Control of NKT Cell Differentiation by Tissue-Specific Microenvironments

Yang Yang, Aito Ueno, Min Bao, Zhongying Wang, Jin Seon Im, Steven Porcelli and Ji-Won Yoon

_J Immunol_ 2003; 171:5913-5920; doi: 10.4049/jimmunol.171.11.5913

http://www.jimmunol.org/content/171/11/5913

References
This article cites 47 articles, 33 of which you can access for free at:
http://www.jimmunol.org/content/171/11/5913.full#ref-list-1

Subscription
Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Control of NKT Cell Differentiation by Tissue-Specific Microenvironments

Yang Yang, Aito Ueno, Min Bao, Zhongying Wang, Jin Seon Im, Steven Porcelli, and Ji-Won Yoon

CD1d-restricted Vα14 NKT cells play an important role in both Th1- and Th2-type immune responses. To determine whether NKT cells develop two functionally distinct subsets that provoke different types of responses, we examined the phenotypes and cellular functions of NK1.1+ and DX5+ T cells. We found that both NK1.1+ and DX5+ T cells are CD1d-restricted Vα14 T cells with identical Ag specificities, phenotypes, tissue locations, and functions. Similar to the NK1.1 marker, the DX5 marker (CD49b) is expressed on mature NKT cells in both NK1.1 allele-positive and allele-negative strains. However, when NK1.1+ and DX5+ NKT cells isolated from different tissues were compared, we found that thymic and splenic NKT cells differed not only in their cytokine profiles, but also in their phenotype and requirements for costimulatory signals. Thymic NKT cells displayed the phenotype of activated T cells and could be fully activated by TCR ligation. In contrast, splenic NKT cells displayed the phenotype of memory T cells and required a costimulatory signal for activation. Furthermore, the function and phenotype of thymic and splenic NKT cells were modulated by APCs from various tissues that expressed different levels of costimulatory molecules. Modulation of NKT cell function and differentiation may be mediated by synergic effects of costimulatory molecules on the surface of APCs. The results of the present study suggest that the costimulatory signals of tissue-specific APCs are key factors for NKT cell differentiation, and these signals cannot be replaced by anti-CD28 or anti-CD40 ligand Abs. The Journal of Immunology, 2003, 171: 5913–5920.

Received for publication February 4, 2003. Accepted for publication September 26, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Y.Y. is a recipient of the Career Development Award from the Juvenile Diabetes Research Foundation International. J.W.Y. holds a Canada Research Chair in Diabetes.

2 Address correspondence and reprint requests to Dr. Ji-Won Yoon, Department of Microbiology and Infectious Diseases, or Dr. Yang Yang, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. E-mail addresses: yoon@ucalgary.ca or yyang@ucalgary.ca

3 Current address: The Chicago Medical School, FUHS, 3333 Green Bay Road, North Chicago, IL 60064

4 Abbreviations used in this paper: αGalCer, α-galactosylceramide; CD1d-KO, CD1d gene-deficient C57BL/6 mice; CD40L, CD40 ligand; I-A-KO, MHC II 1-A gene-deficient C57BL/6 mice; NOD, nonobese diabetic.

Copyright © 2003 by The American Association of Immunologists, Inc. 0022-1767/03/$02.00
Materials and Methods

**Mice and Abs**

All mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained in pathogen-free units of the Animal Resources Center at University of Calgary. CD1 gene-deficient 129 mice were crossed to the C57BL/6 background for seven generations. Deficiency of the CD1 gene was screened by PCR. The PCR primers used for the CD1.1 and CD1.2 genes were 5'-GGTCTGGGGGACAATCTGAAG-3' and 5'-CTG GATCTCAATGGGATCTG-3'. Defective CD1 expression in thymocytes was also examined by FACS. All Abs were purchased from BD Pharmingen Canada (Mississauga, Canada).

**FACS analysis of NKT cells**

Thymocytes, lymph node cells, bone marrow cells, and splenocytes were isolated from 6- to 8-wk-old mice and preincubated with Ab against CD16/32 (2.4G2) in PBS containing 2% FCS. The cells were then incubated with allophycocyanin-labeled αGalCer-CD1d tetramers at room temperature for 40 min, washed, and further incubated with a mixture of anti-TCRβ-FITC (H57-597), anti-NK1.1-PE (PK136), or DX5-PE Abs. The cells were analyzed by FACScan. FITC-labeled anti-CD1d, CD24, CD44, CD62L, and CD69 Abs and allophycocyanin-labeled αGalCer-CD1d tetramers were also used as indicated.

**Isolation and purification of NK1.1 TCR+ DX5 TCR+ and DX5 TCR+ cells.**

Thymocytes and splenocytes from C57BL/6 and AKR mice were incubated with PE-conjugated anti-NK1.1 or DX5 Ab and FITC-conjugated anti-TCRβ-chain Ab. NK1.1+ or DX5+ cells were first enriched by magnetic cell sorting (Miltenyi Biotec, Auburn, CA). NK1.1 TCR+, DX5 TCR+, and DX5 TCR+ cells were further purified by FACS cell sorting. Magnetic cell sorting was also used to deplete NK1.1+ or DX5+ cells from thymocytes or splenocytes.

**Assay for cytokine production**

For ELISAs, purified (>95%) DX5 TCR+, NK1.1 TCR+, and DX5 TCR+ cells were incubated in 96-well plates (1 x 10^5 cells/well) with immobilized Ab to CD3 (10 μg/ml) for 48 h. Ab to CD28 (2 μg/ml) was added as indicated. Cells were also activated by PMA (20 ng/ml) and ionomycin (0.5 μg/ml). For Ag activation, DX5 TCR+, NK1.1 TCR+, and DX5 TCR+ cells (1 x 10^5 cells/well) were incubated in the presence of αGalCer (Kerin Brewery Co., Tokyo, Japan) with or without DX5+ cell-depleted thymocytes or splenocytes as APCs (2 x 10^5 cells/well) for 48 h. Culture supernatant was collected for ELISA (R&D Systems, Minneapolis, MN) to determine the concentrations of IFN-γ and IL-4. Thymocytes, splenocytes, or NK1.1+ cell-depleted or DX5+ cell-depleted thymocytes or splenocytes (1 x 10^5 cell/well) were also activated with αGalCer (100 ng/ml) or immobilized anti-CD3 Ab for 48 h for ELISA.

**Detection of the restricted TCR Vβ-chain usage of NKT cells**

To detect TCR Vα14-Jα281 expression in DX5+ NKT cells, DX5+ TCR+, NK1.1+ TCR+, and DX5 TCR+ cells were isolated and purified from thymocytes and splenocytes from C57BL/6 and AKR mice. Total RNA was extracted from identical numbers of purified DX5 TCR+ cells, NK1.1 TCR+, and DX5 TCR+ cells, and 2.5 μg RNA from each cell was used for cDNA synthesis. TCR Vα14-Jα281 rearrangements were amplified by RT-PCR (Vα14 primer, TAAGACAGCAAGCCTGACAT-3'; Cα primer, 5'-GTCCTCAGGTCCTGGAAGCTG-3'), and PCR products were cloned using Topo-cloning kit (Invitrogen, Carlsbad, CA) and sequenced. RT-PCR reactions specific for Vα14-Jα281 rearrangements were also performed using serially diluted cDNA samples of NKT and T cells isolated from AKR mice. The Jα281 primer sequence and the conditions of RT-PCR reactions were previously described (12). As a control, RT-PCR for β-actin expression was also conducted. The primers for β-actin cDNA amplification were: forward, 5'-GGGCATCTCCTGCTTCGA-3'; and reverse, 5'-GGGCCATCTCCTGCTTCGA-3'.

**Cytokine experiments**

Purified NK1.1+ TCR+ thymocytes or splenocytes were incubated in 96-well plates (2 x 10^5 cells/well) with DX5+ cell-depleted thymocytes or splenocytes (2 x 10^5 cells/well) in the presence or the absence of αGalCer (100 ng/ml) for 2 days. The culture medium was collected and replaced with fresh medium containing IL-2 (20 μg/ml; Takeda Chemical Industries, Osaka, Japan). Cells were cultured for an additional 5 days and then harvested for phenotypic analysis. In control experiments, splenocytes of CD1d-gene deficient mice were cocultured with purified NK1.1 TCR+ thymocytes. For the experiments on signal blockage, purified NK1.1 TCR+ thymocytes (5 x 10^5/well) were cocultured with splenic APCs in the presence of αGalCer (100 ng/ml) and in the presence or the absence of anti-CD40, anti-CD80, or anti-CD86 Ab (10 μg/ml) or a combination of anti-CD80 and anti-CD86 Ab (20 μg/ml). Purified NK1.1+ TCR+ thymocytes were also activated by anti-CD3 (5 mg/ml) or a combination of anti-CD3 and anti-CD28 Abs for 2 days. The proliferation and cytokine production of thymic NKT cells activated by Ab or αGalCer were determined and compared.

**Results**

Both DX5+ and NK1.1+ T cells are responsive to αGalCer

CD1d-restricted NKT cells are characterized by their dominant usage of the Vα14-Jα281 invariant TCR chain (11, 24, 25) and by specificity for the glycolipid, αGalCer (13, 14). As several studies suggested that most mature Vα14 NKT cells do not express DX5 Ag (20–23), we first tested whether the depletion of DX5+ T cells affects the response to αGalCer. We isolated splenocytes from young C57BL/6 mice (8 wk old) and activated the splenocytes with anti-CD3 Ab or αGalCer for 48 h. In a parallel experiment, DX5+ or NK1.1+ cells were removed from the splenocytes using Ab-conjugated microbeads before activation. As shown in Table I, splenocytes of C57BL/6 mice activated with anti-CD3 Ab produced a significant amount of IFN-γ, and IFN-γ production was slightly decreased when DX5+ or NK1.1+ cells were depleted. Splenocytes isolated from CD1d gene-deficient C57BL/6 (CD1d-KO) mice also produced a significant amount of IFN-γ, similar to DX5+ or NK1.1+ cell-depleted splenocytes. In contrast, DX5+ cell-depleted or NK1.1+ cell-depleted splenocytes as well as

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Cells</th>
<th>Treatment</th>
<th>Anti-CD3</th>
<th>Vehicle</th>
<th>αGalCer (100 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFN-γ</strong></td>
<td>Splenocytes</td>
<td>6349 ± 21.2</td>
<td>0±</td>
<td>1005 ± 18.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DX5+ splenocytes</td>
<td>5762 ± 19.5</td>
<td>0±</td>
<td>0±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NK1.1+ splenocytes</td>
<td>5662 ± 104.3</td>
<td>0±</td>
<td>0±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD1d-KO splenocytes</td>
<td>5619 ± 3.5</td>
<td>0±</td>
<td>0±</td>
<td></td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>Splenocytes</td>
<td>214 ± 17.1</td>
<td>0±</td>
<td>160 ± 8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DX5+ splenocytes</td>
<td>19 ± 0.5</td>
<td>0±</td>
<td>16 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NK1.1+ splenocytes</td>
<td>69 ± 1.0</td>
<td>0±</td>
<td>6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD1d-KO splenocytes</td>
<td>48 ± 1.0</td>
<td>0±</td>
<td>0±</td>
<td></td>
</tr>
</tbody>
</table>

For splenocytes (1 x 10^5/well) from C57BL/6 and CD1d-deficient C57BL/6 mice were activated by immobilized anti-CD3 Ab (5 μg/ml) or by αGalCer for 48 h. Cytokine production in the supernatant was measured. DX5+ or NK1.1+ cell-depleted splenocytes were similarly activated. Representative results of three independent experiments are shown. Numbers are the mean ± SD of triplicate samples.

Not detectable.
spleen, and DX5 negative thymocytes. The results of the depletion experiments indicated that splenic DX5+ cells include αGalCer-responder cells; however, it was suggested that DX5+ TCR+ cells were less frequent than NK1.1+ TCR- cells in the thymus (20–22). To determine whether NK1.1+ TCR- cells and DX5+ TCR- cells are different populations in the thymus, we examined the expression of NK1.1 and DX5 markers in Vα14 thymic NKT cells from young C57BL/6 mice. A similar proportion of thymocytes expressed a high level of NK1.1 Ag (0.87%) and a low level of DX5 Ag (0.78%) or were stained by αGalCer/CD1d tetramers (0.90%), and all these thymocytes expressed an intermediate level of TCR (Fig. 1a). DX5 expression was very low, and clear labeling was only achieved with PE-labeled anti-DX5 Ab, whereas fewer cells were detected with FITC-labeled anti-DX5 Ab (data not shown); therefore, PE-labeled anti-DX5 Ab was used for the remainder of this study.

Further studies revealed that Vα14 thymic NKT cells express both NK1.1 and DX5 Ags. Double staining of TCR- thymocytes with anti-NK1.1 and DX5 Abs showed that most NK1.1+ TCR- thymocytes also expressed DX5 Ag (Fig. 1b). Even though the expression level of DX5 in thymocytes was low and variable, when DX5+ thymocytes were depleted from the thymocyte population using DX5-Ab-conjugated microbeads, most of the αGalCer/CD1d tetramer+ and NK1.1+ TCR- thymocytes were concomitantly removed (Fig. 1c), indicating that DX5+ T cells and NK1.1+ T cells are identical Vα14 NKT cells. In addition, although the majority of αGalCer/CD1d tetramer- thymocytes were DX5+ and NK1.1+, a small fraction of αGalCer/CD1d tetramer- thymocytes did not express DX5 (Fig. 1b). We further examined the expression of other NKT cell markers, CD24 and CD44, as NKT cells are known to be CD24- and CD44+ (1–3). When αGalCer/CD1d tetramer- thymocytes were gated, it was clear that most αGalCer/CD1d tetramer+ TCR+ thymocytes were CD24-CD44+, and these cells expressed variable levels of DX5, whereas a small portion of αGalCer/CD1d tetramer- thymocytes were CD24+CD44-, and those cells were DX5+ (Fig. 1d). As CD44 and NK1.1 are both expressed in mature NKT cells (26), it is clear that DX5 is only expressed in mature Vα14 NKT cells. In the spleen of C57BL/6 mice, most of the αGalCer/CD1d tetramer+ T cells also expressed DX5, and NK1.1+ splenic T cells coexpressed DX5 Ag (Fig. 1e). Thus, DX5 is expressed in both thymic and splenic Vα14 NKT cells, and compared with thymic NKT cells, splenic NKT cells expressed a consistent level of DX5.

Thymic and splenic NKT cells differ in their requirements for costimulatory signals and cytokine production

To determine whether DX5+ and NK1.1+ T cells have different functions, we purified NK1.1+ TCR+ and DX5+ TCR+ cells from spleens of C57BL/6 mice and activated these cells with PMA and ionomycin or immobilized anti-CD3 Ab in the presence or the absence of anti-CD28 Ab as a costimulatory signal. DX5+ TCR+ cells were also isolated and activated under identical conditions. We found that splenic NK1.1+ TCR+ and DX5+ TCR+ cells produced equal amounts of IFN-γ and IL-4 under all conditions (Fig. 2a). Both splenic NK1.1+ and DX5+ T cells produced 2-fold more IFN-γ and at least 10-fold more IL-4 than DX5- T cells. In addition, both splenic NK1.1+ and DX5+ T cells produced less IFN-γ and IL-4 in the absence of anti-CD28 Ab, indicating that costimulatory signals are required for the full activation of these splenic NK1.1+ and DX5+ T cells. Thus, NK1.1+ TCR+ and DX5+ TCR+ cells are identical with respect to activation requirements and cytokine production (Fig. 2a). However, when the purified thymic NK1.1+ TCR+ cells were activated, substantial functional differences were seen between splenic and thymic NKT cells. Thymic NKT cells produced ~2- to 3-fold more IFN-γ and 5-fold more IL-4 than splenic NKT cells (Fig. 2a). Therefore, thymic NKT cells not only have a higher capacity for cytokine production, but also a different profile than splenic NKT cells. Furthermore, thymic NKT cells produced similar amounts of cytokines with or without costimulatory signals from anti-CD28 Ab (Fig. 2a), indicating that ligation of TCR is sufficient for the activation of thymic NKT cells.

Differences in the cytokine capacity and requirement of costimulatory signals suggest that thymic and splenic Vα14 NKT cells differ in their requirements for costimulatory signals and cytokine production.
cells might differ in their activation status; therefore, we examined the expression of CD1d, CD44, CD62L, and CD69 in thymic and splenic NK1.1+ T cell of young C57BL/6 mice. Thymic and splenic NK1.1+ T cells expressed identical levels of CD1d, CD44, CD62L, and CD69 Ags were compared. Most thymic and splenic NK1.1+ T cells expressed identical levels of CD1d, but a small number of splenic NK1.1+ T cells expressed a higher level of CD1d. Thymic and splenic NK1.1+ T cells expressed identical high levels of CD44. However, in both C57BL/6 and AKR mice, thymic NK1.1+ T cells showed an activated phenotype, CD62Llow/CD69high, similar to the phenotype of activated T cells, whereas splenic NK1.1+ T cells were CD62Lhigh/CD69low. As splenic NK1.1+ T cells were CD44high, they displayed a phenotype similar to that of memory T cells (Fig. 2b). The differences in phenotypes of thymic and splenic NK1.1+ T cells may be correlated with their different behaviors during activation.

**DX5**

DX5+ T cells in NK1.1+ strains of mice are CD1d-restricted Vα14 NKT cells

As most inbred strains do not express the NK1.1 Ag, we asked whether DX5+ T cells in NK1.1+ strains are CD1d-restricted Vα14 NKT cells. αGalCer/CD1d tetramer staining revealed the presence of Vα14 NKT cells in the thymocytes of AKR, DBA/2, BALB/c, and nonobese diabetic (NOD) mice. Different percentages of the thymocytes were αGalCer/CD1d tetrramer+ ranging from 1.3% in AKR mice to 0.3% in NOD mice. Regardless of the different portions of αGalCer/CD1d tetramer+ NKT cells among thymocytes, most αGalCer/CD1d tetramer+ Vα14 NKT cells expressed various levels of DX5 Ag (Fig. 3a). NOD mice have been known to have a deficient NKT cell population (27, 28); this deficiency can be clearly revealed by DX5 Ab staining, as significantly fewer DX5+ cells were found in NOD than in other strains of mice (Fig. 3a).

We further tested the Ag specificity of splenic and thymic DX5+ T cells of AKR mice. When cultured in the presence of αGalCer for 48 h, both thymocytes and splenocytes produced a significant
were depleted by Ab-conjugated microbeads, IFN-γ and IL-4 production by thymocytes or splenocytes was almost eliminated. When thymocytes and splenocytes were cultured in the presence of immobilized anti-CD3 Ab, the depletion of DX5+ cells also severely reduced both IFN-γ and IL-4 production in thymocytes and IL-4 production in splenocytes. IFN-γ production of splenocytes was slightly decreased by DX5 Ab-mediated depletion, apparently because conventional T cells activated by anti-CD3 produced large amounts of IFN-γ. However, early IL-4 production was DX5+ T cell dependent. These results showed that NK1 allele-negative strains of mice, DX5+ T cells are responsible for αGalCer-induced cytokine production of both thymocytes and splenocytes.

To compare the cellular functions of thymic DX5+ TCR+ with splenic DX5+ TCR+ cells of AKR mice, we activated purified DX5+ T cells with immobilized anti-CD3 Ab and measured IFN-γ production. Thymic DX5+ T cells produced significantly more IFN-γ than splenic DX5+ T cells. Addition of anti-CD28 Ab to the culture resulted in a significant enhancement of IFN-γ production of the activated splenic DX5+ T cells, but not thymic DX5+ T cells (Fig. 3b). Similar to NK1.1+ T cells in C57BL/6 mice, thymic and splenic DX5+ T cells in AKR mice also differ in their requirement for costimulatory signals during activation. In addition, thymic DX5+ T cells showed a phenotype similar to that of activated T cells, whereas splenic DX5+ T cells showed a phenotype similar to that of memory T cells (Fig. 2b), identical with the phenotypes observed in thymic and splenic NK1.1+ T cells in C57BL/6 mice.

**Splenic APCs can modify the phenotype and functional properties of thymic NKT cells**

As thymic and splenic NKT cells develop from the same thymic precursor (29–31), but display different phenotypes and cellular functions, we hypothesized that thymic NKT cells may migrate to the periphery and interact with more diversified APCs, resulting in a change in the phenotype and functional properties of NKT cells. To test this hypothesis, we purified thymic and splenic NK1.1+ T cells from C57BL/6 mice and cocultured these cells with DX5+ cell-depleted thymocytes or splenocytes as APCs in the presence or the absence of αGalCer. After 48 h, the culture supernatant was collected to determine the cytokine profile. Thymic NKT cells produced a large amount of IL-4, but a relatively low level of IFN-γ, when they were cocultured with thymic APCs in the presence of αGalCer. However, coculture with splenic APCs significantly increased IFN-γ production and only slightly affected IL-4 production (Fig. 4a). Similar results were observed when splenic NKT cells were activated with different APCs, although they produced much less IL-4 and slightly less IFN-γ than thymic NKT cells (Fig. 4a). As thymic and splenic NKT cells differ not only in their cytokine production, but also in surface marker expression, we then examined the expression of CD62L in thymic and splenic NKT cells when they were cocultured with different APCs. Thymic NKT were CD62Llow; however, these cells expressed increased levels of CD62L when cultured with splenic APCs for 5 days with or without αGalCer. In contrast, no change in the expression

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Cells</th>
<th>Treatment</th>
<th>Anti-CD3</th>
<th>Vehicle</th>
<th>αGalCer (100 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>Thymocytes</td>
<td>1,726 ± 35.0</td>
<td>0b</td>
<td>0b</td>
<td>878 ± 20.8</td>
</tr>
<tr>
<td></td>
<td>DX5+ thymocytes</td>
<td>52 ± 5.2</td>
<td>0b</td>
<td>0b</td>
<td>32.6 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Splenocytes</td>
<td>11,287 ± 46.0</td>
<td>18 ± 0.4</td>
<td>0b</td>
<td>896.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>DX5+ splenocytes</td>
<td>8,845 ± 48.3</td>
<td>0b</td>
<td>0b</td>
<td>32.6 ± 3.0</td>
</tr>
<tr>
<td>IL-4</td>
<td>Thymocytes</td>
<td>68 ± 7.0</td>
<td>0b</td>
<td>0b</td>
<td>115 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>DX5+ thymocytes</td>
<td>9 ± 1.8</td>
<td>0b</td>
<td>0b</td>
<td>2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Splenocytes</td>
<td>1,342 ± 32.1</td>
<td>11.6 ± 0</td>
<td>0b</td>
<td>332 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>DX5+ splenocytes</td>
<td>18 ± 1.1</td>
<td>0b</td>
<td>0b</td>
<td>45 ± 0</td>
</tr>
</tbody>
</table>

* Splenocytes and thymocytes from AKR mice were activated by immobilized anti-CD3 Ab (5 μg/ml) or αGalCer for 48 h. Cytokine production in the supernatant was measured. DX5+ cell-depleted splenocytes and thymocytes were also activated similarly. Representative results of three independent experiments are shown. Numbers are the mean ± SD of triplicate samples.

* Not detectable.
levels of CD62L were detected in thymic NKT cells cocultured with thymic APCs. In addition, the increased CD62L expression in thymic NKT cells was CD1d dependent, because CD62L expression of thymic NKT cells did not increase when they were cocultured with splenocytes from CD1d-KO mice (Fig. 4b). The expression of CD62L in splenic NKT cells was not significantly changed during activation with different APCs (data not shown).

We then compared the expression levels of CD1d and costimulatory molecules in thymic APCs with those in splenic APCs. We found that splenic APCs expressed higher levels of CD1d and CD86, but similar levels of CD80 compared with thymic APCs (Fig. 5a). To determine whether costimulatory molecules on the surface of APCs play a role in regulation of NKT cell functions, we activated thymic NKT cells purified from C57BL/6 mice with αGalCer presented by splenic APCs in the presence of anti-CD40, -CD80, and -CD86 Abs (40 μg/ml) to block costimulation. The purified thymic NKT cells were also activated by anti-CD3 Ab with or without anti-CD28 Ab. After 48 h, cell proliferation and cytokine production were determined and compared among different groups of activated thymic NKT cells. It was clear that thymic NKT cells proliferated more vigorously when they were activated by anti-CD3 with or without anti-CD28 Ab compared with NKT cells activated with αGalCer and splenic APCs. However, the αGalCer/splenic APCs induced much more IFN-γ, but similar amounts of IL-4 and IL-2 production, compared with those induced by Ab activation (Fig. 5b). Blocking CD40, CD80, and CD86 signals individually did not affect the proliferation of NKT cells, whereas the presence of both anti-CD80 and anti-CD86 Abs in the culture significantly decreased the proliferation of thymic NKT cells. The presence of anti-CD40, -CD80, and -CD86 Abs in the cultures decreased cytokine production. Blocking CD80/CD86 signals simultaneously almost completely eliminated cytokine production, possibly showing synergetic effects of costimulatory signals on the function of NKT cells.

**Discussion**

NKT cells are a small population of T cells that can be clearly distinguished from the mainstream T cell population by the predominant usage of Vα14 TCR and CD1d restriction. Most of the CD1d-restricted NKT cells are known to express NK1.1 Ag and play an important role in immune regulation (5–9). In both NK1.1+ and NK1.1− strains of mice, T cells that express the DX5 Ag were found to have regulatory functions in various immune responses similar to those of NK1.1+ NKT cells. However, DX5+ NKT cells are still considered distinct from NK1.1− NKT cells (16–19, 32, 33), because phenotypic studies suggested that CD1d-restricted Vα14 NKT cells do not express DX5 Ag, and DX5+ T cells are a subset of NKT cells different from NKT cells that express NK1.1 Ag (20–23). As NKT cells evoke a Th1 response in tumor rejection and host defense and a Th2 response in the prevention of autoimmune disease (6, 8–10, 17–19, 28, 32, 33), different regulatory functions may be conducted by subsets of NKT cells. Characterization of DX5+ and NK1.1+ T cells may clarify whether the different functions of NKT cells are due to the development of two different subsets of NKT cells.

We first tested whether depletion of DX5+ cells has any effect on the αGalCer-specific responses, as in both human and mouse, αGalCer is only recognized by CD1d-restricted T cells. We found that DX5+ cells in both thymocytes and splenocytes from either NK1.1+ and NK1.1− strains of mice contain αGalCer-responsive populations. The results of αGalCer/CD1d tetramer staining also clearly revealed that most of NK1.1+ and DX5+ NKT cells are Vα14 NKT cells, and the DX5 Ab-mediated depletion removed most αGalCer/CD1d tetramer+ and NK1.1+ NKT cells. In fact, we have cloned and sequenced TCR cDNA of DX5+ T cells purified from either C57BL/6 and AKR mice and found the predominant usage of Vα14-Jα281 invariant chain in these cells (data not shown). When purified NK1.1+ TCR+ and DX5+ T cells were activated under various conditions, these two populations displayed identical cytokine capacities and profiles. From the results of the functional and phenotype characterizations, we conclude that most NK1.1+ and DX5+ T cells do not represent different subsets of NKT cells. In addition, we found that similar to NK1.1 Ag, DX5 Ag is only expressed in CD24−CD44+ mature NKT cells.

In the present study we found that thymic NKT cells in many strains express DX5 Ag at low levels that can be clearly detected by PE-conjugated Ab and only weakly by FITC-conjugated Ab. FITC-conjugated Ab was used in previous studies that reported negative results of DX5 staining in Vα14 NKT cells (20–22). As
DX5\(^+\) TCR\(^+\) cells demonstrate the functions and phenotypes of \(\text{Va14NKT cells, DX5 Ag can be used as marker to identify and even enrich Va14 NKT cells in NK1.1}^{+}\) strains without affecting the functions of NKT cells. In fact, we are able to readily isolate \(\alpha\text{GalCer/CD1 tetramer}^{+}\) cells from thymocytes of NK1 allele-negative strains, such as BALB/c and NOD mice, using the DX5 marker. However, a small number of T cells in the spleens of young mice express DX5 Ag, but not NK1.1 Ag (32). Therefore, it is difficult to exclude the possibility that a small number of T cells expressing DX5 Ag in the spleen may be different from CD1d-dependent NKT cells. The nature of these cells remains to be determined.

Although NK1.1\(^+\) and DX5\(^+\) NKT cells are not distinct subsets of NKT cells, substantial differences in function between thymic and splenic NKT cells attracted our attention. Under various conditions, thymic NKT cells produced a higher amount of cytokines, especially IL-4, than splenic NKT cells. Apparently thymic NKT cells are able to produce more cytokines and have a cytokine profile biased to Th2 responses. The results of the present study further revealed that thymic and splenic NKT cells from both NK1.1\(^+\) and NK1.1\(^+\) strains of mice differ not only in their cytokine production, but also in the expression of activation markers and requirements for costimulatory signals. Splenic NKT cells required both TCR- and CD28-mediated signals to be fully activated, whereas engagement of CD28 had no detectable effect on the activation of thymic NKT cells. We also found that the engagement of CD40 ligand (CD40L) on the surface of thymic NKT cells did not affect their cytokine production (data not shown), although the CD40 signal was shown to be important for the cytokine profile of splenic NKT cells (34). The effects of costimulatory signals on the cytokine profiles of NKT cells have been studied using peripheral NKT cells, including splenic (34) and hepatic (35) NKT cells. The present study provides the first evidence for different requirements for costimulatory signals between thymic and splenic NKT cells. Importantly, the different costimulatory signal requirement is consistent with different expression patterns of the activation markers between thymic and splenic NKT cells. As we showed in this study, splenic NKT cells are mostly CD4\(^+\)CD62L\(^+\)CD69\(^-\), indicating that splenic NKT cells are memory T cells. The production of a large quantity of cytokines from thymic NKT cells in vitro by TCR ligation is consistent with other studies (21, 26). However, cytokine production from thymic NKT cells in vivo is not readily detected after anti-CD3 Ab or \(\alpha\text{GalCer}^{+}\) injection. Whether this difference between in vitro and in vivo conditions is due to thymus-specific factors that control the production and secretion of cytokines from NKT cells is presently under investigation.

To understand the mechanisms of NKT cell differentiation, we cocultured thymic and splenic NKT cells with APCs from different tissues. It is known that subsets of APCs, such as dendritic cells, enhance IFN-\(\gamma\) production by peripheral NKT cells (36–38). In the present study we found that IFN-\(\gamma\) production, but not IL-4 production, by either thymic or splenic NKT cells can be regulated by different APCs, suggesting that costimulatory signals conferred by splenic APCs enhance IFN-\(\gamma\) production. Interestingly, splenic APCs also enhance CD62L expression in thymic NKT cells after 5 days of culture, even in the absence of \(\alpha\text{GalCer}^{+}\) cells. Because the increased CD62L expression was CD1d dependent, it was likely that engagement of thymic NKT cells with endogenous Ags presented by splenic APCs increases CD62L expression when thymic NKT cells migrate into spleen. CD62L is required for the homing of memory-type T cells to secondary lymphoid organs (39); thus, the increase in CD62L expression may be an important step for NKT cell differentiation when they migrate from thymus to periphery. Diversified functions among NKT cells from different organs have been reported, and it is believed that differential distribution of CD4\(^+\) and CD4\(^+\)CD8\(^-\) NKT cells may be responsible for the functional diversities in the periphery (40–42), because CD4\(^+\) and CD4\(^+\)CD8\(^-\) NKT cells display different cytokine profiles (43, 44). Most NKT cells develop from the thymus, and most thymic NKT cells are CD4\(^+\) (29–31), thus CD4\(^+\) CD8\(^-\) NKT cells may differentiate from CD4\(^+\) NKT cells, but the mechanisms by which NKT cells differentiate remain unclear. A low, but consistent, level of activation by endogenous Ag on the surface of peripheral APCs may also affect CD4\(^+\)CD8\(^-\) double-negative NKT cell differentiation in secondary lymphoid organs, because it has been shown that a large fraction of splenic CD4\(^+\) NKT cells become double-negative NKT cells after repeated activation (45). Further, it has been found that NKT cells in the circulation express different chemokine receptors with distinct cytokine-producing capacities (46), suggesting that migration destinations are associated with the functions of NKT cells. All these data support the idea that the tissue-specific microenvironment determines the differentiation of thymic NKT cells into subsets of peripheral cells that are functionally and phenotypically distinct.

It is unclear how splenic APCs enhance IFN-\(\gamma\) production by thymic NKT cells. In our study we found different expression levels of CD1d and CD86 between thymic and splenic APCs. To determine which signal might be critical for the functional modification of NKT cells, we blocked candidate signaling molecules, CD80 and CD86, individually or together using their specific Abs. Blockage of CD80 or CD86 significantly hampered cytokine production, but not the proliferation of thymic NKT cells in the presence of \(\alpha\text{GalCer}^{+}\) presented by splenic APCs. A similar inhibitory effect was seen when anti-CD40 Ab was added to the culture. In addition, blockage of both CD80 and CD86 resulted in the almost complete elimination of cytokine production and proliferation of thymic NKT cells. In contrast, we found that engagement with anti-CD28 and anti-CD40L Abs had no direct effect on the function of thymic NKT cells. These results suggest that the signal mediated by anti-CD28 or anti-CD40L Ab could not replace the signal mediated by CD80/CD86 or CD40 expressed on splenic APCs. It is possible that multiple signals from APCs may synergistically enhance the function of NKT cells. Another possibility is that the Abs against the molecules on the surface of APCs disrupt structure formation, such as the lipid raft (47), which is required for Ag presentation or other unidentified costimulatory signals. Further studies are needed to clarify the role of complex signals in NKT cell function. However, these results have provided clear evidence that the roles of signals in different tissue-specific microenvironments, consisting of various APC subpopulations, are critical for the development of Th1- or Th2-oriented responses by NKT cells.

In conclusion, both NK1.1\(^+\) and DX5\(^+\) T cells are CD1d-restricted \(\text{Va14NKT cells, and thymic NKT cells differentiate into functionally different NKT cells under tissue-specific microenvironments}. In mice, CD1d-restricted NKT cells preferentially evoke Th1- or Th2-oriented responses by NKT cells.

**Acknowledgments**

We are grateful to Lori Bryant for assistance with flow cytometry, and Dr. Ann Kyle for editorial assistance.

**References**


