Regulation of Secondary Antigen-Specific CD8⁺ T-Cell Responses by Natural Killer T Cells

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Abstract
The physiologic function of natural killer T (NKT) cells in adaptive immunity remains largely unknown because most studies have used NKT cell agonists. In the present study, the role of NKT cells during the secondary effector phase was investigated separately from the primary immunization phase via adoptive transfer of differentiated effector T cells into naive recipients. We found that secondary antitumor CD8⁺ T-cell responses were optimal when NKT cells were present. Tumor-specific CD8⁺ effector T cells responded less strongly to tumor cell challenge in NKT cell–deficient recipients than in recipients with intact NKT cells. NKT cell–mediated enhancement of the secondary antitumor CD8⁺ T-cell response was concurrent with increased number and activity of tumor-specific CD8⁺ T cells. These findings provide the first demonstration of a direct role for NKT cells in the regulation of antigen-specific secondary T-cell responses without the use of exogenous NKT cell agonists such as α-galactosylceramide (α-GalCer). Furthermore, forced activation of NKT cells with α-GalCer during the secondary immune response in suboptimally immunized animals enhanced otherwise poor tumor rejection responses. Taken together, our findings strongly emphasize the importance of NKT cells in secondary CD8⁺ T-cell immune responses.

Introduction
The role of innate immune cells is crucial for induction of complete adaptive immunity associated with T cells. Natural killer T (NKT) cells are considered to function as immunoregulatory cells during the innate phase and are specialized T cells that express T-cell receptors (TCR) and NK lineage cell surface markers. They recognize glycolipids presented by nonpolymorphic CD1d, a nonclassic antigen-presenting molecule (1). The majority of NKT cells have been shown to express an invariant Vα14-Jα18 TCR rearrangement in mice and an invariant Vα24-Jα18 TCR rearrangement in humans (1, 2). α-Galactosylceramide (α-GalCer) is a glycolipid that binds to CD1d and is recognized by the invariant TCRs of NKT cells. Mice injected with α-GalCer show enhanced activation of NKT cells, resulting in the rapid release of both Th1/Th2 cytokines, thereby eliciting a downstream cascade of activation that spreads to dendritic cells (DC), NK cells, and B cells (3–6).

Materials and Methods
Mice and cell lines. C57BL/6 (B6) mice and ovalbumin (OVA)–specific TCR transgenic mice (OT-I) were purchased from The Jackson Laboratory. Mice deficient in CD1d (CD1d−/−) and Vα14⁺ TCR transgenic mice (all in B6) were provided by Dr. A. Bendelac (University of Chicago, Chicago, IL; refs. 14, 15). Jα18−/− mice were gifts from Dr. M. Taniguchi (RIKEN, Yokohama, Japan; ref. 16). Mice were raised in a specific pathogen-free environment at Korea University. The experimental protocols adopted in this study were approved by the Institutional Animal Care and Use Committee of Korea University.

Cell culture and reagents. Cell cultures were maintained in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml gentamicin sulfate, and 50 μmol/L β-mercaptoethanol (all from Life Technologies) and 10% heat-inactivated fetal bovine serum (Hyclone). Chicken OVA was purchased from Sigma-Aldrich, and chicken OVA237-296 peptide (SIINFEKL) was purchased from Genscript. The α-GalCer used in this study was a gift from Dr. A. Bendelac. Complete Freund’s adjuvant (CFA; Mycobacterium tuberculosis) and carboxyfluorescein diacetate succinimidyl ester (CFSE) were obtained from Sigma-Aldrich. Tumor antigens were prepared via more than three freeze/thaw cycles of tumor cells.
peritoneal cells from the immunized mice were transferred i.p. into recipients 2 weeks after immunization, mice were boosted with B16 extract and system was >95%.

were sorted from the spleens of Vα14+ transgenic and OT-I mice via the AutoMACS system (Miltenyi Biotec) using anti-CD4, anti-TCR, anti-CD11c, anti-CD161, anti-CD8α, anti-CD3ε, anti-IL-4, and anti–IFN-γ (all from BD Pharmingen). OVA257-264/H-2Kb tetramers were used to detect OT-I T cells. For intracellular cytokine staining, cells were incubated in culture medium containing Golgi Stop (BD Pharmingen) for 6 h before intracellular staining, fixed, and permeabilized with Cytotox/Cytoperm solution (BD Pharmingen). They were then stained for 45 min with anticytokine mAbs. The cells were analyzed on a FACS Calibur using CellQuest software (BD Biosciences).

In vitro antigen-specific CD8+ T-cell response. For T-cell proliferation assessment by CFSE dilution, OT-I T cells were isolated from naive WT mice by AutoMACS using anti-CD4 mAb-conjugated magnetic beads. Labeled OT-I T cells were subsequently transferred into naive CD1d−/− and CD1d+ mice. Recipients were challenged i.p. with live B16 tumor cells (5 × 10^6 cells per mouse) 2 d after adoptive transfer. Survival was evaluated at the indicated time points after tumor injection. * P < 0.02 versus CD1d− recipients (A). B, OT-I T cells isolated from OVA257-264 peptide-immunized OT-I mice were labeled with CFSE and subsequently transferred into naive CD1d−/− and CD1d+ mice (3 × 10^6 cells per mouse), and then 2 d later, the recipients were challenged with 3 × 10^6 tumor cells. CD8+ donor T cells from naive WT mice were used as a control. The growth rate of s.c. implanted EL-4 or EG.7 tumor cells was monitored. *, P < 0.03; **, P < 0.002 versus CD1d− recipients (B). The data represent one of three independent experiments with similar results.

Cell enrichment by MACS. DCs were obtained from splenocytes of naive mice via MACS using anti-CD11c monoclonal antibody (mAb)-conjugated magnetic beads (Miltenyi Biotec). NKT cells and OT-I T cells were sorted from the spleens of OVA14 transgenic and OT-I mice via the AutoMACS system (Miltenyi Biotec) using α-GalCer/CD1d-tetramer and anti-CD8α mAb-conjugated magnetic beads, respectively, according to the manufacturer’s instructions. The purity of the cells sorted by the AutoMACS system was >95%.

Measurement of antitumor immune responses. B6 mice were immunized i.p. with B16 tumor antigen (equivalent to 5 × 10^5 cells). Vehicle or α-GalCer was coadministered with the antigen when required. Two weeks after immunization, mice were boosted with B16 extract and peritoneal cells from the immunized mice were transferred i.p. into recipient mice. The recipients were challenged i.p. with live B16 tumor cells 2 d after adoptive transfer, and their survival rates were monitored.

OT-I mice were immunized s.c. with 100 μg of OVA peptide emulsified in CFA. At least 2 wk later, splenic OT-I T cells from the immunized mice were sorted and transferred i.v. into recipient mice. For tracking the proliferation after transfer, OT-I T cells were labeled with 5 μM/L CFSE for 15 min before adoptive transfer when required. The recipients were then challenged with EG7 tumor cells (s.c.) or 100 μg α-GalCer peptide (i.v.) at 2 d after adoptive transfer. In reconstitution experiments, recipient mice received i.v. injections of 1 × 10^6 sorted NKT cells at the time of tumor challenge. Tumor volumes were calculated as the means of the products of trisectioned diameters, and tumor weight was measured 15 d after tumor challenge. To monitor CD8+ T-cell responses, peripheral blood lymphocytes (PBL), inguinal LN cells, and splenocytes were collected from the recipients at the indicated time points in each experiment.

Fluorescence-activated cell sorting analysis. Cells preincubated with anti-FcγRII/III mAb 2.4G2 for 30 min were stained with the appropriate combinations of the following mAbs: anti-CD4, anti-TCR, anti-CD11c, anti-CD69, anti-CD8α, anti-CD3ε, anti-IL-4, and anti–IFN-γ (all from BD Pharmingen). OVA257-264/H-2Kb tetramers were used to detect OT-I T cells.

Statistical analysis. Prism software (GraphPad) was used for all statistical analyses. Student’s t tests were used to determine statistically significant differences between two experimental groups. Log-rank tests were used to analyze mouse survival curves.

Results

NKT cell–dependent optimal secondary antitumor immune responses. To rule out the effect of NKT cells on the priming phase of naive T cells, we adoptively transferred the effector cells into naive CD1d−/− or NKT cell–deficient CD1d−/− mice. The adoptive transfer of α-GalCer-immunized mouse-derived effector cells into naive wild-type (WT) mice prolonged survival of the recipients on live tumor challenge when compared with effector cells derived from vehicle-immunized mice (average survival of 19 days versus 40 days). This result confirms the well-known activation by α-GalCer of NKT cell–mediated regulatory processes during the primary immunization phase of an immune response. However, antitumor responses were substantially reduced in CD1d−/− recipients compared with CD1d−/− recipients (Fig. 4A), indicating that NKT cells are important for the augmentation of the antitumor immune response during the secondary phase.

α-GalCer was used as an adjuvant when we immunized donor mice because this adjuvant efficiently produced antigen-specific effector T cells in previous studies (9, 11). The possibility of α-GalCer contamination in the transferred effector cells was highly unlikely due to the following reasons; first, α-GalCer is metabolized quickly in the body (18); second, α-GalCer was used at least 2 weeks before the isolation of effector cells; and third, the cells were washed extensively during cell preparation. To further rule out any possibility of contamination, we analyzed whether the prepared effector cells were contaminated with α-GalCer, which would result in activation of NKT cells in recipients. The effector
cell fraction did not exert any notable effect on purified NKT cells (Supplementary Fig. S1).

Although the effector cells generated by immunization were likely tumor antigen–specific cytotoxic T cells, other cell types, such as B cells, might have also been induced. To determine whether the observed effects were derived from the regulation of antigen-specific cytotoxic T cells, we performed another transfer experiment using OT-I mice and the OVA+ tumor cell line EG.7. OT-I mice were immunized with OVA257-264 peptide mixed with CFA, and effector CD8+ T cells (OT-I T cells) from these mice were purified and adoptively transferred into naive CD1d-/- or CD1d+/- mice that then received a tumor challenge. The subsequent challenge with EG.7 tumors only elicited a vigorous antitumor response in CD1d+/- recipients (Fig. 1B). Similar experiments using a mouse survival model also showed that the absence of NKT cells in recipients substantially reduced the survival rate of mice challenged with EG.7 tumors (Supplementary Fig. S2). The antitumor response of CD1d+/- recipients was antigen specific because the transfer of OT-I T cells to CD1d+/- recipients did not protect the recipients after inoculation with EL-4 tumor cells lacking OVA expression. Donor effector T cells from OT-I mice immunized with OVA protein instead of OVA peptide also exhibited the same results when transferred to naive CD1d-/- or CD1d+/- recipients (data not shown). Because CFA contains components derived from *M. tuberculosis* and there has been a report that Toll-like receptor (TLR)-mediated DC activation could stimulate CD1d expression and modify ligand metabolism in the DCs (19), we evaluated *M. tuberculosis* contamination in OT-I effector T-cell preparations. For this purpose, we isolated OT-I T cells from immunized mice and incubated them with naive splenocytes *in vitro*. OT-I T cells derived from immunized mice were not different from OT-I T cells derived from naive mice in terms of the activation or cytokine secretion of NKT cells and DCs (Supplementary Fig. S3). These results exclude the possibility that contaminating *M. tuberculosis* components could affect NKT cells in the recipients.

![Figure 2. Diminished CD8+ T-cell responses in the absence of NKT cells.](image)

A, PBLs were collected at days 5 (d5) and 11 (d11) after tumor challenge to the recipients treated as in Fig. 1B. OT-I T cells were plotted. B, OT-I T cells induced by s.c. implanted live tumor cells were determined in the PBLs and the inguinal LN at days 5 and 11 after tumor challenge. *, *P* < 0.05; **, *P* < 0.004 versus CD1d-/- recipients. C, CFSE dilution profiles of OT-I T cells. Data were expressed as the mean ± SD of four mice and represent two independent experiments.
NKT cell–dependent numerical increase of CD8+ effector T cells during the secondary immune response. Whereas OT-I T cells were absent in nontransferred mice, these cells were clearly detectable in all OT-I–transferred recipients. Among recipients, CD1d+/C0 mice had roughly twice the frequency of tetramer-positive CD8+ T cells than CD1d/C0/C0 recipients (Fig. 2A). Both CD1d/C0 and CD1d+/C0 recipients had higher numbers of OT-I T cells when they were challenged with EG.7 tumor cells than with EL-4 cells (Fig. 2B).

Next, we analyzed whether this NKT cell–dependent enhancement of the secondary immune response also occurs when antigen-specific T cells are generated early enough to show a memory phenotype. OT-I mice were immunized with OVA peptide/CFA, and 2 months later, OT-I T cells were isolated for the transfer experiment. Primed OT-I T cells showed stronger antitumor activity against EG.7 tumor cells in CD1d+/C0 recipients when compared with CD1d/C0 recipients (Fig. 3A). The number of OT-I T cells in the peripheral blood was also increased in CD1d+/C0 recipients compared with CD1d/C0 recipients (Fig. 3B).

The concurrent NKT cell–dependent antitumor effect and increased number of antigen-specific OT-I T cells led us to directly test whether the presence of NKT cells could support the division of primed effector T cells. CFSE-labeled OT-I effector T cells were stimulated with OVA peptide-pulsed DCs in the presence of graded numbers of NKT cells. The expansion of effector T cells was completely antigen dependent because there was no obvious effector T-cell division on stimulation with antigen-free CD1d/C0 DCs regardless of NKT cell number (Fig. 3C and D).

Additionally, the number of IFN-γ–producing effector T cells increased proportionally with the number of cocultured NKT cells when primed OT-I T cells were stimulated with antigen-pulsed CD1d+/C0 DCs but not with CD1d/C0 DCs (Fig. 4A). Considering that NKT cell–dependent effector T-cell expansion/activation occurs in the absence of artificial NKT cell ligands during the secondary response, it is likely that natural ligands for NKT cells produced by DCs can stimulate NKT cells in physiologic...
conditions. In support of this idea, we were able to detect NKT cell–produced IFN-γ and IL-4 after in vitro stimulation of NKT cells with CD1d+/− DCs, but not with CD1d−/− DCs, in the absence of α-GalCer, although the overall magnitudes were lower than those obtained with α-GalCer stimulation (Fig. 4B). Activation of antigen-presenting cells (APC) by OT-I T cells in this process seemed critical for inducing NKT cell–dependent enhanced OT-I T-cell proliferation. In the absence of NKT cells, DCs only exhibited increased surface expression of CD1d and the costimulatory molecule B7.2 when they presented OVA peptides to effector T cells. However, the expression of MHC class I and II was not affected by the same conditions (Fig. 4C). When these DCs were further isolated and incubated again with isolated NKT cells, DCs preincubated with OT-I T cells in the presence of OVA antigen were able to activate NKT cells more strongly than those incubated with OT-I T cells without OVA antigen (Fig. 4D). These results suggest that activated APCs up-regulated CD1d metabolism and further support the stimulation of OT-I T-cell proliferation via the activation of NKT cells.

To rule out the influence of immune responses against tumor cell–derived antigens other than OVA, recipient mice were challenged with SIINFEKL peptide antigens instead of tumor cells; this enabled the monitoring of in vivo immune responses of OT-I T cells against a specific antigen. We labeled OT-I T cells with CFSE, adoptively transferred them, and then measured the number and cell division of the CFSE-labeled cells. Whereas primed OT-I T cells in the unchallenged recipients did not undergo cell division, the same OT-I T cells in antigen-challenged CD1d−/− recipients showed substantial cell division but relatively weak expansion when compared with cells implanted in CD1d+/− recipients. These findings are similar to those obtained in the tumor challenge experiments (Fig. 5A and B). The total number of OT-I T cells in CD1d+/− recipients was about twice that in CD1d−/− recipients when primed OT-I T cells were transferred (Fig. 5C).

The absence of invariant NKT (iNKT) cells but not CD1d expression itself was the primary reason for inefficient antitumor activity in CD1d−/− recipients; Jo18−/− recipients, which do not have iNKT cells, also exhibited substantially reduced antitumor activity that was comparable with that of the CD1d−/− recipients. However, the antitumor activity was similar to that of the CD1d+/− recipients when Jo18−/− mice received Vα14+ NKT cells at the time of tumor challenge. Implantation of NKT cells alone in the absence of effector OT-I T cells in Jo18−/− recipients resulted in a similar level of tumor growth when compared with CD1d+/− recipients that did not receive OT-I effector T cells (Fig. 6A).

**Exogenous NKT cell activation during adaptive immune responses further enhances antitumor activity.** Lastly, we evaluated the effect of exogenous NKT cell activation to determine whether there is any additional enhancement of antitumor activity by NKT cell activation with α-GalCer during the secondary T-cell response in the same tumor rejection model (Fig. 6B). To observe synergistic effects of artificial NKT cell activation, B6 mice were immunized with B16 tumor cell extracts plus 250 ng of α-GalCer; this induced suboptimal immune responses that were insufficient to protect mice against subsequent live tumor cell challenge (Supplementary Fig. S4). Two weeks after immunization, live tumor cells were injected into mice that had been treated with vehicle or α-GalCer (1 μg/mouse) 1 day before tumor injection to artificially

![Figure 4.](image-url)
stimulate NKT cells. Although mice immunized with suboptimal quantities of α-GalCer exhibited somewhat prolonged survival compared with nonimmunized mice, all mice in this group still succumbed eventually. However, when NKT cells were activated immediately before tumor challenge in suboptimally immunized mice, a strong antitumor response was observed, with 50% of the animals remaining tumor-free >90 days after tumor challenge.

Administration of α-GalCer alone before tumor challenge did not affect the survival of nonimmunized mice or mice immunized with vehicle. Thus, protective immunity was conferred not by α-GalCer–activated NKT cells themselves but rather by antigen-specific effector T cells that had been optimized by the NKT cells.

**Discussion**

Although a large body of evidence suggests an immunoregulatory role for NKT cells in innate immunity (20–22), it has yet to be clearly shown that NKT cells are involved in the secondary adaptive immune responses of antigen-specific effector T cells. To determine the regulatory role of NKT cells in adaptive immunity, we transferred tumor-specific effector T cells into naive recipients with differential levels of NKT cell activity; these animals included CD1d−/−, CD1d+/−, and Jα18−/− mice. During the secondary immune response, effector cells from mice immunized with B16 tumor extracts and effector OT-I cells prepared by immunization with OVA257-264 peptide or whole OVA protein both showed increased antitumor activity on adoptive transfer to NKT cell–deficient recipients. Although antitumor activities in the recipients were related to the levels of effector T cells after tumor cell challenge, CD1d−/− recipients showed significantly prolonged survival rates compared to CD1d+−/− mice.
compared with CD1d<sup>−/−</sup> and Jα18<sup>−/−</sup> recipients. These results were surprising, as they suggested that not only antigen-specific T cells but also NKT cells were highly involved in the antigen-specific secondary adaptive immune response.

The NKT cell–dependent enhanced secondary immune response was not restricted to effector T cells freshly generated by recent immunization events; it also occurred in effector T cells generated several months earlier, thus were closer to a memory phenotype (Fig. 3). It is therefore likely that NKT cell–dependent enhanced adaptive immune responses are not restricted to effector cells at a certain differentiation stage.

NKT cells influenced effector CD8<sup>+</sup> T cells not only quantitatively but also qualitatively, as shown by the higher ratio of IFN-γ–producing OT-I T cells observed on restimulation with CD1d<sup>+/+</sup> DCs when compared with CD1d<sup>−/−</sup> DCs in the presence of an increasing number of NKT cells. Taken together, these findings suggest that NKT cells could promote the numerical expansion and functional activation of antigen-specific effector CD8<sup>+</sup> T cells via CD1d on APCs during secondary immune responses.

Saliö and colleagues (19) showed that TLR-mediated activation of APCs could modify their lipid biosynthetic pathway and up-regulate lipid antigen-loaded CD1d, thus resulting in activation of CD1d-restricted iNKT cells in humans. In our study, antigen-specific effector T cells increased the expression of CD1d on antigen-presenting DCs without TLR stimulation; these DCs could then activate NKT cells directly. These results suggest that not only TLR-mediated stimulation but also the interaction with antigen-specific effector T cells can potentiate APCs for NKT cell activation via enhanced CD1d expression or modified ligand metabolism. Because NKT cells are normally negatively regulated by MHC class I molecules on DCs, stress-induced DC maturation might overcome MHC class I–mediated NKT cell inhibition (23) to allow activation.

From this perspective, we determined whether the antitumor activity of preexisting tumor-specific effector T cells could be further enhanced in cases where NKT cells are artificially activated by α-GalCer during the secondary immune response. On activation before challenge with live tumor cells, NKT cells greatly enhanced antitumor immunity of tumor-specific CD8<sup>+</sup> T cells (Fig. 6B). This finding shows that α-GalCer treatment alone during the effector phase of an ongoing immune response can strengthen otherwise weak immune responses; this result has implications for efforts to increase the strength of weak antigen-targeting responses such as the tumor-specific CTL pathway in human cancer patients.

It can be argued that if NKT cells invariably optimize secondary immune responses, NKT cell–deficient mice should have significant defects in immunity. Generally, however, NKT cell–deficient mice are immune competent (24–26). There are also numerous reports suggesting activating or suppressing roles for NKT cells in many immune responses only on activation by specific ligands. Such findings suggest several possibilities. First, NKT cell–dependent modulation of the secondary immune response could comprise a delicate adjustment of the immune reaction. Thus, in many experimental settings, immune response measurements were not sensitive enough to distinguish differences in immune responses in the presence or absence of NKT cells. Second, it is possible that there are certain immunologic conditions where NKT cells significantly contribute to the optimal secondary immune response. Our adoptive transfer setting could be one of these cases. Third, NKT cells might be differentially involved in the regulation of the secondary immune response of different subsets of immune effector cells. In this context, Griseri and colleagues (27) showed that adoptive transfer of islet-specific CD8<sup>+</sup> T cells into NKT cell–enriched V<sup>α</sup>14<sup>+</sup> transgenic recipients exacerbated type I diabetes. They also showed that NKT cells impaired the differentiation of anti-islet CD4<sup>+</sup> T cells into Th1 effector cells, and that eventually the autoreactive CD4<sup>+</sup> T cells become anergic (28). It thus seems that NKT cells activate autoreactive effector CD8<sup>+</sup> T cells and suppress CD4<sup>+</sup> effector T cells in this specific disease model. It will be of interest to analyze whether NKT cell–dependent modulation of the secondary immune response occurs not only in CD8<sup>+</sup> effector T cells but also in other immune effector cells such as CD4<sup>+</sup> T cells and B cells.

This study is the first report to compare the natural function of NKT cells between the primary immunization phase and the secondary effector phase of an immune response under physiologic conditions without using any artificial NKT cell ligands. It is currently accepted that NKT cells are innate-like lymphocytes that regulate several immune responses (1). Our data provide new support for the view that NKT cells can regulate immune responses during the adaptive phase as well as the innate phase and might also be used in novel approaches for the development of new antitumor immunotherapeutic and vaccine strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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