, Lakewood, NJ) and 0.1% (vol/vol) DNase I (fraction IX; Sigma-Aldrich). Digestion products were filtered with mesh by using an 18-gauge needle to obtain single-cell suspensions. Spleens were mechanically disrupted and passed through mesh strainers to obtain single-cell suspensions. Red blood cells in both tissue types were removed by means of incubation for 5 minutes at 25°C in lysis buffer (Sigma-Aldrich) before rewashing. Whole cell numbers were obtained by counting in a hemacytometer under $\times 100$ magnification with a light microscope.

Flow cytometric analysis

Cells were preincubated with a blocking mAb to the FcR γ receptor (2.4G2) and washed before staining. Cells were stained with allophycocyanin-conjugated antibody to T-cell receptor β (H57-597 [109212]; BioLegend) and phycoerythrin-conjugated anti-CD1d tetramers loaded with PBS57 (National Institutes of Health/National Institutes of Allergy and Infectious Diseases MHC Tetramer Core Facility, Atlanta, Ga) to identify NKT cells in addition to fluorescein isothiocyanate-conjugated anti-CD38 (90 [102705]; BioLegend), allophycocyanin e780-conjugated anti-CD4 (RM4-5 [47-0042-82]; eBioscience, San Diego, Calif) to further characterize NKT cell subsets. Lung cells were stained additionally with phycoerythrin-Texas red-conjugated antibody to CD45 (MCD4517; Invitrogen, Carlsbad, Calif) to identify hematopoietic cells. Cells were analyzed on a FACSCanto (BD Biosciences, San Jose, Calif) with FlowJo 8.8.7 software (TreeStar, Ashland, Ore).

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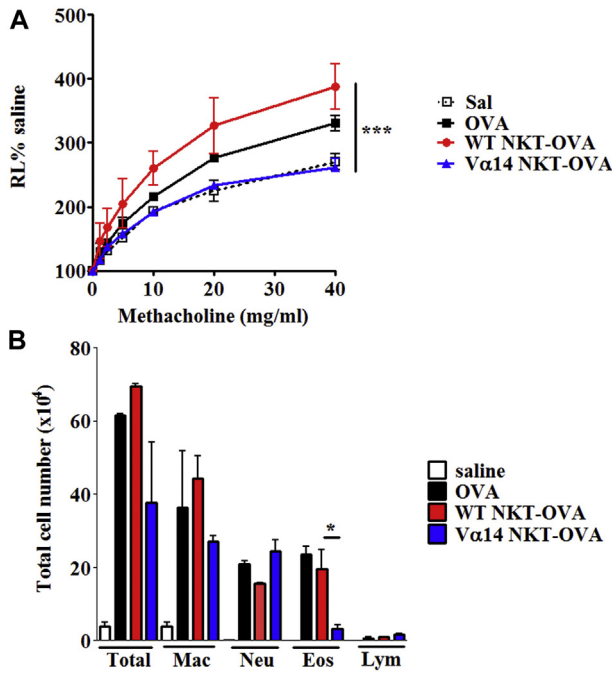


FIG E1. DN V α 14 Tg NKT cells showed a direct suppressive effect on CD4⁺ T-cell proliferation. V α 14 Tg NKT cells showed suppressive activity against allergen-induced AHR. NKT cells were purified from splenocytes of WT BALB/c and V α 14 Tg mice by using magnetic cell sorting. Purified NKT cells were adoptively transferred into OVA-sensitized BALB/c recipient mice ($n = 4-6$ mice per group) by means of intravenous injection (5×10^5 cells per mouse) 1 hour before the first OVA challenge (day 7). Changes in lung resistance (*RL*) on challenge with increasing concentrations of methacholine were measured in recipient mice after 3 consecutive days (days 7–9) of OVA challenge (**A**), and cells in bronchoalveolar lavage fluid were collected and analyzed (**B**). *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. * $P \leq .05$ and *** $P < .001$, 2-way ANOVA (Fig E1, A) or the Student 2-tailed *t*-test (Fig E1, B).

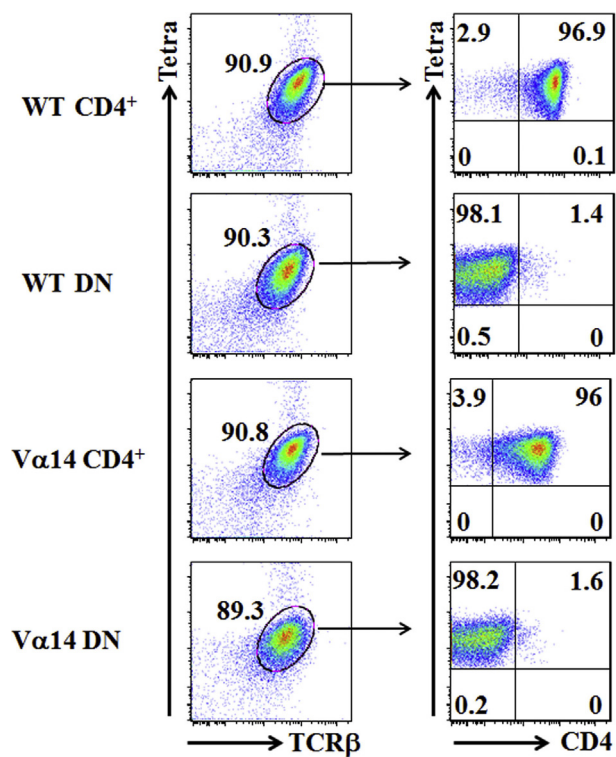


FIG E2. WT and Vα14 Tg splenocytes were stimulated with α-GalCer to expand the NKT cell population. After 96 hours of stimulation, splenocytes were stained with CD1d tetramers, T-cell receptor (*TCR*) β, and CD4 and sorted into CD4⁺ and DN subpopulations.

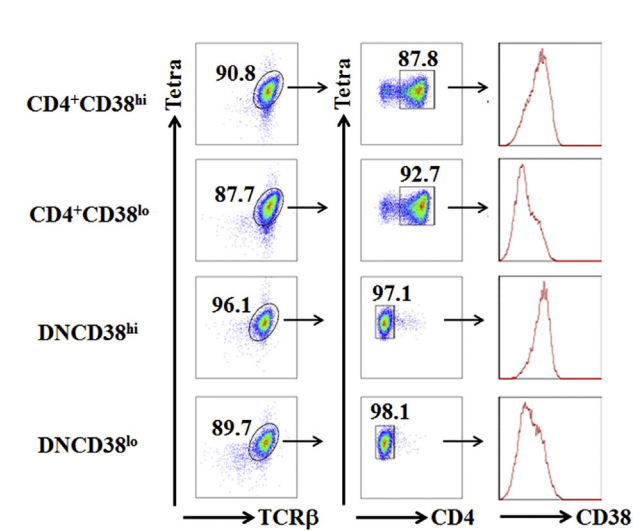


FIG E3. WT BALB/c splenocytes were stimulated with α -GalCer (100 ng/mL) to expand the NKT cell population. After 96 hours of stimulation, the splenic T-cell receptor (*TCR*) β^+ CD1d tetramer-positive NKT cell population was gated on and then 4-way sorted into CD4⁺CD38^{hi}, CD4⁺CD38^{lo}, DN CD38^{hi}, and DN CD38^{lo} subsets by using the BD FACSARIA II cell sorter.

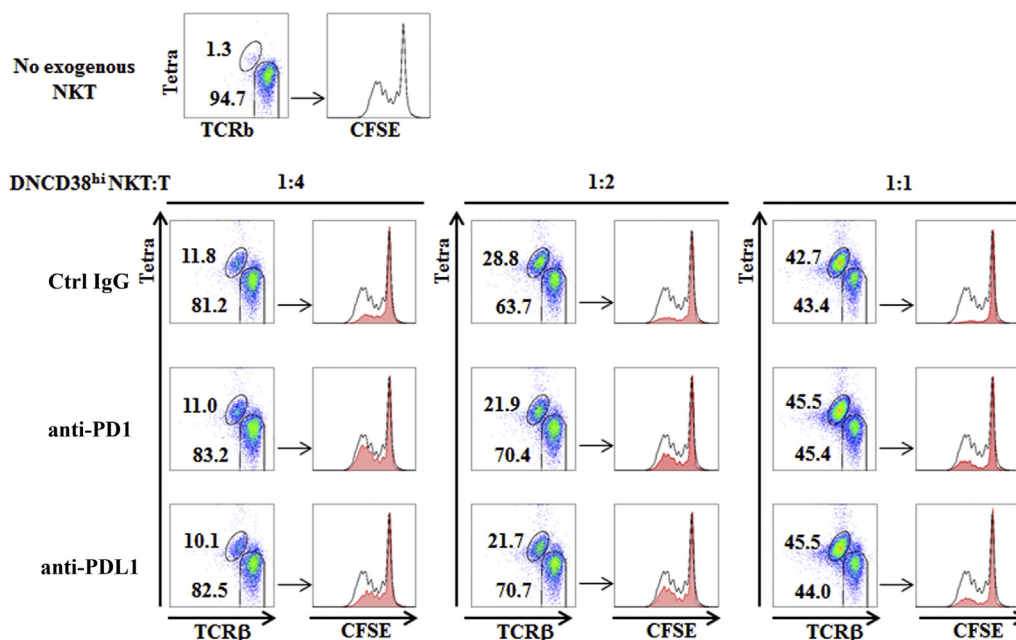


FIG E4. Blockade of programmed cell death protein 1 (*PD-1*) and programmed death ligand 1 (*PD-L1*) partially rescue the suppressive effect of DN CD38^{hi} NKT cells on CD4⁺ T-cell response. WT BALB/c splenocytes were stimulated with α -GalCer (100 ng/mL) to expand the NKT cell population. After 96 hours of stimulation, splenic DN CD38^{hi} T-cell receptor (*TCR*) β^+ CD1d tetramer-positive NKT cells were sorted with a BD FACSARIA II cell sorter. Carboxyfluorescein succinimidyl ester (*CFSE*)-labeled CD4⁺ T cells were used as responders and stimulated with ConA (0.45 μ g/mL). DN CD38^{hi} NKT cells were added to the culture at the indicated NKT/T-cell ratios in the presence of 10 μ g/mL control IgG, anti-PD-1, or anti-PD-L1 blocking antibody. Color-filled histograms show the CFSE dilution of CD4⁺ T cells after 5 days of coculture (gated on TCR β^+ CD1d tetramer-negative T cells), and black histograms represent the control (no exogenous NKT cell coculture).

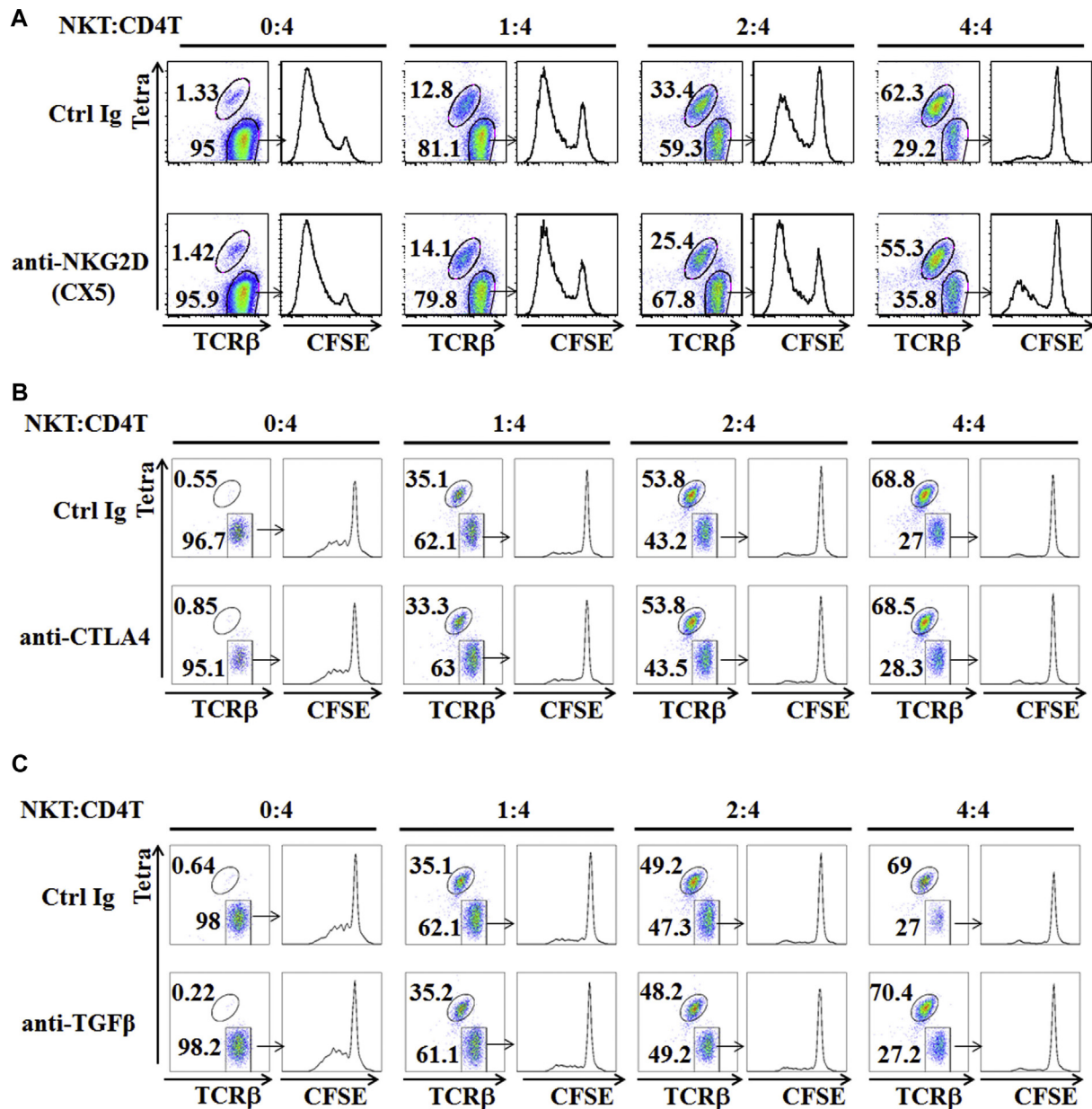


FIG E5. The suppressive effect of DN CD38^{hi} NKT cells is minimally dependent on NKG2D but not cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) or TGF- β . WT BALB/c splenocytes were stimulated with α -GalCer (100 ng/mL) to expand the NKT population. After 96 hours of stimulation, splenic DN CD38^{hi} T-cell receptor (TCR) β ⁺ CD1d tetramer-positive NKT cells were sorted with a BD FACSaria II cell sorter. Carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ T cells were used as responders and stimulated with ConA (0.45 μ g/mL). DN CD38^{hi} NKT cells were added to the culture at the indicated NKT/T-cell ratios in the presence of 10 μ g/mL control IgG and anti-NKG2D blocking antibody (CX5; **A**), anti-CTLA-4 blocking antibody (**B**), or anti-TGF- β blocking antibody (**C**). Histograms show the CFSE dilution of CD4⁺ T cells after 5 days of coculture (gated on TCR β ⁺ CD1d tetramer-negative T cells).

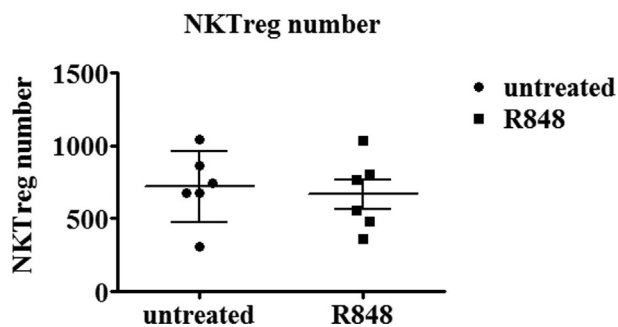


FIG E6. TLR7 stimulation cannot expand suppressive DN CD38^{hi} NKT cells *in vivo*. WT BALB/c mice were treated with resiquimod (R848, 100 μ g per mouse) administered intraperitoneally at 2 weeks of age. Lung cells were isolated from R848-treated or mock-treated mice at 4 weeks of age. NKT cells from lungs were then analyzed for CD4 and CD38 expression, and absolute numbers of DN CD38^{hi} NKT cells in the lung were calculated.