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NKT Cell-Dependent Regulation of Secondary Antigen-Specific, Conventional CD4⁺ T Cell Immune Responses

YoungHyun Shin,* Changwan Hong,* Hyunji Lee,* Jung Hoon Shin,* Seokmann Hong,[†] and Se-Ho Park*

NKT cells are considered to be innate-like regulatory cells. However, their regulatory functions in adaptive immune responses have not been studied in detail. In this study, we investigated the immunoregulatory functions of NKT cells during the secondary phase of an Ag-specific CD4⁺ T cell response. When compared with OVA-specific effector CD4⁺ T cells adoptively transferred into NKT cell-deficient naive CD1d^{-/-} mice, the same T cells transferred into naive CD1d^{+/-} mice exhibited substantially stronger immune responses on OVA challenge. The enhanced immune response of the transferred CD4⁺ T cells in the presence of NKT cells correlated with an increase in their proliferation *in vivo*. In addition, T cells transferred into CD1d^{+/-} recipients showed enhanced cytokine productions relative to T cells in CD1d^{-/-} recipients. To elucidate the physiological relevance of the regulatory role of NKT cells in a disease setting, OVA-specific asthma was induced in recipient mice after adoptive transfer of OVA-specific CD4⁺ T cells. CD1d^{+/-} recipients showed stronger asthmatic phenotypes in all indications when compared with CD1d^{-/-} recipients. Taken together, these results suggest that NKT cells are critical for the regulation of Ag-specific, conventional CD4⁺ T cells during the secondary phase of an adaptive immune response. *The Journal of Immunology*, 2010, 184: 000–000.

Natural killer T cells are a heterogeneous subset of T cells that express both TCRs and NK-lineage cell surface markers, such as CD161, CD16, Ly49A, and Ly49C (1). However, most NKT cells are distinct from conventional $\alpha\beta$ T cells in that their TCRs display limited diversity and recognize glycolipids presented by nonpolymorphic CD1d, a nonclassical Ag-presenting molecule, rather than peptide-MHC complexes (2). Previous studies have suggested that NKT cells play immunoregulatory roles during the innate immune phase (3). However, recent studies have shown that cytokines rapidly produced by NKT cells can regulate the function of dendritic cells (DCs), NK cells, and B cells, as well as of conventional CD4⁺ and CD8⁺ T cells, suggesting that NKT cells are involved in the generation of successful adaptive immune responses (4–8). Several studies have demonstrated that stimulation of NKT cells with α -galactosylceramide (α -GalCer) *in vivo* significantly increases immune responses against coinjected Ags (9–11). Fujii et al. (10) suggested that injection of α -GalCer rapidly stimulates full maturation of DCs and that this stimulatory effect accounts for the induction of combined CD4⁺ Th1 and CD8⁺ T cell immunity to coadministered proteins. In addition, it has been reported that adaptive, CD4⁺ T cell-mediated antitumor immune re-

sponses during the early effector phase are critically dependent on NKT cells (12).

Ag-specific, adaptive T cell responses in both defense and pathogenesis consist of a relatively weak primary immune response and a stronger secondary immune response mediated by activated or memory T cells on Ag rechallenge. Although it has recently been shown that a successful CD8⁺ T cell-mediated secondary immune response to Ag is dependent on the presence of NKT cells (13), it is not clear whether NKT cells also contribute to CD4⁺ T cell-mediated secondary immune responses.

In this study, we investigated the role of NKT cells in the regulation of CD4⁺ T cell-dependent secondary immune responses by adoptively transferring primed CD4⁺ T cells into both NKT cell-present and -deficient naive recipients. In addition, we used an OVA-induced murine asthma model to investigate the contribution of NKT cells in the induction of a CD4⁺ T cell-mediated disease. We provide evidences demonstrating that, in the presence of NKT cells, Ag-specific CD4⁺ T cells exhibit enhanced proliferation, cytokine production, and ability to mediate OVA-induced murine asthma on secondary Ag challenge during the effector phase of the immune response.

Materials and Methods

Mice, culture media, and reagents

Six- to 8-wk-old BALB/c or C57BL/6 CD1d^{+/-} and CD1d^{-/-} mice (14) were bred under specific pathogen-free conditions at Korea University. The experimental protocols adopted in this study were approved by the Institutional Animal Care and Use Committee of Korea University. OT-II TCR and DO11.10 TCR transgenic (Tg) mice, as well as CD45.1 congenic mice, were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 V α 14 TCR Tg mice and α -GalCer were provided by Dr. A. Bendelac (University of Chicago, Chicago, IL) and J α 18^{-/-} mice (BL/6 background) were generous gifts from Dr. M. Taniguchi (Chiba University, Chiba, Japan) (15). CFA; *Mycobacterium tuberculosis*, OVA, and CFSE were purchased from Sigma-Aldrich (St. Louis, MO). Alum was purchased from Pierce (Rockford, IL). Chicken OVA_{323–339} peptide (H-2^d restricted; amino acid sequence ISQAVHAHAIEINEAGR) was purchased from GenScript (Piscataway, NJ). Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin,

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Abbreviations used in this paper: α -GalCer, α -galactosylceramide; AHR, airway hyperresponsiveness; DCs, dendritic cells; iNKT, invariant NKT; Tg, transgenic; WT, wild-type.

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10 µg/ml gentamicin sulfate, 50 µM β-mercaptoethanol (all from Life Technologies, Rockville, MD) and 10% heat-inactivated FBS (Hyclone, Logan, UT). IL-4, IL-5, and IFN-γ concentrations were measured using the OPTIA Mouse IL-4, IL-5 and IFN-γ enzyme-linked immunosorbent assay kit (BD Pharmingen, San Diego, CA). Reagents for detecting mouse IL-13 were purchased from R&D Systems (Minneapolis, MN).

FACS analysis

FACS staining was performed as previously described (13). Briefly, cells preincubated with the anti-FcγRII/III mAb 2.4G2 for 30 min were stained with the appropriate combinations of the following mAbs: anti-CD4, anti-TCR-β, anti-DO11.10 TCR, anti-CD45.1, anti-CD3e, anti-IL-4, and anti-IFN-γ (all from BD Pharmingen). For intracellular cytokine staining, cells were incubated in culture medium containing Golgi Stop (BD Pharmingen) for 6 h, followed by intracellular staining, fixation, and permeabilization with Cytofix/Cytoperm solution (BD Pharmingen). The cells were then stained with anticytokine mAbs for 45 min. Cells were analyzed on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

In vivo and ex vivo Ag-specific CD4⁺ T cell responses

DO11.10 and OT-II Tg mice were immunized s.c. or i.p. with 100 µg OVA protein emulsified in CFA or adsorbed in alum, respectively. Two weeks later, splenic CD4⁺ T cells from immunized mice were sorted using anti-

CD4-coated magnetic beads (Miltenyi Biotec, Auburn, CA), labeled with 5 µM CFSE for 10 min and transferred i.v. into recipients (5×10^6 cells/mouse). To ensure that the same number of donor cells was transferred into each recipient, the number of donor cells in peripheral blood of the recipients was monitored 18 h after adoptive transfer. Among the recipients, only the mice showing similar levels of donor cells were subjected to further Ag challenge. Appropriate recipient mice were rechallenged i.v. with 100 µg OVA protein 1 d after adoptive transfer. Splenic CD4⁺ T cell responses were analyzed at time points between 3–5 d after Ag challenge. For ex vivo experiments, splenocytes from the recipients were collected 1 d after adoptive transfer without Ag challenge and were subsequently cultured with 100 µg/ml OVA for 3–7 d.

Measurement of airway hyperresponsiveness in OVA-induced asthma

Mice were immunized i.p. with 100 µg OVA in alum. After 14 d, CD4⁺ T cells isolated with anti-CD4-coated magnetic beads (Miltenyi Biotec) were adoptively transferred i.v. into CD1d^{+/+} and CD1d^{-/-} recipients (5×10^6 cells/mouse). The next day and for 5 consecutive days thereafter, all recipient mice were exposed to an aerosol of 5% OVA for 30 min. For some experiments, differentiated CD4⁺ Th2 cells were transferred. For this purpose, OVA-specific CD4⁺ Th2 cells were generated from splenocytes of DO11.10 TCR Tg mice as previously described (16, 17). The cells were then transferred i.v. into CD1d^{+/+} and CD1d^{-/-} recipients (5×10^6 cells/mouse). One day later and for 4 consecutive days thereafter, mice were exposed to an aerosol of 5% OVA for 30 min. Control mice received T cells i.v. but were exposed to PBS rather than OVA for 30 min. Twenty-four hours after the last Ag challenge, airway hyperresponsiveness (AHR) responses were analyzed by

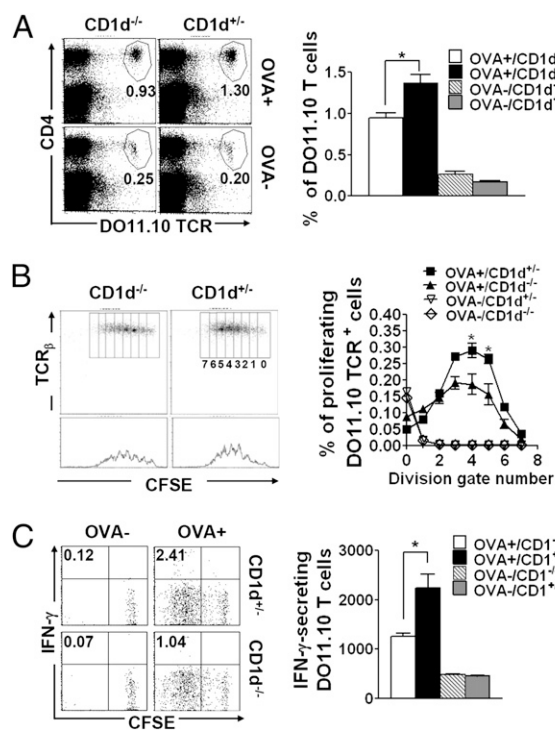


FIGURE 1. The requirement of NKT cells for the optimal secondary immune response of CD4⁺ Th1 cells derived from DO11.10 Tg mice against Ag restimulation in vivo. DO11.10 mice were immunized s.c. with 100 µg OVA emulsified in CFA. After 2 wk, primed CD4⁺ T cells were sorted with anti-CD4 Ab, labeled with CFSE, and transferred into naive CD1d^{-/-} and CD1d^{+/+} mice (5×10^6 cells/mouse). The next day, the recipients were challenged with 100 µg OVA. Four days later, splenocytes were prepared from the recipients. The response of OVA-specific CD4⁺ T cells was assessed by cell surface staining and flow cytometry. OVA-unchallenged CD1d^{+/+} and CD1d^{-/-} recipients were used as controls. **A**, The total percentage of CD4⁺ DO11.10 TCR⁺ cells, regardless of their cell division, was determined (**p* < 0.05). **B**, CFSE profiles of CD4⁺ DO11.10 TCR⁺ cells; the numbered gates indicate individual cell divisions. Summary of the varying proportions of cells in each cycle of division between undivided (D₀) and maximally divided cells (D₇) gated in the left panel. **p* < 0.05 versus primed DO11.10 → CD1d^{-/-} groups (-▲-). **C**, The number of IFN-γ-secreting cells among CD4⁺ DO11.10 TCR⁺ cells was determined by intracellular staining (**p* < 0.05). Data are expressed as the mean ± SEM of three mice and represent three independent experiments.

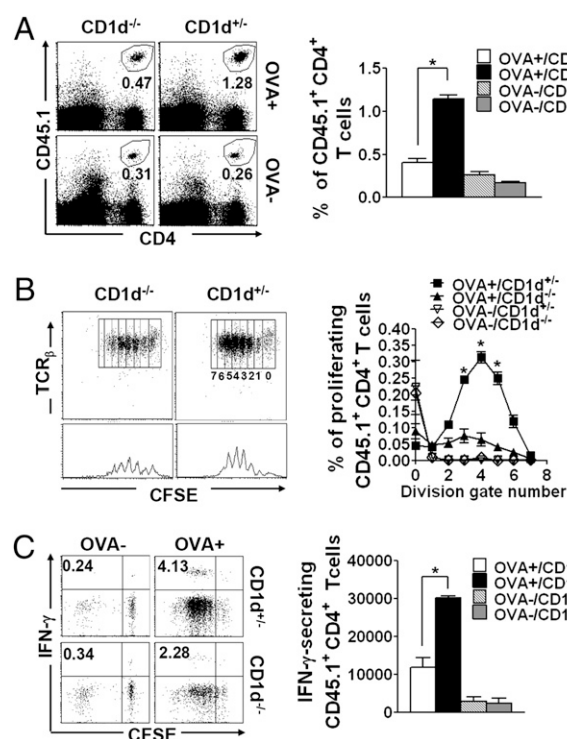


FIGURE 2. The requirement of NKT cells for the optimal secondary immune response of CD4⁺ Th1 cells derived from OT-II Tg mice against Ag restimulation in vivo. CD45.1/OT-II mice were immunized s.c. with 100 µg OVA emulsified in CFA. Primed CD4⁺ T cells were isolated and transferred and recipients were challenged with 100 µg OVA as described Fig. 1. **A**, The total percentage of CD4⁺CD45.1⁺ cells was determined (**p* < 0.05). **B**, CFSE profiles of CD4⁺CD45.1⁺ cells; the numbered gates indicate individual cell divisions. Summary of the varying proportions of cells in each cycle of division between undivided (D₀) and maximally divided cells (D₇) gated in the left panel. **p* < 0.05 versus primed CD4⁺ OT-II T cells → CD1d^{-/-} groups (-▲-). **C**, The number of IFN-γ-secreting cells among OVA-specific CD4⁺ T cells was determined by intracellular staining (**p* < 0.05). Data are expressed as the mean ± SEM of three mice and represent three independent experiments.

placing conscious mice in a whole-body plethysmograph and measuring methacholine-induced airflow obstruction as previously described (18). Single-cell suspensions of bronchial lymph nodes were prepared as previously described (12) and then cultured with 100 μ g/ml OVA in RPMI 1640 culture medium. After 5 d of culture, the levels of cytokines in culture supernatants were determined using an enzyme-linked immunoassay kit (BD Pharmingen).

Coculture of NKT cells with Th1 or Th2 cells in vitro

To measure activation and cytokine production, OT-II T cells were isolated with anti-CD4-coated magnetic beads (Miltenyi Biotec) and subsequently differentiated into Th1 and Th2 cells using several cytokines, as previously described (19–21). DCs were sorted from spleens of CD1d^{+/-} and CD1d^{-/-} mice using anti-CD11c-coated magnetic beads (Miltenyi Biotec) and then pulsed with OVA_{323–339} peptide. Next, OT-II-derived Th1 and Th2 cells were cocultured with OVA peptide-pulsed DCs in the presence of NKT cells enriched from V α 14 Tg mice. NKT cells were sorted from the spleens of V α 14 Tg mice via the MACS system (Miltenyi Biotec) using α -GalCer/CD1d-tetramer magnetic beads, according to the manufacturer's instructions. The purity of the NKT cell population was >90. Twenty-four hours later, the activation status of CD4⁺ Th1 and Th2 cells was assessed by measuring intracellular levels of IFN- γ and IL-4, respectively.

Statistical analysis

Prism software (GraphPad, San Diego, CA) was used for all statistical analyses. The Student *t* test was used to determine whether differences between two experimental groups reached statistical significance. Throughout the text, figures, and legends the following symbols are used to denote statistical significance: **p* < 0.05, ***p* < 0.01.

Results

NKT cells enhance the activation of Ag-specific CD4⁺ Th1 cells during secondary immune responses in vivo

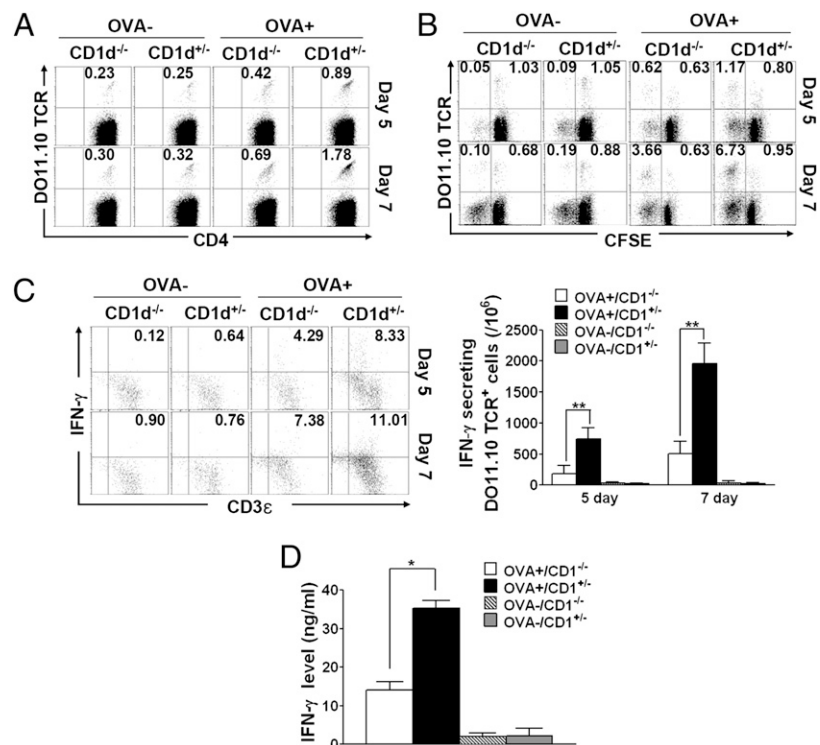
Recently, we demonstrated that NKT cells play an essential role during the secondary immune response of CD8⁺ T cells by inducing the expansion and activation of effector CD8⁺ T cells (13). Based on these results, we hypothesized that NKT cells would also contribute to the secondary immune response of CD4⁺ T cells. To test our hypothesis, we examined the activity of primed effector CD4⁺

T cells during a secondary immune response in the presence or absence of NKT cells. Although both unchallenged recipients showed low but similar levels of CD4⁺ DO11.10 donor T cells, the percentage of donor T cells after OVA challenge in CD1d^{+/-} recipients was substantially higher than in CD1d^{-/-} recipients (1.37 ± 0.17 versus 0.94 ± 0.10 , **p* < 0.05) (Fig. 1A). The increased frequency of donor T cells observed in CD1d^{+/-} recipients on Ag challenge appeared to be the result of increased cell division because these cells exhibited a more profound dilution of CFSE when compared with T cells in CD1d^{-/-} recipients (Fig. 1B). In addition, the presence of NKT cells in recipient mice resulted in a 2-fold increase in the relative numbers of IFN- γ -secreting CD4⁺ DO11.10 T cells in OVA-challenged recipients (Fig. 1C).

To exclude the possibility that our observations were attributed to the genetic background of the recipient mice, we next used another OVA-specific OT-II TCR Tg model on a C57BL/6 genetic background. The number of CD45.1⁺CD4⁺ cells in OVA-challenged CD1d^{+/-} mice was ~2-fold higher than in CD1d^{-/-} recipients ($1.14\% \pm 0.063$ versus $0.403\% \pm 0.08$, **p* < 0.05) (Fig. 2A). In addition, the numbers of cell divisions (Fig. 2B) and of IFN- γ -secreting donor cells (Fig. 2C) in response to OVA challenge were higher in CD1d^{+/-} recipients than in CD1d^{-/-} recipients. Collectively, our results using two genetically different OVA-specific TCR Tg models clearly demonstrate that NKT cells are important for the activation of secondary immune responses by CD4⁺ Th1 effector cells in vivo.

We performed similar experiments using J α 18^{-/-} mice as recipients to examine whether the phenomenon observed with the Ag-specific CD4⁺ T cell response was due to the lack of type 1 invariant NKT (iNKT) cells. As expected, the number of transferred OT-II T cells in OVA-challenged J α 18^{-/-} mice was lower than in OVA-challenged wild-type (WT) recipients (Supplemental Fig. 1A). In addition, the numbers of cell divisions (Supplemental Fig. 1B) and of IFN- γ -secreting donor cells (Supplemental Fig. 1C) were higher after OVA challenge in WT recipients as compared with J α 18^{-/-} recipients. Taken together, these results clearly demonstrate that

FIGURE 3. The requirement of NKT cells for the optimal secondary immune response of CD4⁺ Th1 cells derived from DO11.10 Tg mice against Ag restimulation ex vivo. Primed CD4⁺ T cells were obtained from DO11.10 mice as described in Fig. 1 and were transferred into naive CD1d^{-/-} and CD1d^{+/-} mice (5×10^6 cells/mouse). The next day, splenocytes were prepared from each recipient and were treated with 100 μ g/ml OVA for 3–7 d. OVA-specific CD4⁺ T cell responses were assessed by cell surface staining and flow cytometry. **A**, The total percentage of CD4⁺ DO11.10⁺ cells in the absence or presence of OVA was determined. **B**, Then, 5–7 d after incubation with OVA, proliferating CD4⁺CD3⁺ cells were analyzed. Numbers within plots indicate the percentage of positive cells. **C**, The percentage and number of IFN- γ -secreting cells among DO11.10 TCR⁺ CD4⁺ T cells were determined by intracellular staining (***p* < 0.01). **D**, The levels of IFN- γ in culture supernatants were determined using an enzyme-linked immunoassay (**p* < 0.05). Data are expressed as the mean $\pm$ SEM of three mice and represent three independent experiments.



type 1 iNKT cells are important for the activation of secondary immune responses by CD4⁺ Th1 effector cells.

NKT cells enhance the activation of Ag-specific CD4⁺ Th1 cells during secondary immune responses ex vivo

Next, we further examined the effect of NKT cells during the secondary immune response of Th1 CD4⁺ cells using an ex vivo system. The proportion and division of OVA-specific DO11.10 T cells in CD1d^{+/-} recipients were substantially higher than in CD1d^{-/-} recipients when restimulated with OVA for 5 and 7 d (Fig. 3A, 3B). In addition, the number of IFN- γ -secreting DO11.10 T cells was also 3–4 times higher in CD1d^{+/-} recipients on OVA restimulation (Fig. 3C). Moreover, the levels of IFN- γ were higher in culture supernatants of splenocytes isolated from CD1d^{+/-} recipients (Fig. 3D). These results were confirmed using OT-II Tg mice as the source of donor cells (data not shown).

Altogether, our results demonstrate that NKT cells play an essential role in inducing the expansion and Th1-skewed IFN- γ secretion of primed CD4⁺ Th1 effector cells, indicating that NKT cells are necessary for optimal secondary immune responses of CD4⁺ Th1 effector cells.

NKT cells enhance the activation of Ag-specific CD4⁺ Th2 cells during secondary immune responses in vivo

In addition to the essential role that NKT cells have in regulating secondary Th1 immune responses, it is of interest whether NKT cells can influence secondary Th2 immune responses. To evaluate this possibility, we performed similar experiments as described in Fig. 1 using alum as an adjuvant instead of CFA because alum has been known to skew CD4⁺ T cell differentiation toward the Th2 type (22). CD45.1⁺/OT-II mice were immunized with OVA adsorbed in alum. Two weeks later, primed CD4⁺ effector T cells were isolated, labeled with CFSE, and transferred into naive CD1d^{-/-} and CD1d^{+/-} mice. As shown in Fig. 4A, the percentage of CD4⁺ CD45.1⁺ donor T cells in OVA-challenged CD1d^{+/-} mice was higher than in CD1d^{-/-} recipients (0.94 ± 0.04 versus 0.65 ± 0.1 , $*p < 0.05$). In addition, CD4⁺ CD45.1⁺ T cells proliferated significantly in CD1d^{+/-} recipients when compared with CD1d^{-/-} recipients (Fig. 4B). The presence of NKT cells during the secondary immune response enhanced the number of IL-4-secreting CD4⁺ CD45.1⁺ T cells in OVA-challenged recipients (Fig. 4C). These results clearly show that NKT cells can enhance the secondary responses of both Th1- and Th2-differentiated, Ag-specific CD4⁺ effector T cells.

Interaction between APCs and NKT cells is required for the enhanced response of effector CD4⁺ T cells

We next analyzed whether NKT cells require CD1d-mediated stimulation to boost CD4⁺ T cell function during a secondary immune response. For this purpose, CD4⁺ T cells were isolated from naive OT-II Tg mice and further differentiated into Th1 or Th2 cells in vitro as described in the *Materials and Methods*. These differentiated Th1 or

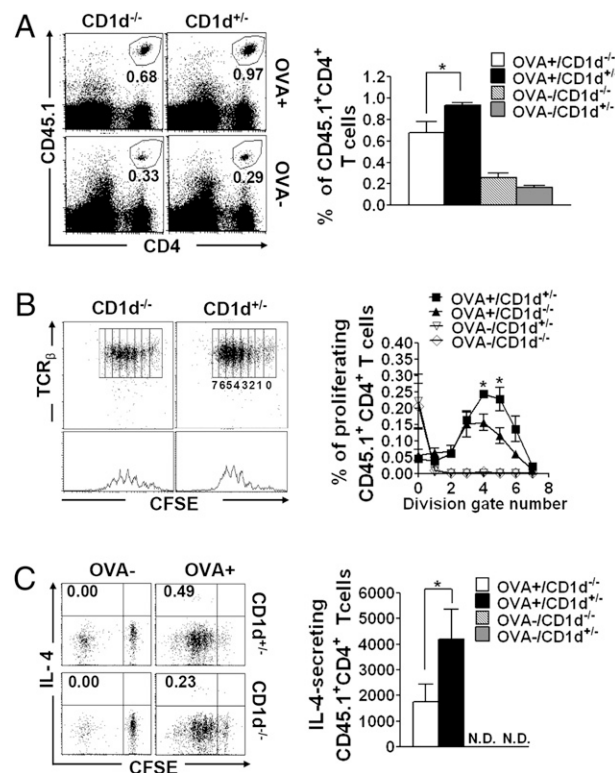
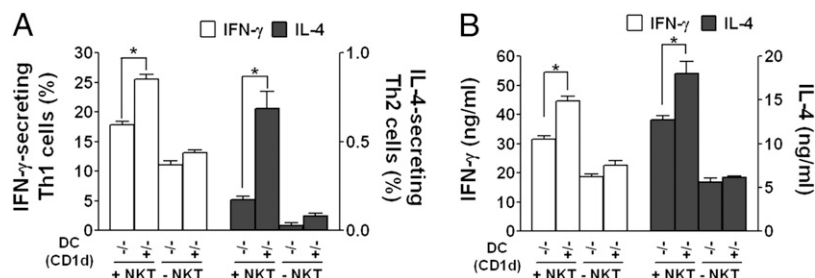


FIGURE 4. The requirement of NKT cells for the optimal secondary immune response of CD4⁺ Th2 cells against Ag restimulation in vivo. CD45.1/OT-II Tg mice were immunized i.p. with 100 μ g OVA adsorbed in alum. After 2 wk, primed CD4⁺ T cells were sorted with anti-CD4 Ab, labeled with CFSE, and transferred into naive CD1d^{-/-} and CD1d^{+/-} mice (5×10^6 cells/mouse). The next day, the recipients were challenged with 100 μ g OVA and 3–5 d later splenocytes were prepared from recipients. **A**, The total percentage of CD4⁺ CD45.1⁺ cells was determined ($*p < 0.05$). **B**, CFSE profiles of CD4⁺ CD45.1⁺ cells; the numbered gates indicate individual cell divisions. Summary of the varying proportions of cells in each cycle of division between undivided (D_0) and maximally divided cells (D_7) gated in the left panel. $*p < 0.05$ versus primed CD4⁺ T cells $\rightarrow$ CD1d^{-/-} groups ($-\Delta$). **C**, The number of IL-4-secreting cells among OVA-specific CD4⁺ T cells was determined by intracellular staining ($*p < 0.05$). Data are expressed as the mean $\pm$ SEM of three mice and represent three independent experiments.

Th2 cells were cultured for 24 h in vitro with or without NKT cells and OVA_{323–339} peptide-pulsed (OVA⁺) DCs derived from either CD1d^{+/-} or CD1d^{-/-} mice. The percentage of cytokine-producing effector T cells and the amount of cytokines produced (IFN- γ in Th1-differentiated OT-II cells and IL-4 in Th2-differentiated OT-II cells) were higher in the presence of Ag-presenting CD1d^{+/-} DCs (Fig. 5A, 5B). These results suggest that NKT cells need to interact with CD1d on the APCs to stimulate Ag-specific CD4⁺ T cells on recognition of Ag.

FIGURE 5. NKT cells enhance the production of cytokines by effector CD4⁺ T cells in vitro. Differentiated CD4⁺ Th1 and Th2 cells were incubated for 24 h with peptide-pulsed CD1d^{+/-} or CD1d^{-/-} DCs in the presence or absence of NKT cells. **A**, One day after incubation, cytokine secretion was analyzed in CD45.1⁺ CD4⁺ cells. The percentage of IFN- γ - or IL-4-secreting cells among Th1 CD4⁺ T cells or Th2 CD4⁺ T cells was determined by intracellular staining ($*p < 0.05$). **B**, IFN- γ and IL-4 levels in culture supernatants were determined using an enzyme-linked immunoassay ($*p < 0.05$).



Diminished CD4⁺ Th2 cell responses in the absence of NKT cells during a secondary immune response in the OVA-induced asthma model

To evaluate the effect of NKT cells on the secondary phase of a CD4⁺ T cell immune response in a chronic inflammatory condition, we used a murine model of asthma because NKT cells have been shown to play a key role in the development of an asthmatic phenotype, which includes AHR and cytokine production (IL-4, IL-5, and IL-13) (22–25). After producing asthmatic T cells by immunization of WT mice with OVA protein adsorbed in alum, CD4⁺ Th2 cells were sorted and adoptively transferred into CD1d^{+/-} or CD1d^{-/-} naive mice. When recipients were challenged with OVA, the extent of AHR, which is a hallmark of an asthmatic response, was magnified in CD1d^{+/-} mice (Fig. 6A). In addition, the production of Th2 cytokines, which are necessary for the development of asthma (26), was enhanced in the presence of NKT cells (Fig. 6B), suggesting that NKT cells are important for the development of a CD4⁺ Th2 cell-dependent secondary immune response to asthma.

Furthermore, we adoptively transferred OVA-specific Th2 cells differentiated from DO11.10 Tg mice into CD1d^{+/-} or CD1d^{-/-} naive mice and monitored the extent of AHR on OVA challenge. As shown in Fig. 6C, CD1d^{+/-}, but not CD1d^{-/-}, mice exhibited an enhanced AHR. Taken together, these results demonstrate that NKT cells play a critical role in the development and maintenance of asthma during the secondary phase of an immune response.

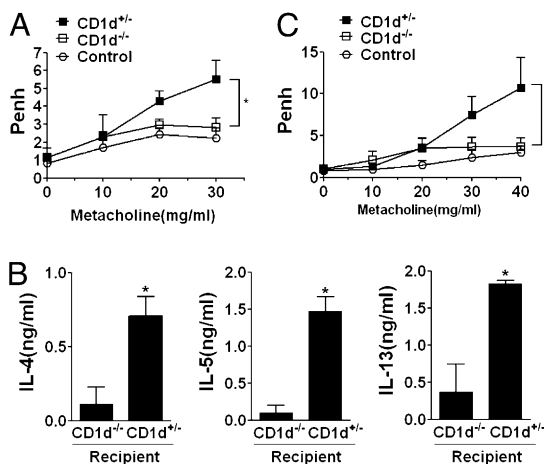


FIGURE 6. The absence of NKT cells during the secondary immune stage of murine asthma decreases AHR. *A*, WT mice were immunized i.p. with 100 μ g OVA adsorbed in alum. After 2 wk, primed CD4⁺ T cells were sorted from splenocytes with anti-CD4 Ab and transferred into naive CD1d^{+/-} and CD1d^{-/-} mice. The recipients were then challenged with an aerosol of 5% OVA for 5 d and AHR to inhaled methacholine was measured 24 h after the last OVA challenge. Control mice also received primed CD4⁺ T cells but were challenged with PBS instead of OVA (* p < 0.05). *B*, Cells from bronchial lymph nodes were prepared 24 h after the measurement of AHR and were restimulated (5×10^5 cells) with 100 μ g/ml OVA for 5 d. Supernatants were collected and the levels of IL-4, IL-5, and IL-13 were analyzed by an enzyme-linked immunoassay (* p < 0.05). *C*, OVA_{323–339}-specific Th2 cells derived from DO11.10 TCR Tg mice were generated in vitro as described in the *Materials and Methods*. CD4⁺ Th2 cells were injected i.v. into naive CD1d^{+/-} and CD1d^{-/-} mice. The recipients were then challenged with an aerosol of 5% OVA for 4 d and AHR to inhaled methacholine was measured 24 h after the last OVA challenge. Control mice also received primed CD4⁺ Th2 cells but were challenged with PBS instead of OVA (* p < 0.05). Data are expressed as the mean $\pm$ SEM of four mice and represent two independent experiments.

Discussion

NKT cells have long been considered to be regulators of innate immune responses (3, 4). Recently, however, it has been shown that NKT cells also play an immunoregulatory role in the secondary phase of an adaptive immune response by mediating the production of cytokines and enhancing the numerical increase of Ag-specific, conventional CD8⁺ T cells (13). In addition, we and others previously reported that NKT cells have a pivotal role in the establishment of CD4⁺ T cell-mediated adaptive immune responses (9–12). However, it was not clear when and how NKT cells contributed to the adaptive immune response of Ag-specific CD4⁺ T cells because these previous studies were not designed to distinguish between the role of NKT cells during the primary and secondary immune phases.

We examined the role of NKT cells during the secondary phase of an adaptive immune response mediated by Ag-specific, conventional CD4⁺ T cells. For this purpose, we performed similar adoptive transfer experiments as previously described by us to show the importance of NKT cells in the secondary immune response of CD8⁺ T cells (13). In the current study, we used both CFA and alum as adjuvants because it has been reported that CFA is strongly biased toward priming Th1-mediated responses, whereas alum is associated with the induction of Th2-mediated responses (22, 27). Depending on the type of adjuvant used, Th1- or Th2-skewed CD4⁺ T cells were induced in mice immunized with OVA protein. These CD4⁺ effector T cells were transferred into CD1d^{+/-} and CD1d^{-/-} mice and the recipients were subsequently challenged with OVA to monitor Th1 or Th2 immune responses. In addition, our results using J α 18^{-/-} mice clearly demonstrate that type 1 iNKT cells are important for the activation of secondary immune responses by CD4⁺ effector cells. Interestingly, secondary immune responses, such as CD4⁺ T cell expansion and cytokine production (IFN- γ for Th1 and IL-4 for Th2), were significantly enhanced in CD1d^{+/-} recipients both in vivo and ex vivo. In particular, we observed an increase in the number of IFN- γ -secreting CD4⁺ Th1 cells and IL-4-secreting CD4⁺ Th2 cells when CD4⁺ T cells were restimulated with CD1d^{+/-} DCs in the presence of NKT cells (Fig. 5). Furthermore, the expression of activation marker CD86 on DCs was higher in OVA-challenged CD1d^{+/-} recipient mice than in OVA-challenged CD1d^{-/-} recipient mice, suggesting that NKT cells boost APC functions in vivo (data not shown). However, further studies are needed to understand fully how NKT cells contribute to the secondary CD4⁺ T cell response by facilitating APC functions in vivo. Nevertheless, our results suggest that NKT cells play an essential role in promoting the numerical expansion and functional activation of both Ag-specific Th1 and Th2 cells during the secondary adaptive immune response.

We further examined the in vivo relevance of NKT cells in the induction of adaptive immune responses by CD4⁺ T cells. For this purpose, we used an OVA-induced asthma model (26, 28, 29). Previously, Lisbonne et al. (28) reported that WT mice treated with CD1d-specific mAb before allergen sensitization and challenge failed to develop AHR. In addition, Akbari et al. (26) showed that both NKT cell-deficient CD1d^{-/-} and J α 18^{-/-} mice developed neither AHR nor airway eosinophilia, and adoptive transfer of WT NKT cells into J α 18^{-/-} mice before allergen challenge restored airway inflammation and AHR. These results strongly suggest that the presence of NKT cells augments the development of asthma. Although these studies suggest that NKT cells are necessary for the development of asthma, they did not clearly show when and how NKT cells contribute to the development of this disease. Because the development of asthma is the result of multiple effector steps,

we performed adoptive transfer experiments to separate primary and secondary immune responses. The fact that OVA-primed CD4⁺ T cells derived from WT mice and in vitro-differentiated Th2 CD4⁺ T cells commonly exhibited an enhanced AHR on OVA-challenge when transferred into CD1d^{+/-} recipients but not when transferred into NKT cell-deficient CD1d^{-/-} recipients clearly suggests that a NKT cell-dependent enhanced secondary adaptive immune responses by Th2 cells is responsible at least, in part for the pathogenesis of the murine model of asthma.

The results of our current and previous studies (13) clearly suggest that NKT cells can modulate conventional CD4⁺ T cell responses (both Th1 and Th2) by enhancing the established Ag-specific response at the secondary phase. In addition, our results define a natural function of NKT cells in the modulation of T cell-dependent immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental FIGURE S1. The requirement of type 1 NKT cells for the optimal secondary immune response of CD4⁺ Th1 cells derived from OT-II Tg mice against antigen re-stimulation *in vivo*.

OT-II mice were immunized s.c. with 100 μ g OVA emulsified in CFA. Primed CD4⁺ T cells were isolated and transferred, and recipients were challenged with 100 μ g OVA as described in Fig. 2. (A) The total percentage of OT-II T cells was determined (**p* < 0.05). (B) CFSE profiles of OT-II T cells; the numbered gates indicate individual cell divisions. Summary of the varying proportions of cells in each cycle of division between undivided (D₀) and maximally divided cells (D₇) gated in the left panel. * *p* < 0.05 versus primed CD4⁺ OT-II T cells $\rightarrow$ J α 18^{-/-} groups (-▲-). (C) The number of IFN- γ -secreting cells among OVA-specific CD4⁺ T cells was determined by intracellular staining (**p* < 0.05). Data are expressed as the mean $\pm$ SEM of four mice and represent two independent experiments.

