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*J Immunol* 2010; 184:2095-2106; Prepublished online 20 January 2010;
doi: 10.4049/jimmunol.0901348
http://www.jimmunol.org/content/184/4/2095

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Invariant NKT Cells Preferentially Modulate the Function of CD8α+ Dendritic Cell Subset in Inducing Type 1 Immunity against Infection

Antony George Joyee, Jude Uzonna, and Xi Yang

Although studies suggest that NKT cell (NKT) activation modulates the function of dendritic cells (DCs) in inducing T cell responses, it is unknown whether this modulating effect is biased to a DC subset. We previously reported that NKT activation could modulate DC function in inducing protective T cell immunity to Chlamydia pneumoniae, an intracellular bacterial infection. In this study, we investigated the effect of NKT activation on DC subsets, using multiple approaches, including gene knockout mice, α-galactosylceramide stimulation, adoptive transfer of invariant NKT (iNKT), and functional analysis of DC subsets in both in vitro and in vivo settings. We found a preferential modulating effect of iNKTs on the CD8α+ DC subset. Specifically, we found that iNKT-deficient mice, compared with wild-type (WT) mice, showed reduced CD8α+ DC expansion with lower CD40 expression and IL-12 production, whereas enhancing iNKT activation in WT mice or adoptive transfer of iNKTs to Jo18−/− mice resulted in increased function of CD8α+ DCs in inducing type 1 immune responses. Further, DC-iNKT coculture experiments showed a direct CD40L-dependent enhancing effect of iNKTs on IL-12p70 production by CD8α+ DCs. More importantly, CD8α+ DCs from Jo18−/− mice, compared with those from WT mice, showed significantly reduced ability to activate IFN-γ-producing T cells in vitro and to induce type 1 immunity and protection in vivo. Moreover, a similar CD8α+ DC subset alteration was found in the Jo18−/− mice following Leishmania major infection. Our data provide the first direct evidence that iNKTs preferentially promote the functional development of a subset of DC to generate protective immunity against infections. The Journal of Immunology, 2010, 184: 2095–2106.

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Received for publication April 29, 2009. Accepted for publication December 11, 2009.

This work was supported by operating grants from the Canadian Institutes for Health Research and the Manitoba Health Research Council (to X.Y.). A.G.J. is a recipient of a Postdoctoral Fellowship Award from the Manitoba Health Research Council. X.Y. is the Canada Research Chair in Infection and Immunity.

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Abbreviations used in this paper: α-GalCer, α-galactosylceramide; DC, dendritic cell; dLN, draining lymph node; EB, elementary body; IFU, inclusion-forming unit; iNKT, invariant NKT cell; KO, gene knockout; KO CD8α+ DC, CD8α+ DCs isolated from Jo18−/− mice following C. pneumoniae infection; MFI, mean fluorescence intensity; NK, NKT cell; p.i., postinfection; SK-EB, sonicated killed C. pneumoniae EB; WT, wild-type; WT CD8α+ DCs, CD8α+ DCs isolated from WT mice following C. pneumoniae infection; veh, vehicle.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901348
FIGURE 1. Reduced expansion and maturation of the CD8α+ DC subset in Jα18−/− mice following C. pneumoniae infection. Jα18−/− and WT (C57BL/6) mice were killed at specified days after intranasal infection with C. pneumoniae (3 × 10³ IFUs), and the DCs isolated from the spleens were analyzed. A, DCs in the Jα18−/− mice show a reduced proportion of the CD8α+ DC subset as analyzed on days 3 and 7 p.i.; living cells were selected according to forward- and side-scatter properties. CD11chi MHC IIhi cells were gated for analysis of conventional DC-DC subsets. Representative dot plots with the percentage of the CD8α+ DC subpopulation are shown (left), as well as the graphical summary for the percentage of the CD8α+ DC subset within the DCs (right). B, Jα18−/− mice show reduced numbers of splenic CD8α+ DCs p.i. Total splenocytes were stained for DC markers and analyzed. The absolute numbers of whole DCs (CD11chi MHC IIhi), CD8α+ DCs (CD11chi CD8αhi), and CD8α− DCs (CD11chi CD8α2) in the spleen following C. pneumoniae infection are shown. Total DC number per spleen was calculated as % CD11chi MHC IIhi cells × total number of cells per spleen/100. Similarly, the numbers for CD8α+ DC and CD8α− DC were calculated. C, CD8α+ DC in lung dLN; Total LN cells were stained, and the percentage of CD8α+ DCs on
this study, we extended our investigation on the modulating effect of iNKTs activation on DCs to the level of DC subsets. We found that iNKTs preferentially modulate the CD8a+ DC subset, con-
sequently augmenting the generation of protective type 1 T cell immune responses after C. pneumoniae infection. Moreover, a similar alteration of phenotype and cytokine production by
CD8a+ DC subset was found in Jc18−/− mice following Leish-
mania major infection. Our data highlight the role played by
iNKTs in skewing the function of a subset of DCs for enhanced immunity against infections.

Materials and Methods

**Mice**

C57BL/6 and BALB/c mice were purchased from Charles River Canada (Montreal, Canada) or bred at the University of Manitoba animal care facility. Breeding pairs of Jc18−/− (iNKT knockout [KO]) mice with B6 back ground were kindly provided by Dr. Masaru Taniguchi, RIKEN Research Center for Allergy and Immunology, Yokohama City, Japan. These mice were bred and maintained at the pathogen-free animal care facility, University of Manitoba (Winnipeg, Canada). Mice 8–10 wk old were used in the study. All experiments were performed in compliance with the guidelines issued by the Canadian Council of Animal Care.

**Organism, infection, and quantitative assessment of infection**

The culture, propagation of C. pneumoniae (AR-39), and determination of infectivity, as measured by inclusion-forming units (IFUs) of the bacterial preparation, were performed as described previously (13). Highly purified elementary body (EB) preparations were obtained by Renografin gradient separation. For in vitro antigenic restimulation in culture, a sonicated killed C. pneumoniae EB (SK-EB) preparation was used (13). For in vivo infection, mice were mildly sedated with isoflurane and intranasally inoculated with 3 × 10^5 IFUs C. pneumonia in 40 μl PBS. At predetermined days after inoculation, the mice were euthanized and the lungs were asepto-
ically isolated and processed for quantitatively assessing C. pneumonia as described (13).

**α-GalCer administration**

α-GalCer was provided by Kirin Brewery (Gunma, Japan). Mice received a single injection (i.v.) of 4 μg α-GalCer diluted in a 0.2-ml volume of PBS. Control mice were injected with an identical volume of solution alone (0.025% polysorbate 20 in PBS). Mice were infected with C. pneumoniae 2 h after α-GalCer or vehicle injection. DC isolation, purification of DC subsets, and cytokine production analysis

**Spleens**

Spleens were harvested from the Jc18−/− mice and control animals postinfection (p.i.) and processed into single-cell suspensions. Total DCs were prepared using CD11c-magnetic microbeads and MACS columns, as described previously (14). For preparation of splenic DCs, splenocytes were first incubated with a mixture of Abs to deplete T, B, and NK cells to enrich DCs. The DC subsets were separated according to CD8a expression by incubation with anti-CD8a-coupled microbeads followed by two passages over a MACS column (Miltenyi Biotec, Auburn, CA). The CD8a cells were further enriched for DCs by depleting any remaining CD8+ cells and then incubating with anti-CD11c-coupled microbeads and positive selection over MACS column. Flow cytometric analysis revealed that each purified fraction con-
tained >96% CD8a+CD11c+ and CD8a+CD11c− cells (not shown). The DC subsets isolated from different groups of mice were cultured in complete RPMI 1640 medium containing 10% heat-inactivated FBS, 25 μg/ml gentamicin, 2 mM l-glutamine, and 5 × 10^{-5} 2-ME (Kodak, Rochester, NY) in 96-well plates at 1 × 10^6 cells/well for 72 h, and the supernatants were measured for IL-12p70 by ELISA, using unlabeled (capture) and biotinylated (detection) Abs (purchased from BD Pharmingen, San Diego, CA), as previously described (13, 14).

Flow cytometry

To analyze the expression of various surface markers, freshly isolated splenic DCs were stained with anti-CD11c-PE, anti-CD8-PerCep, anti-MHC class II-allophycocyanin (I-A/E), anti-CD40-FITC, anti-CD80-FITC, and anti-CD86-FITC or with respective isotype controls (eBio-
science, San Diego, CA). The proportion of CD8a+ DCs among the gated CD11c+ MHC II+ cells was analyzed. For intracellular cytokine staining, DCs were stimulated with SK-EB (10^6 IFUs) for 6 h in complete RPMI 1640 medium at 37°C, and for the last 4 h of incubation, monensin (eBioscience) was added to accumulate the cytokine intracellularly. Cultured cells (10^6 cells) were washed and incubated with FcR blocking Abs (anti-16/32; eBioscience) for 15 min at 4°C to block nonspecific staining. The cell surface markers were first stained, and the cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer’s instructions, followed by intracellular staining with anti-IL-12 (p40/p70)-allophycocyanin (C15.6.6) mAbs (BD Biosciences, San Jose, CA) or with corresponding isotype control Abs in permeabilization buffer (BD Pharmingen). Finally, the cells were washed, resuspended in Dulbecco’s PBS containing 2% FCS and 1 mM EDTA, and analyzed by flow cytometry. Sample data were collected using a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using WinMDI software, version 2.8 (Scripps Research Institute, La Jolla, CA).

**NKT-DC coculture**

iNKTs were isolated to high purity (>96%) from T cells enriched by negative selection and then with PE-mCD4/PBS57 ligand tetramer (pro-
vided by National Institute of Allergy and Infectious Disease MHC Tet-
tramer Core Facility, Atlanta, GA) and anti-PE beads (Miltenyi), as described previously (14). DC subsets (10^6 cells) were cultured with NKTs (5 × 10^3) in the presence or absence of SK-EB (10^6 IFUs) in 96-well plates, with or without 10 μg/ml each of anti-IFN-γ (R4-6A2) or CD40L

gated CD11c+ MHC II+ cells (after excluding B220/CD3+ cells) is shown. Also shown are flow cytometry images (left) and a graphical summary for the number of CD8a+ DCs in LN (right). D, Altered DC costimulatory molecule expression pattern of CD40, CD80, and CD86 in Jc18−/− mice. For cos-
timulatory molecule expression on DC subsets, freshly isolated splenic DCs were stained for DC markers and for molecules CD40, CD80, and CD86. At day 3 p.i., expression of the respective molecules was analyzed. Analysis was performed on gated CD8a+(CD11c+CD8a+) and CD8a− DC (CD11c+CD8a−) subsets. Shown are representative histograms (left) with the percentages of positive cells marked and a graphical summary of the data (right). E, Intracellular cytokine analysis for IL-12 production by DC subsets (gated CD11c+ cells). Shown are representative flow cytometry images (left). The percentages of IL-12-producing cells among CD8a+ and CD8a− DC populations were calculated and graphically summarized (right). F, IL-12p70 production by a CD8a+ DC subset from WT and Jc18−/− mice. The CD8a+ and CD8a− DC subsets purified from WT and KO mice at day 3 p.i. were placed in culture for 72 h, and the concentration of IL-12p70 in the supernatants was measured by ELISA. Results are expressed as mean ± SD. At least three independent experiments with four mice in each group were performed, and one representative experiment is shown. *p < 0.05; **p < 0.01; ***p < 0.001.

**Table I. Similar basal level of surface molecule expression on CD8a+ and CD8a− DC subsets in WT and KO mice**

<table>
<thead>
<tr>
<th>Surface Markers</th>
<th>WT</th>
<th>Jc18−/−</th>
<th>WT</th>
<th>Jc18−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40 %</td>
<td>44.0 ± 2.8</td>
<td>43.6 ± 1.1</td>
<td>9.8 ± 1.2</td>
<td>9.9 ± 1.6</td>
</tr>
<tr>
<td>MFI</td>
<td>19.5 ± 1.4</td>
<td>19.2 ± 2.4</td>
<td>10.3 ± 0.7</td>
<td>19.2 ± 0.6</td>
</tr>
<tr>
<td>CD80 %</td>
<td>20.0 ± 0.9</td>
<td>20.6 ± 1.8</td>
<td>7.3 ± 0.9</td>
<td>6.7 ± 1.2</td>
</tr>
<tr>
<td>MFI</td>
<td>17.1 ± 1.2</td>
<td>18.0 ± 0.8</td>
<td>11.3 ± 1.0</td>
<td>10.7 ± 0.8</td>
</tr>
<tr>
<td>CD86 %</td>
<td>14.8 ± 0.6</td>
<td>14.2 ± 1.5</td>
<td>4.4 ± 0.6</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>MFI</td>
<td>5.8 ± 0.4</td>
<td>5.5 ± 0.4</td>
<td>3.6 ± 0.2</td>
<td>3.9 ± 0.3</td>
</tr>
</tbody>
</table>

Expression of costimulatory surface markers CD40, CD80, and CD86 on DC subsets of naive WT and Jc18−/− mice (three mice per group). Data are shown as mean ± SD of three mice in each group.

---

**Table II. C. pneumoniae infection**

<table>
<thead>
<tr>
<th>Organism, infection, and quantitative assessment of infection</th>
<th>WT</th>
<th>Jc18−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. pneumoniae (AR-39)</td>
<td>10^6 IFUs</td>
<td>10^6 IFUs</td>
</tr>
<tr>
<td>Control solution</td>
<td>0.025% polysorbate 20 in PBS</td>
<td>0.025% polysorbate 20 in PBS</td>
</tr>
<tr>
<td>Infection</td>
<td>10^6 IFUs</td>
<td>10^6 IFUs</td>
</tr>
</tbody>
</table>

*Note: IFUs = international fluorescent units.*
(1B1) mAb. Concentrations of IL-12p70 in the supernatants were measured by ELISA.

**NKT adoptive transfer**

For adoptive transfer, splenic iNKTs were isolated and first washed in protein-free PBS and then injected into the tail vein of syngeneic naive C57BL/6 mice (1 × 10⁶ cells/mouse). Two hours after adoptive transfer, the mice were intranasally inoculated with 3 × 10⁶ IFUs *C. pneumoniae* in 40 μl PBS. At indicated time points p.i., DCs were isolated and analyzed.

**DC-T cell coculture**

The ability of DC subsets to activate *C. pneumoniae* Ag-specific T cell responses was assessed using a DC/T cell coculture system as described (14). T cells were isolated from the spleens of *C. pneumoniae*-immunized mice, using negative selection by depleting non-T cells (14). CD8α⁺ and CD8α⁻ DC subsets isolated from *C. pneumoniae*-infected Jx18⁻/⁻ mice and wild-type (WT) mice were cocultured with purified T cells (DC/T cell ratio, 1:10) in 200 μl complete RPMI medium, with or without SK-EB (10⁴ IFUs/ml), in 96-well plates for 48 h. Ag-specific effector responses by CD8 and CD4 T cells were analyzed by intracellular cytokine staining for IFN-γ and flow cytometry, as described (13). In the indicated experiments, purified anti–IL-12 mAbs (clone C15.6; BD Bioscience), antagonistic anti-CD40 mAbs (clone HMO-3; BD Bioscience), or anti-CD40L mAbs (MR-1; eBioscience) were added to the designated DC-T cell coculture wells at 10 μg/ml to block endogenous cytokine activity and/or CD40-CD40L interaction.

The naïve T cell priming ability of the DC subsets was examined using another DC-Cd4 T cell coculture system, as previously described (14). A CD8α⁺ DC subset isolated from NKT-deficient CD1d⁻/⁻ and WT BALB/c mice p.i. was cocultured with CD4 T cells isolated from the spleens of naive D011.10 OVA-specific TCR αβ transgenic mice (BALB/c background) in 96-well plates in the presence of OVA (100 μg/ml) in complete RPMI medium for 48 h, and the concentrations of IFN-γ and IL-4 in the supernatants were measured by ELISA.

**Adaptive transfer of CD8α⁺ DCs and challenge infection**

Splenic CD8α⁺ DCs were isolated from WT and Jx18⁻/⁻ mice, as described above. The cells were first washed in protein-free PBS and then injected into the tail vein of syngeneic naive WT recipient mice (0.5 × 10⁶ DCs/mouse). The mice were intranasally inoculated with 3 × 10⁶ IFUs *C. pneumoniae* in 40 μl PBS 2 h after the adoptive transfer. The mice were killed at a specified time, and the lungs were aseptically collected and processed for the quantitatively assessment of *C. pneumoniae* in vivo growth, as described previously (13).

**In vitro restimulation assays and cytokine measurements**

To analyze cytokine production, single-cell suspensions were prepared from draining (mediastinum) lymph nodes and cultured at a concentration of 5 × 10⁶ cells/ml alone or with SK-EB (10⁴ IFUs/ml). The 72-h supernatants were analyzed for IFN-γ and IL-4 by ELISA.

**Leishmania major infection and analysis of DC subset and T cell response**

For infection with *L. major*, mice were injected with stationary-phase promastigote parasites (5 × 10⁶ in 50 μl PBS suspension) in both the hind footpads, as described (15). The popliteal draining lymph nodes (dLNs) and spleens were collected at specified time points p.i. for analysis of DC phenotype and T cell cytokine production.

**Statistical analysis**

Data were analyzed using an unpaired, two-tailed Student t test (Prism 4; GraphPad, San Diego, CA). A p value <0.05 was considered significant.

**Results**

The CD8α⁺ DC subset in Jx18⁻/⁻ mice shows reduced expansion, altered costimulatory molecule expression, and reduced IL-12 cytokine production following *C. pneumoniae* infection.
To elucidate the effect of iNKTs on DC subsets, we analyzed CD8α+ and CD8α− DC subsets in Jx18−/− and WT mice following C. pneumoniae infection. We found that Jx18−/− mice, compared with WT mice, showed marked reduction in CD8α+ DCs at an innate phase time point (day 3 p.i.) and a later point following infection (day 7 p.i.) (Fig. 1A, 1B). The percentage (Fig. 1A) and absolute number (Fig. 1B) of CD8α+ DCs in infected WT mice were significantly higher than those in Jx18−/− mice. However, there was no significant difference between WT and KO in the numbers of CD8α− DCs at both time points, although this subpopulation also increased in number on day 7 p.i. (Fig. 1B). In contrast to this p.i. change, the numbers and the proportion of CD8α+ DCs in uninfected Jx18−/− and WT mice were similar (Fig. 1A, 1B). In addition, we also found significantly reduced accumulation of CD8α+ DCs in the lung dLNs of Jx18−/− mice compared with WT mice p.i. (Fig. 1C).

We further analyzed the expression of costimulatory molecules on DC subsets p.i. We found that CD8α+ DCs of Jx18−/− mice showed significantly reduced expression of CD40 and CD80 molecules than was found in WT mice (Fig. 1D). The most notable difference was a much reduced expression of CD40 on CD8α+ DCs in Jx18−/− mice compared with WT mice (p < 0.001). In contrast, CD8α− DCs in the WT and Jx18−/− mice showed similar levels of CD40 expression (p > 0.05). Interestingly, both CD8α+ and CD8α− DC subsets showed a higher CD86 expression in Jx18−/− mice than in WT mice (p < 0.05). The levels of tested costimulatory surface molecules were similar in the uninfected Jx18−/− and WT mice (Table I). The data suggest that iNKTs preferentially modulate the expansion and costimulatory surface marker expression of the CD8α+ DC subset following C. pneumoniae infection.

Considering the critical importance of IL-12 production by DCs in its function to induce type 1 T cell response, we further examined the spontaneous cytokine production pattern of the DC subsets isolated from C. pneumoniae-infected mice. We found that a significantly higher percentage of CD8α+ DCs than CD8α− DCs produced IL-12 (36.4 ± 2.9 versus 16.7 ± 1.6; p < 0.01) in the WT mice, as evaluated by intracellular cytokine analysis (Fig. 1E). However, the IL-12–producing CD8α+ DCs were dramatically reduced in the Jx18−/− mice. In contrast, IL-12 production by CD8α− DCs was similar in the two types of mice. Further, cytokine analysis following culture of CD8α+ and CD8α− DC subpopulations showed significantly lower levels of IL-12p70 in the supernatants of cultured CD8α+ DCs from Jx18−/− mice, compared with those from WT mice (Fig. 1F). As a control, no detectable IL-12p70 was measured in the culture supernatants of either CD8α+ or CD8α− DCs isolated from naive WT or Jx18−/− mice (data not shown). Therefore, iNKTs alter not only CD8α+ DC surface marker expression but also their IL-12 production, which is functionally related to DC function.

**NKT activation by α-GalCer enhances CD40 expression and IL-12 production by the CD8α+ DC subset p.i.**

In line with data from the comparison of Jx18−/− and WT mice, we found that CD8α+ DCs from WT mice treated with α-GalCer prior to infection (enhanced iNKT activation) showed higher CD40 expression (Fig. 2A) and IL-12 production (Fig. 2B), compared with vehicle-treated controls. As a control, treatment of Jx18−/− mice with α-GalCer had no effect on DC surface marker and IL-12 production (data not shown); thus the changes in DCs in WT mice following α-GalCer treatment are mediated by NKTs. In aggregate, these findings obtained from the models of both iNKT
deficiency and enhanced iNKT function suggest that iNKTs exert a preferential influence on CD8α+ DC subset activation and cytokine production following C. pneumoniae infection.

Adoptive transfer of iNKTs promotes the activation of CD8α+ DCs in Jα182/2 mice following Cpn infection

We further performed adoptive transfer experiments to confirm the differential modulating effect of iNKTs on a DC subpopulation in vivo. Freshly isolated iNKTs were adoptively transferred to Jα182/2 mice immediately prior to C. pneumoniae infection, and the DC phenotypic changes and IL-12 production were analyzed at day 3 p.i. Indeed, we found that the iNKT recipients showed a selective increase in the CD8α+ DC subset with increased IL-12 production (Fig. 3A, 3B). These results provided direct in vivo evidence that iNKTs preferentially promote the activation of CD8α+ DCs following C. pneumoniae infection.

NKT-deficient mice after NKT adoptive transfer show the development of protective type 1 T cell responses against infection

As we reported previously, the Jα182/2 mice failed to develop type 1 immunity against C. pneumoniae infection (13, 14). We therefore tested the effect of iNKT restoration in these mice on their protective immunity against C. pneumoniae challenge infection. We found that the expansion of CD8α+ DCs in the iNKT recipient KO mice, as shown above (Fig. 4), correlated well with the development of protective immune responses against infection. The iNKT recipients showed reduced body weight loss (Fig. 4A) and, more importantly, significantly reduced C. pneumoniae growth in their lungs, compared with the mice without iNKT transfer (Fig. 4B). Moreover, the iNKT restoration resulted in the development of a type 1 cytokine production pattern with higher levels of IFN-γ but lower levels of IL-4 production by lung dLN cells (Fig. 4C). Specific T cell cytokine analysis showed enhanced CD8 and CD4 T cell responses (higher IFN-γ production) in the iNKT recipients (Fig. 4D). These data from iNKTs supplementary in vivo experiments further confirmed the importance of iNKTs in modulating the CD8α+ DC subset and demonstrated the linkage between iNKT-mediated changes in CD8α+ DCs and the development of type 1 polarized protective immunity following C. pneumoniae infection.

iNKTs selectively enhance IL-12p70 production by the CD8α+ DC subset through CD40–CD40L interaction and IFN-γ production

After confirming the preferential modulating effect of iNKTs on theCD8α+ DC subset by the adoptive transfer approach, we did further experiments to directly examine the modulating effect of NKTs on DC subsets, especially the molecular basis using an in vitro NKT-DC coculture approach. We cocultured CD8α+ and CD8α- DC subsets from naive WT mice, with NKTs in the presence of SK-EB, and determined the level of bioactive IL-
iNKTs selectively enhance IL-12p70 production by the CD8α⁺ DC subset, and the effect is dependent on CD40–CD40L interaction and IFN-γ. The effects of iNKTs on DC subsets were directly analyzed using a coculture system. CD8α⁺ DCs or CD8α⁻ DCs and iNKTs were freshly isolated from naive mice, and the DC subsets were cultured separately in the presence or absence of iNKTs with C. pneumoniae SK-EB. Bioactive IL-12p70 levels in 48-h culture supernatants were determined by ELISA. The involvement of CD40L and IFN-γ in DC IL-12 production induced by iNKTs was analyzed using blocking Abs, as described in Materials and Methods. The results are mean ± SD. Data are representative of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

12p70 in the culture supernatants. We found that IL-12p70 production by cultured CD8α⁺ DCs was dramatically increased (>5-fold) owing to the presence of NKTs (Fig. 5). However, IL-12p70 production by CD8α⁻ DCs did not change significantly when cocultured with NKTs. IL-12 was not detectable in cultures of NKTs alone, with or without SK-EB stimulation (data not shown). We have previously shown that CD40–CD40L interaction and IFN-γ production by NKTs play a critical role in the modulating effect of NKTs on DCs at the whole DC (not subsets) level (14), so we further extended our analysis to determine the contribution of CD40L and IFN-γ to the modulating effect of NKTs on DC subsets. This was done using blocking Abs for these molecules in NKT-CD8α⁺ DC and NKT-CD8α⁻ DC cocultures. We found that NKT-mediated enhancement of IL-12p70 production by CD8α⁺ DCs was greatly inhibited upon blockade of CD40–CD40L signaling, whereas IFN-γ blockade showed less but also significantly reduced IL-12p70 production (Fig. 5). These data further confirm that NKTs mediate a preferential modulating effect on CD8α⁺ DCs, particularly on bioactive IL-12 production, and demonstrate the significant contribution of CD40L–CD40 interaction and IFN-γ production to this modulating process.

iNKT-mediated effect on CD40 expression and IL-12 production by CD8α⁺ DCs enhances the ability of CD8α⁺ DCs for polarizing type 1 CD8 and CD4 T cell response

Although data from iNKT adoptive transfer experiments (Figs. 3, 4) have shown a close association of the modulating effect of iNKTs on the CD8α⁺ DC subset with enhanced type 1 T cell responses, a study was needed to confirm direct linkage between the modulating effect of iNKTs on CD8α⁺ DCs and type 1 cytokine production by Ag-specific T cells. We therefore further examined the ability of the DC subsets isolated from Jx18⁻/⁻ and WT mice in directing T cell cytokine response, using a DC-T coculture approach. Specifically, we examined the effect of iNKTs on the ability of DCs to promote IFN-γ production by C. pneumoniae-specific T cells. T cells isolated from C. pneumoniae-immunized mice were subjected to coculture with DC subsets isolated from C. pneumoniae-infected WT and Jx18⁻/⁻ mice in the presence of SK-EB stimulation, and the cytokine production pattern of CD8 and CD4 T cells was analyzed. Notably, CD8α⁺ DCs isolated from WT mice following C. pneumoniae infection (WT CD8α⁺ DCs) induced a large proportion of CD8 T cells to produce IFN-γ, whereas the ability of the CD8α⁺ DCs from the Jx18⁻/⁻ mice to activate Chlamydia-specific CD8 T cells for IFN-γ production was significantly reduced (Fig. 6A). Similarly, WT CD8α⁺ DCs also induced more IFN-γ-producing CD4 T cells than did CD8α⁺ DCs isolated from Jx18⁻/⁻ mice following C. pneumoniae infection (KO CD8α⁺ DCs) (Fig. 6B). However, the CD8α⁻ DCs isolated from WT and KO mice did not differ significantly in inducing Ag-driven production of IFN-γ by CD8 and CD4 T cells (Fig. 6A, 6B). In the absence of C. pneumoniae Ag, the percentage of IFN-γ–producing cells in DC-T cell cocultures was below 1% or undetectable in showing the specificity of T cell effector response. These results demonstrate that iNKT-mediated preferential modulation of the CD8α⁺ DC subset in phenotype and IL-12 production indeed has functional implications for Ag-specific adaptive T cell responses.

To further examine the functional importance of iNKT-mediated increase of CD40 expression and IL-12 production by CD8α⁺ DCs in inducing C. pneumoniae-specific T cell responses, we added neutralizing Abs to CD40 and IL-12 in the same DC-T coculture system. We found that neutralization of CD40 or IL-12 in the coculture significantly inhibited IFN-γ production by CD8 and CD4 T cells induced by CD8α⁺ DCs (Fig. 6C, 6D). Coblockade of both CD40 and IL-12 resulted in further inhibition of IFN-γ–producing T cell responses, which suggested that these molecules synergistically play a critical role in eliciting type 1 T cell responses by CD8α⁺ DCs. CD8α⁺ DCs from Jx18⁻/⁻ mice showed significant decreases of these molecules compared with those from WT mice (Fig. 1); this might be the molecular basis for the reduced capacity of CD8α⁺ DCs from Jx18⁻/⁻ mice in directing type 1 T cell responses.

Moreover, the T cell priming ability of CD8α⁺ DCs was analyzed in a Th2 OVA model system using a DC-CD4 T cell–OVA coculture approach (14). In this case, naive CD4 T cells isolated from naïve DO11.10 transgenic mice (with TCR specific for OVA peptide) were subjected to coculture with DCs isolated from naive C. pneumoniae-infected WT (BALB/c) and NKT-deficient CD1d-KO mice, in the presence of OVA, and the cytokine production was analyzed. As shown in Fig. 6E, WT CD8α⁺ DCs induced significantly higher Th1 (IFN-γ) than did those from KO mice. The CD8α⁺ DC isolated from KO mice induced more Th2 (IL-4) response (Fig. 6F) in this system. Collectively, these in vitro experiments demonstrated that iNKT-mediated changes in DC subpopulations in turn affect the pattern of T cell responses.

Adoptive transfer of CD8α⁺ DCs from WT mice, but not those from Jx18⁻/⁻ mice, enhanced protective type 1 immunity in vivo

The results thus far demonstrated that NKTs preferentially modulated CD8α⁺ DCs, leading to alterations in surface markers, cytokine production, and in vitro function. Finally, we performed adoptive transfer experiments to directly examine the functional relevance of iNKT-mediated modulation of CD8α⁺ DCs during infection, in inducing protective immunity in vivo against challenge C. pneumoniae infection. CD8α⁺ DCs isolated from C. pneumoniae-infected Jx18⁻/⁻ and WT mice following infection (day 3 p.i.) were adoptively transferred (i.v.) to naïve C57BL/6 mice, and the recipients were challenged with intranasal C. pneumoniae infection. Mice that received no cell transfer (PBS...
alone) with the same challenge infection served as controls. We found that the WT CD8α+ DC recipient mice exhibited lower body weight loss and faster recovery (Fig. 7A) than did the KO CD8α+ DC recipients and the controls. Consistently, C. pneumoniae loads in the lungs of WT CD8α+ DC recipients were significantly lower (~100-fold) than those in the control mice without adoptive transfer (Fig. 7B). However, there was no significant difference in lung chlamydial loads between KO CD8α+ DC recipients and controls (Fig. 7B). The histological analysis also showed less pathological change in the lungs of WT CD8α+ DC recipients, whereas the change in KO CD8α+ DC recipient mice was similar to that in the controls (Fig. 7C). In correlation with these findings, the WT CD8α+ DC recipients, unlike the mice receiving KO CD8α+ DCs, showed significantly enhanced type 1 cytokine response, with higher levels of IFN-γ but reduced IL-4 production by the LN cells, compared with the PBS controls (Fig. 7D). Intracellular cytokine staining on the LN T cells showed significantly higher IFN-γ-producing CD8 T cells in the WT CD8α+ DC recipients than in the KO CD8α+ DC recipients. A similar trend was observed for IFN-γ production by CD4 T cells, although the difference did not reach statistical significance (Fig. 7E). These results showed the functional effect of NKT-mediated modulation of the CD8α+ DC subset in vivo. Collectively, our findings demonstrate an important role for NKTs in modulating CD8α+ DCs to direct strong type 1 protective immunity against C. pneumoniae infection in vivo.

**Preferential modulating effect of NKTs on the CD8α+ DC subset and Th1 responses in Leishmania major infection**

Finally, to examine whether the phenomenon of NKT modulation of the CD8α+ DC subset is specific to C. pneumoniae infection or a more general phenomenon, we tested the effect of NKTs on the DC subset in a different model, L. major parasitic infection. It has been reported that iNKTs play a crucial role in an early stage of protective immunity against infection with Leishmania major (16). Following footpad infection with L. major (at day 3), we analyzed DC phenotypic changes and costimulatory molecule expression. Interestingly, we found a significantly lower proportion of CD8α+...
DCs in the dLNs of Jα18−/− mice, compared with WT mice (Fig. 8A). There was an ~7-fold difference in the numbers of CD8α+ DCs in dLNs between the two groups of mice (Fig. 8B). Further, the costimulatory molecule expression—in particular, CD40 on DC—was significantly different. The Jα18−/− mice, compared with the WT mice, showed much reduced CD40 expression, and the difference was more pronounced with the CD8α+ DC population (Fig. 8C). Similar changes in the proportion of the CD8α+ DC subset and the costimulatory molecule expression were observed in the spleen at day 6 p.i. (data not shown). In addition, CD8α+ DC in Jα18−/− mice also showed lower IL-12 production, as analyzed by intracellular staining (Fig. 8D).

To ascertain whether the change in DC phenotype influences T cell immune response, we also analyzed the T cell cytokine response to infection (day 6 p.i.). We found significantly lower IFN-γ production by T cells in Jα18−/− mice compared with WT mice (Fig. 8E), which clearly correlated with the DC subset change p.i. Overall, these findings in different infection models show a similar impact of NKTs on the activation of CD8α+ DC and T cell responses.

**Discussion**

Although recent studies suggest that NKT activation could modulate the function of DCs in inducing T cell responses, it has remained unknown whether this modulating effect is biased to a DC subset. The most significant finding of this study is that NKTs preferentially modulate the functional ability of the CD8α+ DC subset in vivo. This conclusion was generated from data from four different experimental approaches. First, the Jα18−/− mice showed reduced expansion of and lower IL-12 production by CD8α+ DCs following C. pneumoniae infection (Fig. 1). Second, the administration of NKT enhancer, α-GalCer, promoted CD8α+ DC expansion with increased IL-12 production during C. pneumoniae infection (Fig. 2). Third, adoptive transfer of iNKTs to Jα18−/− recipient mice, which led to CD8α+ DC expansion with higher IL-12 production, enhanced type 1 T cell responses and accelerated
within DCs (prepared and analyzed for the CD8+ DCs in the presence of soluble populations is shown.) In the presence of soluble Leishmania Ag and analyzed by intracellular cytokine staining. Graphical summary of the percentage of IL-12–producing cells among CD8+ DCs from DCs (17). Our flow cytometry analysis also confirmed this (data not shown). Therefore, the intrinsic difference between CD8α+ and CD8α− DCs in CD1d expression may partially explain the preferential modulation of CD8α+ DCs by NKTs. The higher CD1d expression by CD8α+ DCs may make this DC subset more efficient in presenting endogenous and/or exogenous lipid Ags and the subsequent activation of NKTs. Through the bidirectional interaction between NKTs and DCs and a positive feedback loop, the activated NKTs may have a higher chance to influence the CD8α+ DC subset. Indeed, we have found that CD1d blockade dramatically inhibited cytokine production (IL-12p70) by DCs in NKT-DC cocultures (data not shown). Next, the intrinsic difference between CD8α+ DCs and CD8α− DCs in CD40 expression and in the response to CD40 ligation could also contribute to preferential modulation of CD8α+ DCs. It has been shown that CD40 triggering is required for IL-12 production by CD8α+ DCs, but not CD8α− DCs (18). We have reported that CD40L–CD40 interaction is critical for the modulating effect of NKTs on DCs during C. pneumoniae infection. WT and Jα18−/− mice were infected with C. pneumoniae (Fig. 4). Finally, we demonstrated that changes in the CD8α+ DC subset mediated by NKT activation had functional implication in directing T cell immunity in vitro and in vivo experiments. We found that CD8α+ DCs from Jα18−/− mice, compared with those from WT mice, exhibited reduced ability to reactivate IFN-γ-producing C. pneumoniae–specific CD8 and CD4 T cells. In addition, the CD8α+ DCs from WT mice relative to those from NKT-deficient Jα18−/− mice were more efficient in inducing IFN-γ production when cocultured with OVA peptide-specific naïve CD4 T cells from DO11.10 mice (Fig. 6E, 6F). Importantly, we found that CD8α+ DCs from Jα18−/− mice, compared with those from WT mice, showed significantly reduced ability to induce in vivo type 1 immunity and protection in adoptive transfer experiments (Fig. 7). The present finding advanced our recently reported results showing that NKTs promoted the function of DCs in inducing type 1 T cell immunity, in a whole DC level (14). Moreover, a similar alteration of phenotype and cytokine production by CD8α+ DC subset was found in Jα18−/− mice following L. major infection. To our knowledge, this report is the first demonstrating a preferential modulation of CD8α+ DCs in a functional implication in host defense against infections.

Why do NKTs preferentially influence CD8α+ DCs? It has been shown that CD8α+ DCs express higher basal levels of CD1d than do CD8α− DCs (17). Our flow cytometry analysis also confirmed this (data not shown). Therefore, the intrinsic difference between CD8α+ and CD8α− DCs in CD1d expression may partially explain the preferential modulation of CD8α+ DCs by NKTs.
the activation and potency of NKTs to provide stimulatory feedback signals to DCs (19). Therefore, preferential interaction between NKTs and the IL-12–producing CD8α− DCs consequently resulted in an enhanced ability of the latter to prime and polarize CD8 and CD4 T cells in a type 1 direction. In addition, the modulating effect of NKTs on CD8α+ DCs, especially on CD40 expression and IL-12 production by this DC subset, can further strengthen the capacity of CD8α+ DCs in promoting type 1 T cell response. Numerous studies have shown the role of CD40 and IL-12 in enhancing CD4 and CD8 T cell responses in different settings, including infections (20–22). Indeed, in vitro neutralization experiments also demonstrated the importance of CD40/IL-12 in the induction of type 1 T cell responses (Fig. 6C, 6D). More importantly, our data showed the functional effect of NKT-mediated modulation of CD8α+ DCs in inducing protective immunity in vivo. Notably, our findings also suggest that NKT-mediated modulation helps in the induction of protective CD8+ T cell response against C. pneumoniae infection through enhancing the function of CD8α+ DCs. Altogether, our data present a clear linkage between NKT-mediated preferential CD8α+ DC subset functional changes and the development of enhanced type 1 polarized protective immunity. A possibility difficult to address at present is that the various DC subsets might differ in terms of being infected by C. pneumoniae and/or in Ag uptake, subsequently leading to differential DC subset modulation. We still have technical difficulty in measuring chlamydial Ags in the splenic DCs isolated from C. pneumoniae-infected mice, using PCR and Western blot analysis (14). This needs to be addressed in future studies. However, we believe the potential difference in Ag uptake is unlikely to be the major reason for the observed difference, because several recent studies have shown a similar capacity of CD8α+ and CD8α− DCs in uptaking pathogens in other model systems (23–25). Moreover, even in some earlier studies that demonstrated a difference in this regard, the CD8α+ DC subset, rather than the CD8α− DC subset, is the one showing lower Ag uptake (26, 27).

The present findings may have broader implications for understanding cellular mechanisms in the development of immunity in infections or disease conditions. In addition to showing the significant effect of NKTs on the function of the CD8α+ DC subset in Chlamydia infection, we also found an iNKT-mediated enhanced activation of CD8α+ DCs that correlated with enhanced Th1 response in a parasitic infection with Leishmania. These additional data suggest that the phenomenon of iNKT modulation of the CD8α+ DC subset may be more general in the host defense against infections, although the modulating effect may vary with different infections, depending on the extent and nature of initial NKT activation and other factors. Because the protective immunity in both chlamydial and leishmanial infections is a type 1 immune response, and because CD8α+ DCs are known to preferentially induce or promote Th1 responses (2, 3), it would be important to test the effect of NKTs on DCs in infections needing a type 2 response for protection. More importantly, caution must be observed in generalizing the modulating property of iNKTs on the basis of our results, particularly considering that iNKTs are known to play diverse roles in different infections (28).

What is the possible linkage between the current findings in a mouse model and human diseases? Recent studies have suggested that a BDCA-3+ DC subset in humans, which also expresses DNGR-1, may represent a DC lineage equivalent to that of the mouse CD8α− DC (29–31). Striking patterns and close matches between the mouse CD8α− DC subset and the human BDCA-3+ DC subset, attributing some functional equivalence to these DC subsets, have been observed (30). Our results, which show the functional effect of iNKTs in modulating CD8α+ DCs, raises the question of whether human iNKTs have a preferential modulating effect on the BDCA-3+ DC subset.

In conclusion, our findings provide the first direct evidence that NKTs promote the activation of CD8α+ DCs and the functional ability of this DC subset to generate type 1 T cell immune response against infection. The data provide a novel explanation for the role of NKTs in augmenting protective type 1 immunity in microbial infections and may have implications for immune strategies targeting DC subsets and NKT–DC interaction for the control of infection and other diseases.

Disclosures
The authors have no financial conflicts of interest.

References


