

Natural Killer T Cells Are Essential for the Development of Contact Hypersensitivity in BALB/c Mice

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Contact hypersensitivity (CHS) has been widely used to study cutaneous immune responses, as a prototype of delayed-type hypersensitivity. Although natural killer T (NKT) cells have been assumed to have an important role in CHS, their role is controversial. Here, we report the role of NKT cells in the sensitization phase of CHS, by promoting the survival and maturation of dendritic cells (DCs) in the draining lymph nodes (LNs). The CHS response was attenuated with *Cd1d1*^{-/-} and *Traj18*^{-/-} BALB/c mice in which NKT cells were absent. In the draining LNs, the number of effector T cells and cytokine production were significantly reduced with NKT cell-deficient mice. NKT cells activated and colocalized with DCs in the draining LNs after sensitization. The number of migrated and mature DCs was reduced in NKT cell-deficient mice 72 hours after FITC application. In *in vitro* experiments, activated NKT cells enhanced bone marrow-derived DC (BMDC) survivability via tumor necrosis factor (TNF) production from BMDCs. In addition, TNF production from BMDCs was partially suppressed by the neutralizing anti-CD54 or CD154 antibodies. Our data demonstrate that DC-NKT interaction has a pivotal role in the sensitization phase of CHS.

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INTRODUCTION

Contact hypersensitivity (CHS) has been widely used to study cutaneous immune responses, as a prototype of delayed-type hypersensitivity mediated by antigen-specific T cells (Tomura *et al.*, 2010; Egawa and Kabashima, 2011; Honda *et al.*, 2013). During the sensitization phase of CHS, hapten-bearing cutaneous dendritic cells (DCs), such as epidermal Langerhans cells and dermal DCs, migrate into skin-draining lymph nodes (LNs). An essential step in the development of CHS is the completion of DC maturation and DC presentation of antigens to naïve T cells in the LNs. In the subsequent challenge phase, re-exposure to the cognate hapten results in the recruitment of antigen-specific T cells and other nonantigen-specific leukocytes (Honda *et al.*, 2013).

Natural killer T (NKT) cells are characterized by the expression of an invariant antigen receptor encoded by V α 14-J α 18 in mice and V α 24-J α 18 in humans (Lantz and Bendelac, 1994; Prussin and Foster, 1997). NKT cells recognize self- or non-self glycolipid ligands in conjunction with the monomorphic major histocompatibility complex (MHC)-like molecule CD1d (Brigl and Brenner, 2004). In addition, they mediate intermediary functions that link the innate and acquired immune systems, regulating protective and regulatory responses by their rapid secretion of large amounts of cytokines such as IL-4 and IFN- γ after activation (Taniguchi *et al.*, 2003; Bendelac *et al.*, 2007).

Although NKT cells have been assumed to have an important role in CHS, their role is controversial. Previous studies have demonstrated that the CHS response is attenuated in NKT cell-deficient mice (Campos *et al.*, 2003; Nieuwenhuis *et al.*, 2005; Askenase *et al.*, 2011). Meanwhile, other studies have shown that the CHS response is enhanced in NKT cell-deficient mice (Goubier *et al.*, 2013). A previous report demonstrated that NKT cells in the liver secreted IL-4, which stimulated B-1 B cells to initiate T-cell recruitment for CHS (Campos *et al.*, 2003). DCs in the draining LNs have an essential role in the development of CHS (Kripke *et al.*, 1990). Therefore, it is of great importance to clarify the relationship between NKT cells and DCs in the draining LNs during CHS.

In this study, we demonstrated that the CHS response was attenuated in NKT cell-deficient mice compared with wild-type (WT) mice. In the draining LNs, we found that NKT cells

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Abbreviations: α -GalCer, α -galactosylceramide; AV, annexin V; B6, C57BL/6; BMDC, bone marrow-derived dendritic cell; CD40L, CD40 ligand; CHS, contact hypersensitivity; DC, dendritic cell; DNBS, dinitrobenzene sulfonic acid; LN, lymph node; MHC, major histocompatibility complex; NKT, natural killer T; PI, propidium iodide; TNF, tumor necrosis factor; WT, wild type

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activated DCs after sensitization, and that NKT cells accumulated in the vicinity of DCs. In addition, NKT cells promoted DC survival partially via tumor necrosis factor (TNF) production *in vitro*. This DC–NKT interaction seemed to be essential for the development of the sensitization phase of CHS.

RESULTS

Suppression of CHS response in NKT cell-deficient mice

To investigate the role of NKT cells in cutaneous acquired immune responses, we used DNFB-induced CHS as a model. CHS responses in *Cd1d1*^{−/−} and *Tra18*^{−/−} mice were attenuated compared with that in BALB/c WT mice both 24 and 48 hours after the challenge (Figure 1a). The attenuated CHS responses in *Cd1d1*^{−/−} and *Tra18*^{−/−} mice were confirmed using an additional hapten oxazolone (see Supplementary Figure S1 online). Histology of the ears 48 hours after the challenge showed considerable lymphocyte infiltration and edema in the dermis of sensitized BALB/c WT mice, which were less apparent in both sensitized *Cd1d1*^{−/−} and *Tra18*^{−/−} mice (Figure 1b). The histological scores of *Cd1d1*^{−/−} and *Tra18*^{−/−} mice were lower than those of BALB/c WT mice (Figure 1c).

To clarify the action phase of NKT cells in CHS, we used an adoptive transfer-induced CHS model. The BALB/c WT recipients of the T cells from sensitized BALB/c WT mice showed an enhanced CHS response, whereas the BALB/c WT recipients of the T cells from sensitized *Cd1d1*^{−/−} and *Tra18*^{−/−} mice showed an impaired CHS response (Figure 1d). On the other hand, the BALB/c WT recipients of

the T cells from sensitized BALB/c WT mice showed a similar response to that of the *Cd1d1*^{−/−} or *Tra18*^{−/−} recipients of the T cells from sensitized BALB/c WT mice (Figure 1d). To evaluate whether the attenuated CHS in NKT cell-deficient mice reflected the lack of NKT cells, we performed the reconstitution assay. The attenuation of CHS response in NKT cell-deficient mice was fully restored by the reconstitution of NKT cells (Figure 1e). Although we cannot exclude a possibility that the restored CHS response in reconstituted NKT-deficient animals is due to artificial activation of NKT cells during sorting with α -galactosylceramide (α -GalCer)-loaded CD1d dimers (not due to activation by the hapten and/or subsequent inflammation), the above data indicate that NKT cells have important roles in the sensitization phase of CHS.

We also analyzed the compositions of skin-draining LN cells after sensitization. Five days after sensitization, the total cell numbers in the skin-draining LNs of NKT cell-deficient mice were lower than those of BALB/c WT mice (Figure 2a). In addition, the number of total CD4⁺ and CD8⁺ T cells (Figure 2b and c), CD44[−] naïve (Figure 2d) and CD44⁺ CD62L⁺ central memory T cells (Figure 2e), and CD44⁺ CD62L[−] effector memory T cells (Figure 2f) in the skin-draining LNs of NKT cell-deficient mice were lower than those of BALB/c WT mice. In contrast, the number of each T-cell subset in the LNs without sensitization was comparable between NKT cell-deficient mice and BALB/c WT mice (Figure 2b–f). To confirm whether the decrease of these cells in NKT cell-deficient mice was directly derived from the lack

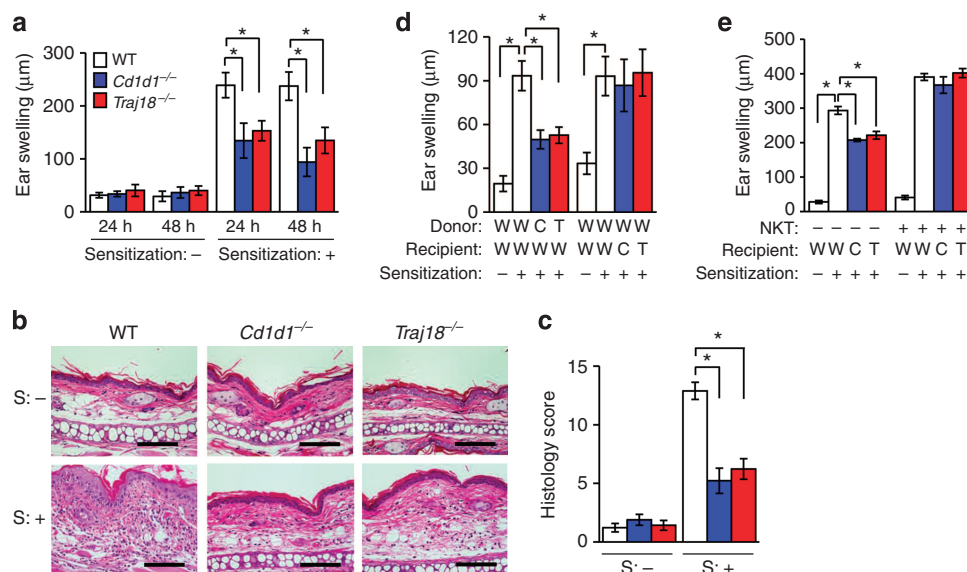


Figure 1. Natural killer T (NKT) cells are essential for the sensitization phase in contact hypersensitivity (CHS). (a) Wild-type (WT) BALB/c mice, *Cd1d1*^{−/−} mice, and *Tra18*^{−/−} mice (*n* = 5 per group) were sensitized with or without DNFB, and ear swelling was measured 24 and 48 hours after challenge with DNFB. (b) Hematoxylin and eosin staining of the ears of sensitized BALB/c WT, *Cd1d1*^{−/−}, and *Tra18*^{−/−} mice 48 hours after challenge with DNFB. Bar = 100 μm. (c) Samples were scored for the severity and character of the inflammatory response using a subjective grading scale. The total histology score was calculated as the sum of scores. (d) T cells from DNFB-sensitized BALB/c WT, *Cd1d1*^{−/−}, and *Tra18*^{−/−} mice were adoptively transferred to induce CHS (*n* = 5 per group). (e) NKT cells purified from the liver were transferred 1 day before sensitization with or without DNFB, and ear swelling was measured 24 hours after challenge with DNFB (*n* = 3 per group). C, *Cd1d1*^{−/−}; T, *Tra18*^{−/−}; W, WT. All data are presented as the mean ± standard error of the mean (SEM), and each data point is representative of at least three experiments with similar results. **P* < 0.05 versus corresponding mice.

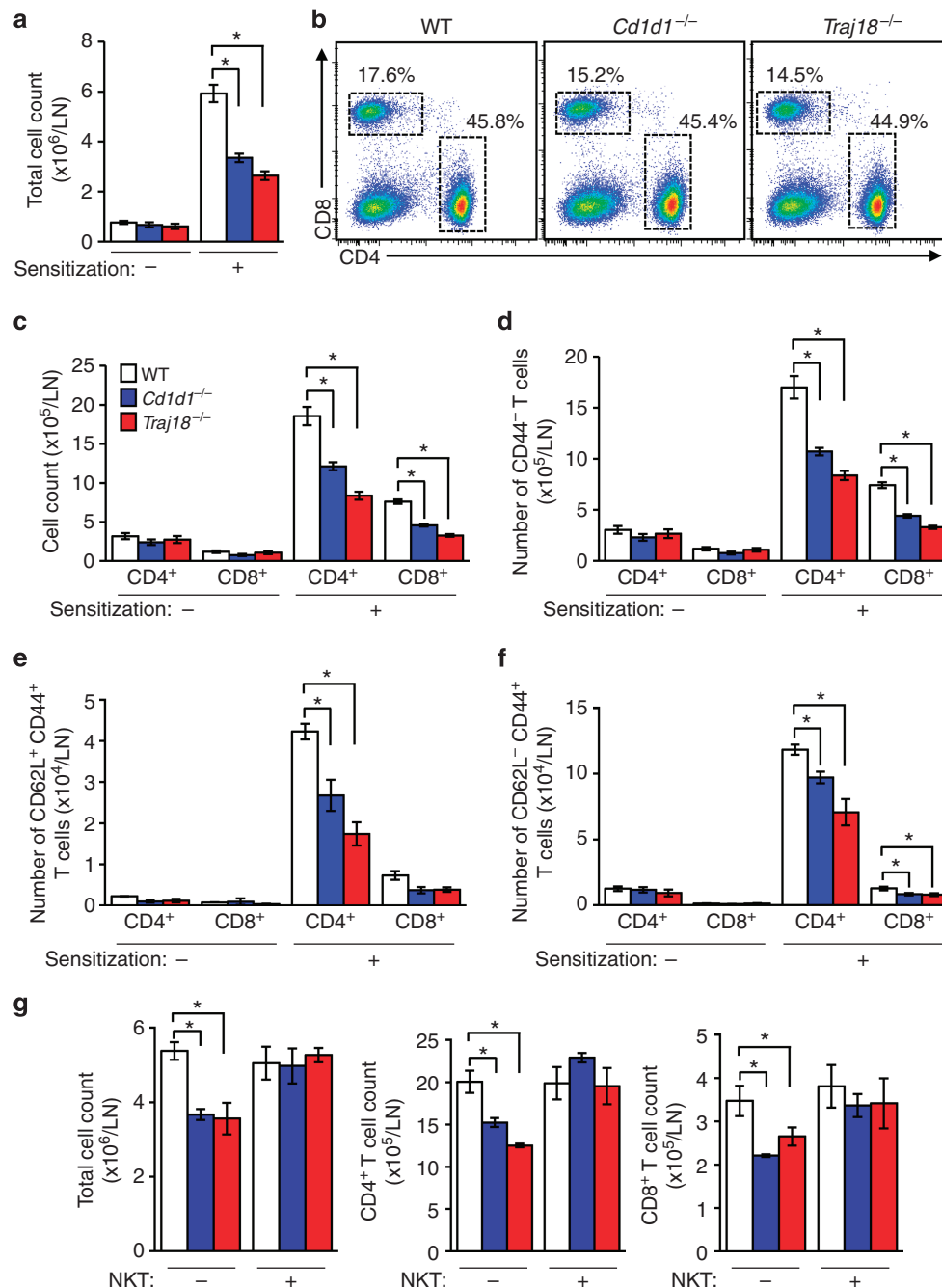


Figure 2. Impaired development of T-cell subset in $Cd1d1^{-/-}$ and $Traj18^{-/-}$ mice after sensitization. (a–f) Skin-draining axillary lymph node (LN) cells were collected 5 days after DNFB application to the abdomens of BALB/c wild-type (WT), $Cd1d1^{-/-}$, and $Traj18^{-/-}$ mice ($n=5$ per group). The number of total LN cells (a) and representative flow cytometric plots of LN cells (b) 5 days after sensitization are shown. The number of $CD4^+$ and $CD8^+$ T cells (c), $CD44^-$ naïve (d), $CD44^+$ $CD62L^+$ central memory (e), and $CD44^+$ $CD62L^-$ effector memory (f) subsets of $CD4^+$ and $CD8^+$ T cells are shown. (g) Natural killer T (NKT) cells purified from liver were transferred to BALB/c WT, $Cd1d1^{-/-}$, and $Traj18^{-/-}$ mice ($n=3$ per group) 1 day before application with FITC. LN cells were collected 3 days after FITC application to the abdomens. The number of total LN cells (g, left), $CD4^+$ T cells (g, middle), and $CD8^+$ T cells (g, right) are shown. All data are presented as the mean $\pm$ standard error of the mean (SEM) and are representative of three independent experiments with similar results. * $P<0.05$ versus corresponding mice.

of NKT cells, we adoptively transferred NKT cells into BALB/c WT or NKT cell-deficient mice. The decreased numbers of total cells, $CD4^+$, and $CD8^+$ T cells in the skin-draining LNs of the NKT cell-deficient mice were fully recovered by the

reconstitution of NKT cells (Figure 2g). These results suggest that NKT cells affected the proliferation of T cells and T-cell priming was restored by NKT cell reconstitution in NKT cell-deficient mice in the draining LNs during sensitization.

Impaired cytokine production in draining LNs of NKT cell-deficient mice after DNFB application

To evaluate the effect of NKT cells on T-cell proliferation and cytokine production, the skin-draining LN cells from BALB/c WT, *Cd1d1*^{-/-}, and *Tra18*^{-/-} mice were challenged in the presence or absence of 2, 4-dinitrobenzene sulfonic acid (DNBS) *in vitro*. The incorporation of [³H] thymidine was lower in NKT cell-deficient mice than in BALB/c WT mice (Figure 3a). In addition, the levels of IFN- γ , IL-13, and IL-17A in the culture supernatant in the presence of DNBS were markedly reduced in the LN cells of NKT cell-deficient mice compared with those of BALB/c WT mice (Figure 3b–e). These results indicate that cell proliferation and cytokine production of skin-draining LN cells are impaired in NKT cell-deficient mice.

Increased number and activation of NKT cells in the skin-draining LNs after sensitization

Next, we examined whether NKT cells accumulated in the skin-draining LNs and colocalized with DCs after sensitization. The number of NKT cells in the skin-draining LNs of BALB/c WT mice 5 days after sensitization was increased compared with that of BALB/c WT mice without sensitization (Figure 4a). As CD69 is known as an activation marker of NKT cells (Nishimura *et al.*, 2000), we analyzed the number of CD69⁺ NKT cells and the expression level of CD69 on NKT cells in the skin-draining LNs. Both the number of CD69⁺ NKT cells and the expression levels of CD69 on NKT cells in the skin-draining LNs after sensitization were increased compared with those of untreated BALB/c WT mice (Figure 4b).

Immunohistochemical studies showed that NK1.1⁺ T-cell receptor- β ⁺ NKT cells were localized in the vicinity of CD11c⁺ DCs in the paracortex area of the skin-draining LNs in CD11c-yellow fluorescent protein mice 5 days after sensitization (Figure 4c). To confirm this, we also showed that CD1d dimer⁺ NKT cells were close to CD11c⁺ DCs in the skin-draining LNs after sensitization (Figure 4d). These results suggest that activated NKT cells accumulate and colocalize with DCs in the draining LNs after sensitization.

Reduced number and maturation of DCs in the skin-draining LNs after FITC application in NKT cell-deficient mice

One of the essential steps in the sensitization phase of CHS is the migration and maturation of hapten-bearing cutaneous DCs into the skin-draining LNs. To assess the effect of NKT cells on DC migration and/or maturation, we used FITC-induced cutaneous DC migration as a model. Migrated DCs from the skin were detected as CD11c⁺ MHC class II⁺ FITC⁺ cells. Although the number of total DCs and CD11c⁺ MHC class II⁺ FITC⁺ Langerin^{+/+} DCs that migrated from the skin to the draining LNs 24 hours after FITC application was comparable between *Tra18*^{-/-} mice and BALB/c WT mice (Figure 5a), the number of DCs and CD11c⁺ MHC class II⁺ FITC⁺ Langerin^{+/+} DCs migrating into the draining LNs 72 hours after FITC application was significantly reduced in *Tra18*^{-/-} mice compared with BALB/c WT mice (Figure 5b). On the other hand, the number of CD11c⁺ MHC class II⁺ FITC⁻ resident DCs 24 and 72 hours after FITC application were comparable in *Tra18*^{-/-} mice and in BALB/c WT mice (Figure 5a and b). These reductions of migrated DCs were fully

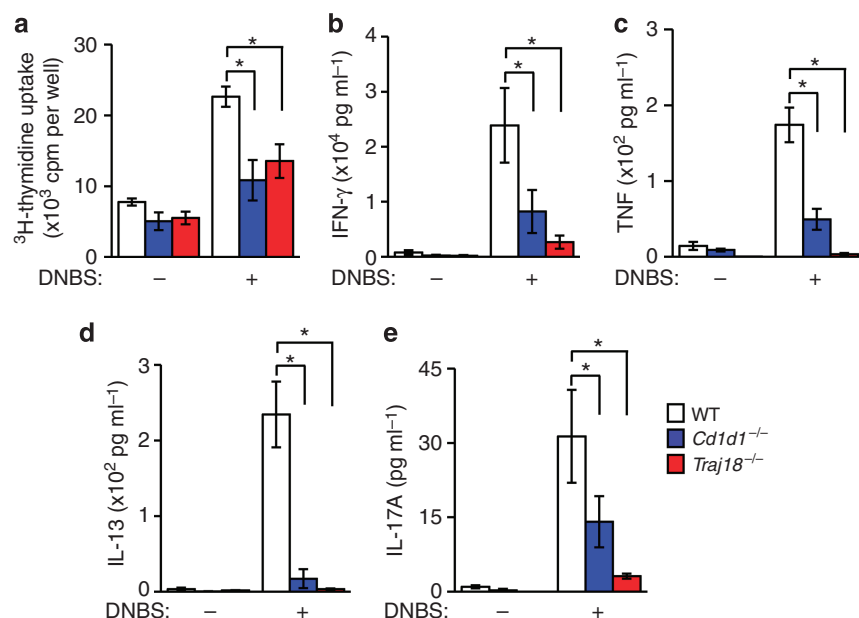


Figure 3. Reduced cell proliferation and cytokine production in the draining lymph nodes (LNs) of sensitized *Cd1d1*^{-/-} and *Tra18*^{-/-} mice. (a–e) 2, 4-Dinitrobenzene sulfonic acid (DNBS)-induced lymphocyte proliferation and cytokine production. Cells were collected from the LNs of BALB/c wild-type (WT), *Cd1d1*^{-/-}, and *Tra18*^{-/-} mice 5 days after DNFB application and cultured for 3 days with or without 100 μ g/ml DNBS. (a) Cell proliferation was measured by [³H] thymidine incorporation ($n=5$ mice per group). (b–e) The amount of IFN- γ , tumor necrosis factor (TNF), IL-13, and IL-17A in the culture medium was measured by ELISA (IFN- γ and IL-13) and cytometric bead array (TNF and IL-17A; $n=5$ mice per group). All data are presented as the mean $\pm$ standard error of the mean (SEM) and are representative of three independent experiments with similar results. * $P<0.05$ versus corresponding mice.

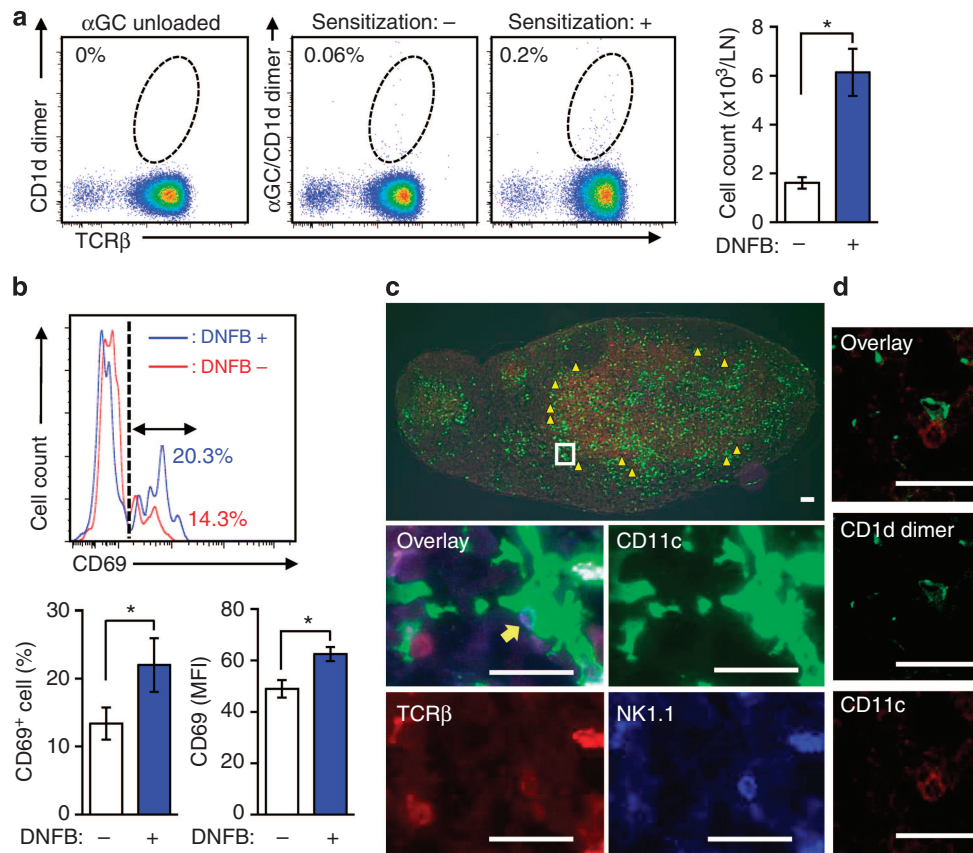


Figure 4. Increase in the number and activation of natural killer T (NKT) cells after sensitization. (a, b) Skin-draining axillary lymph node (LN) cells were collected 3 days after DNFB application on the abdomens of BALB/c wild-type (WT) mice ($n=5$ per group). NKT cells were identified by their expression of T-cell receptor- β (TCR β) and reactivity with the α -galactosylceramide (α GC)-loaded CD1d dimer (a, left panels), and the numbers of NKT cells in draining LNs after sensitization are shown (a, right panel). (b) The expression levels of CD69 on NKT cells are shown; the numbers below the bracketed lines indicate the percentage of CD69 $^{+}$ cells. The percentages of CD69 $^{+}$ NKT cells (lower left panel) and the expression levels of CD69 on NKT cells (lower right panel) are shown. (c, top) Visualization of double immunofluorescence for TCR β (red) and NK1.1 (blue) of LNs from DNFB-sensitized mice whose CD11c $^{+}$ dendritic cells (DCs) expressed enhanced yellow fluorescent protein (green). Bar = 100 μ m. (c, middle and lower) Enlargement of the outlined area above, CD11c (middle right), TCR β (lower left), NK1.1 (lower right), and showing the overlay of TCR β and NK1.1 (middle left). Bar = 25 μ m. NKT cells were localized in the vicinity of DCs (yellow arrowheads in the upper panel and yellow arrow in the middle left panel). (d) Visualization of α GC-loaded CD1d (d, middle), CD11c staining (d, lower), and double immunofluorescence for α GC-loaded CD1d dimer (green) and CD11c (red) (d, top) of LNs from DNFB-sensitized BALB/c mice. Bar = 25 μ m. All data are presented as the mean $\pm$ standard error of the mean (SEM) and are representative of three independent experiments with similar results. * $P<0.05$ versus corresponding mice. MFI, mean fluorescence intensity.

recovered by the reconstitution of NKT cells into NKT cell-deficient mice (see Supplementary Figure S2 online). Similar findings were observed in *Cd1d1* $^{-/-}$ mice (data not shown). These data suggest that NKT cells are able to promote the survivability of hapten-pulsed DCs.

We then analyzed the expression levels of co-stimulatory molecules on migrated DCs 72 hours after FITC application. The expression levels of CD40, CD80, and CD86 on CD11c $^{+}$ MHC class II $^{+}$ FITC $^{+}$ migrated DCs were lower in *Tra18* $^{-/-}$ mice than in BALB/c WT mice (Figure 5c).

Attenuated apoptotic cell death of DCs via TNF- α from activated NKT cells

The migrated and matured cutaneous DCs will die 48 hours after their arrival in the draining LNs (Winzler *et al.*, 1997; De Smedt *et al.*, 1998; Angeli *et al.*, 2006). However, the

mechanism of cell death after migration remains largely unclear. As shown in Figure 5a and b, the number of migrated DCs in NKT cell-deficient mice was reduced at 72 hours but not at 24 hours after FITC application. Therefore, we hypothesized that NKT cells promoted the survival of migrated DCs in the draining LNs. To test this hypothesis, we evaluated the effect of NKT cells on the survival of DCs *in vitro*. The live cells were determined as annexin V (AV) $^{-}$ propidium iodide (PI) $^{-}$ and the apoptotic cells as AV $^{+}$ PI $^{-}$ (Figure 6a). The number of total bone marrow-derived DCs (BMDCs) and AV $^{-}$ PI $^{-}$ live BMDCs were significantly increased and the number of AV $^{+}$ PI $^{-}$ apoptotic BMDCs was reduced by cocultivation with α -GalCer-activated NKT cells but not by nonactivated NKT cells (Figure 6a). These results suggest that activated NKT cells enhance the survivability of BMDCs.

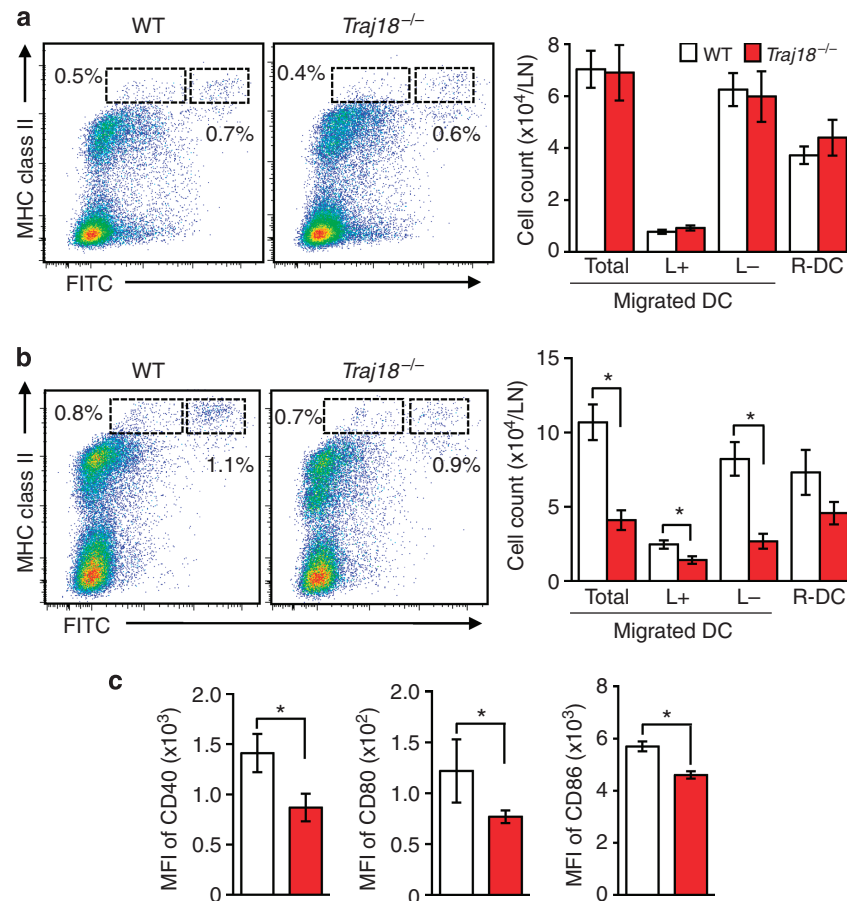


Figure 5. Reduced number and impaired maturation of dendritic cells (DCs) in natural killer T (NKT) cell-deficient mice. (a, b) The number of CD11c⁺ major histocompatibility complex (MHC) class II⁺ DC subsets in the draining lymph nodes (LNs) of BALB/c wild-type (WT) and *Traj18*^{-/-} mice (*n* = 5 per group) 24 hours (a) and 72 hours (b) after the application of FITC. (c) The expression levels of CD40, CD80, and CD86 on FITC⁺ CD11c⁺ MHC class II⁺ DCs of BALB/c WT and *Traj18*^{-/-} mice. All data are presented as the mean $\pm$ standard error of the mean (SEM) and are representative of three independent experiments with similar results. **P* < 0.05 versus corresponding mice. L, Langerin; MFI, mean fluorescence intensity; R-DC, resident DC.

TNF- α derived from DCs is known to promote the survival of DCs (Ludewig *et al.*, 1995; Lehner *et al.*, 2012). Therefore, we examined TNF- α expression in BMDCs and found that the number of TNF- α ⁺ BMDCs was increased by cocultivation with α -GalCer-activated NKT cells (Figure 6b). In addition, BMDCs secreted higher levels of TNF in the coculture with activated NKT cells compared with incubation with BMDCs or NKT cells alone (Figure 6b). On the other hand, there were only marginal TNF- α ⁺ NKT cells when compared with DCs in the single culture or the cocultivation with BMDCs (see Supplementary Figure S3 online). In addition, the expression levels of co-stimulatory molecules including CD40, CD80, and CD86 on BMDCs 48 hours after cocultivation with α -GalCer-activated NKT cells were markedly increased (Figure 6c).

To explain the ability of activated NKT cells to boost TNF production by BMDCs, we used the neutralizing anti-CD40 ligand (CD40L, CD154), ICAM-1 (CD54), IL-2, IFN- γ , or IL-4 antibody. The neutralizing anti-CD40L or anti-ICAM-1 antibody suppressed TNF production by BMDCs at least in part

(Figure 6d). On the other hand, the neutralizing anti-IL-2, IFN- γ , or IL-4 antibody did not suppress TNF production (see Supplementary Figure S4 online). Consistently, the neutralizing anti-CD40L and ICAM-1 antibodies but not anti-IL-2, IFN- γ , and IL-4 antibodies suppressed DC survivability enhanced by activated NKT cells (see Supplementary Figure S5a, b online).

The number of AV⁻ PI⁻ live BMDCs was significantly reduced by treatment with the neutralizing anti-TNF- α antibody, and the number of AV⁺ PI⁻ apoptotic BMDCs in the coculture with activated NKT cells was blocked by the neutralizing anti-TNF- α antibody (Figure 6e). To confirm the enhancement of DC survivability by activated NKT cells, we next used LN-derived DCs and cocultured with NKT cells activated with phorbol-12-myristate-13-acetate and ionomycin. The number of total LN-derived DCs and AV⁻ PI⁻ live LN-derived DCs was significantly increased by cocultivation with activated NKT cells. In addition, the number of AV⁻ PI⁻ live LN-derived DCs was significantly reduced by treatment with the neutralizing anti-TNF- α antibody (Figure 6f).

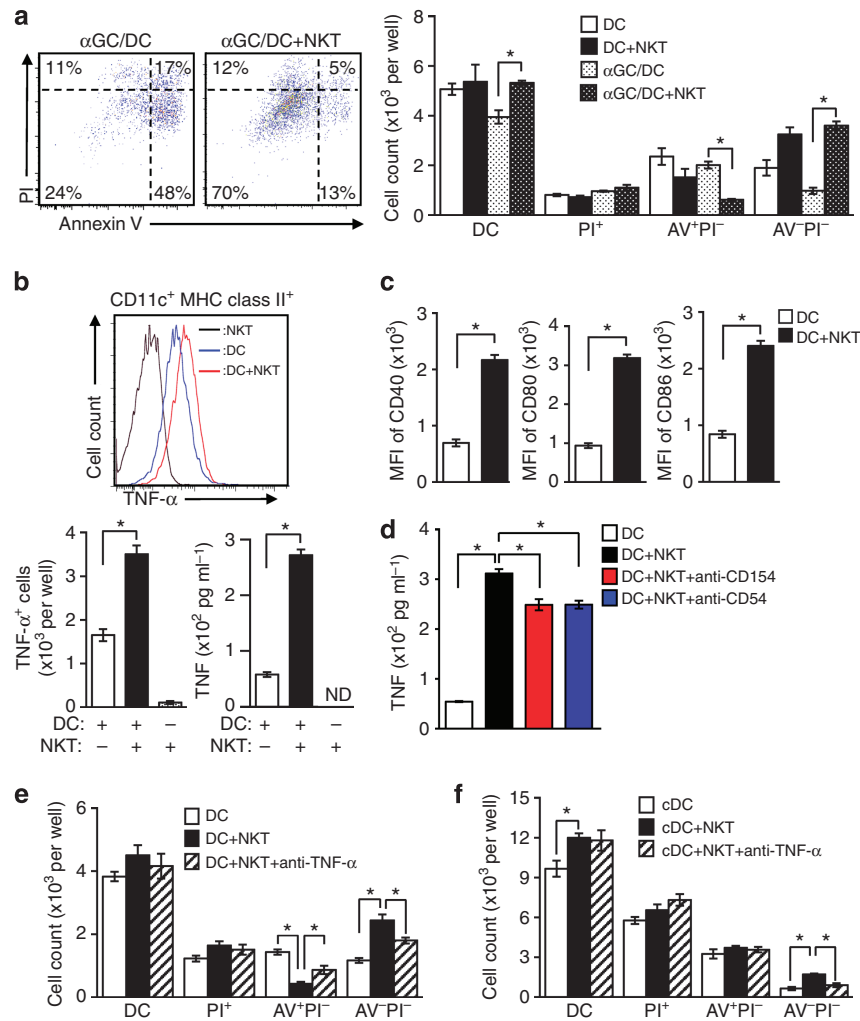


Figure 6. Reduced apoptotic cell death of dendritic cells (DCs) following culture with activated natural killer T (NKT) cells. (a) α -Galactosylceramide (α GC)-loaded or unloaded bone marrow-derived DCs (BMDCs) induced from C57BL/6 (B6) mice were incubated in medium alone (black dot columns or open columns, respectively), in the presence of NKT cells (white dot columns or black columns, respectively) for 48 hours. The expression of annexin V (AV) and propidium iodide (PI) gated on the CD11c⁺ major histocompatibility complex (MHC) class II⁺ population (left panels) and the number of total BMDCs, dead DCs (PI⁺ cells), apoptotic DCs (AV⁺PI⁻ cells), and viable DCs (AV⁻PI⁻ cells) are shown (right panel). (b) Flow cytometric analysis of tumor necrosis factor (TNF)- α cells after the incubation of α GC-loaded BMDCs with or without NKT cells, or NKT cells alone, and the amount of TNF in the culture medium as measured by cytometric bead array are shown. ND, not detected. (c) The expression levels of CD40, CD80, and CD86 on CD11c⁺ MHC class II⁺ BMDCs incubated with or without NKT cells activated with phorbol-12-myristate-13-acetate (PMA) and ionomycin for 48 hours. (d) The amount of TNF in the culture medium after incubation of BMDCs with or without NKT cells activated with PMA and ionomycin in the presence of the neutralizing anti-CD154 antibody, anti-CD54 antibody, or isotype control for 48 hours is shown. (e) BMDCs were incubated with or without NKT cells activated with PMA and ionomycin in the presence of anti-TNF- α antibody or isotype control for 24 hours and analyzed by flow cytometry. (f) DCs derived from draining lymph nodes (LNs) were incubated with or without NKT cells in the presence of anti-TNF- α antibody or isotype control for 48 hours and analyzed by flow cytometry. cDC, LN-derived DCs. All data are presented as the mean $\pm$ standard error of the mean (SEM) and are representative of three independent experiments with similar results. * P < 0.05 versus corresponding mice. MFI, mean fluorescence intensity.

We next examined whether activated hapten-specific T cells also enhanced DC survivability. We collected activated hapten-specific T cells from draining LNs 5 days after hapten application. The number of AV⁺PI⁻ DCs is decreased by cocultivation of activated hapten-specific T cells (see Supplementary Figure S6a online). On the other hand, cocultivation of nonactivated T cells did not decrease the number of AV⁺PI⁻ DCs (see Supplementary Figure S6b online). These findings suggest that activated hapten-specific T cells are also able to enhance DC survivability, but not T cells in the steady states *in vitro*.

DISCUSSION

We demonstrated herein that the CHS response was attenuated in *Cd1d1*^{-/-} and *Traj18*^{-/-} mice compared with that in BALB/c WT mice. In the draining LNs, the number of effector T cells and cytokine production was significantly reduced in NKT cell-deficient mice. NKT cells activated and colocalized with DCs in the draining LNs after sensitization. The number of migrated DCs in NKT cell-deficient mice was reduced at 72 hours but not at 24 hours after FITC application. In *in vitro* experiments, activated NKT cells enhanced BMDC survivability by promoting TNF production by BMDCs.

CD40L (CD154) and ICAM-1 (CD54) had a partial but a significant role in TNF production by DCs and DC survivability induced by cocultivation of NKT cells. This DC–NKT interaction has a pivotal role in the sensitization phase of CHS.

Consistent with our study, there are some reports that NKT cells have a critical inductive role during CHS (Campos *et al.*, 2003; Nieuwenhuis *et al.*, 2005; Askenase *et al.*, 2011). However, these studies focused on the role of NKT cells during the elicitation phase. Sensitization with picryl chloride (Campos *et al.*, 2003) or DNFB (Askenase *et al.*, 2011) rapidly activates hepatic NKT cells to release IL-4, which is instrumental for B1 B-cell production of hapten-specific immunoglobulin M, required for the elicitation of CHS in BALB/c mice. One possible reason that we did not find any differences during the elicitation phase of the CHS response between BALB/c WT and NKT cell-deficient mice is the difference in the protocol. In these reports, the role of NKT cells was analyzed by repeating sensitization at multiple sites with a high dose of haptens, instead of the usual CHS protocol, which is single hapten application (Otsuka *et al.*, 2011). Repeated hapten application is known to elicit a Th2-type response, which is used as an atopic dermatitis model (Otsuka *et al.*, 2013). Therefore, there is a possibility that the authors might have examined the role of NKT cells in the skin Th2 immune reaction.

On the other hand, other studies have shown that the CHS response is enhanced in NKT cell-deficient mice. It has been reported that NKT cells suppress the CD8⁺ T-cell response, which leads to an enhanced CHS response in NKT cell-deficient mice (Goubier *et al.*, 2013). The relative importance of these two opposite functions during CHS may depend on the mouse strain. The CHS response using DNFB is strongly impaired in BALB/c mice deficient in IL-4, B cells, or NKT cells (Askenase *et al.*, 2011), whereas it normally develops in C57BL/6 (B6) mice with these deficiencies (Goubier *et al.*, 2013). This indicates that NKT cells may have a pivotal role in CHS to DNFB in BALB/c but not in B6 mice.

We have previously reported that mast cells directly regulate DC activation in the dermis; indeed, the maturation and migration of DCs are regulated by TNF- α on mast cells (Otsuka *et al.*, 2011). Consistently, mast cell-associated TNF- α can make important contributions to the migration of hapten-bearing DCs during the initial stages of the sensitization phase of CHS (Suto *et al.*, 2006). In this study, we demonstrated that TNF- α derived from DCs via contact with activated NKT cells had an essential role in enhancing the survivability of DCs. Taken together, TNF- α had an essential role in the sensitization phase of CHS in the different steps. Consistent with these reports, the CHS response was markedly reduced in TNF-deficient mice (Pasparakis *et al.*, 1996).

NKT cells have dual functions not only during CHS but also for DC survivability. Repetitive antigen stimulation of NKT cells leads to increased production of IL-21, which leads to IL-21-dependent DC death (Wan *et al.*, 2013). In contrast, a single injection of α -GalCer into mice activates DCs, as reflected by increased MHC class II and IL-12 expression (Hermans *et al.*, 2003). Herein, we report that a single stimulation of NKT cells enhanced DC survivability, which

is the opposite result to that of repeated stimulation (Rogers *et al.*, 2004). This dual function of NKT cells for DC survivability might also be one of the possible reasons for the discrepancy in the role of NKT cells during CHS.

We demonstrated that activated hapten-specific T cells but not nonsensitized T cells were also able to enhance DC survivability *in vitro*. Therefore, *in vitro* properties of activated NKT cells to enhance DC survivability are not limited to this subset. We consider that activated lymphocytes (including NKT cells and conventional T cells) may promote DC survivability in a context-dependent manner. It might be intriguing to address this issue in the future study.

In summary, we demonstrated an important step involving NKT cells in the development of CHS in the draining LNs. Activated NKT cells promoted TNF- α expression in DCs, which enhanced the survivability of DCs. These NKT-DC networks in the draining LNs have an important role during the development of CHS.

MATERIALS AND METHODS

Animals

B6 and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). *Tra18-* and *Cd1d1-*deficient mice on a BALB/c background were kindly provided by Dr M. Taniguchi at RIKEN (Yokohama, Japan; Cui *et al.*, 1997) and Dr L. Van Kaer at Vanderbilt University School of Medicine (Nashville, TN, USA; Mendiratta *et al.*, 1997), respectively. In other experiments, we used CD11c-yellow fluorescent protein mice (kindly provided by Dr Nussenzweig; Lindquist *et al.*, 2004), the DCs of which express the yellow fluorescent protein. Eight- to ten-week-old female mice bred in specific pathogen-free facilities at Kyoto University (Kyoto, Japan) were used for all experiments. All experimental procedures were approved by the Institutional Animal Care and Used Committee of Kyoto University Graduate School of Medicine (Kyoto, Japan).

Reagents, antibodies, and intracellular staining

We purchased DNFB from Nacalai Tesque (Kyoto, Japan) and DNBS from Alfa Aesar (Ward Hill, MA). FITC-, PE-, PE-Cy7-, APC-, APC-7-, and Pacific Blue-conjugated anti-CD4, anti-CD8, anti-CD44, anti-CD62L, anti-T-cell receptor- β , anti-B220, anti-CD69, anti-NK1.1, anti-CD11c, anti-CD40, anti-CD80, anti-CD86, and anti-MHC class II were purchased from eBioscience (San Diego, CA), and Alexa Fluor 488 goat anti-mouse IgG1 was purchased from Invitrogen (Carlsbad, CA).

APC α -GalCer-loaded CD1d dimer (BD Biosciences, San Jose, CA) for NKT cell detection was prepared as previously described (Watarai *et al.*, 2008). For Langerin (CD207) staining, cells were fixed and permeabilized with cytofix/cytoperm solution (BD Biosciences) and stained with biotin-conjugated anti-Langerin antibody. For intracellular staining of TNF- α , isolated NKT cells and BMDCs were stimulated with 50 ng/ml phorbol-12-myristate-13-acetate and 1 μ M ionomycin in the presence of Golgi-stop (BD Biosciences) and stained with anti-TNF- α antibody using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions.

Samples were measured on an FACS Fortessa flow cytometer (BD Biosciences) using the FACSDiva software (BD Biosciences). Analysis was performed using the FlowJo software (Tree Star, Ashland, OR).

Histology and immunohistochemistry

The sections were stained with hematoxylin and eosin, and the histological scoring was evaluated as reported (Nakajima *et al.*, 2010). In brief, the samples were scored for the severity and character of the inflammatory response using a subjective grading scale. Responses were graded as follows: 0, no response; 1, minimal response; 2, mild response; 3, moderate response; and 4, marked response. The slides were blinded, randomized, and reread to determine the histology score. All studies were read by the same dermatopathologist using the same subjective grading scale. The total histology score was calculated as the sum of scores, including inflammation, neutrophils, mononuclear cells, edema, and epithelial hyperplasia.

For immunohistochemistry, the sections were fixed overnight in 4% paraformaldehyde, cryoprotected in 20% sucrose, and frozen in the frozen section compound FSC 22 (Leica, Wetzlar, Germany). The immunohistochemical staining experiments were performed by using PE-conjugated anti-mouse T-cell receptor- β (eBioscience), APC-conjugated anti-mouse NK1.1 (eBioscience), and PE-conjugated anti-mouse CD11c (eBioscience). Alexa 488 α -GalCer-loaded CD1d dimer for NKT cell detection was prepared as previously described (Watarai *et al.*, 2008). Fluorescence images were obtained using a BIOREVO BZ-9000 system (Keyence, Osaka, Japan).

Lymphocyte proliferation assay and cytokine production

RPMI 1640 (Sigma-Aldrich, Taufkirchen, Germany) containing 10% heat-inactivated fetal calf serum (Invitrogen), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (Mediatech, Manassas, VA), 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin was used as the culture medium.

For DNBS-dependent proliferation, single-cell suspensions were prepared from skin-draining axillary LNs of mice 5 days post sensitization with DNFB on the abdomen. One million LN cells were cultured with or without 100 μ g/ml sodium DNBS for 72 hours, pulsed with 0.5 μ Ci [3 H]thymidine for the last 24 hours, and subjected to liquid scintillation counting. For the measurement of cytokine production, the culture supernatants were collected 72 hours post incubation.

The amounts of IFN- γ and IL-13 were measured by ELISA (BD Biosciences). The amounts of TNF and IL-17A were measured using a cytometric bead array system (BD Biosciences) according to the manufacturer's instructions.

CHS protocol

Mice were sensitized with 25 μ l of 0.5% (w/v) DNFB in acetone/olive oil (4/1) or 3% oxazolone (Wako Pure Chemical Industries, Osaka, Japan) in ethanol on abdominal skin. On day 5, the ears were challenged by the application of 20 μ l of 0.3% DNFB or 1.5% oxazolone. For adoptive transfer, LN cells were prepared from the inguinal, axillary, brachial, and cervical LNs of one mouse sensitized with 50 μ l of DNFB on abdominal and dorsal skin and 20 μ l of DNFB on the ears 5 days previously. Whole T cells from LNs were purified magnetically by negative selection using a Pan T-cell isolation kit II (Miltenyi Biotech, Bergisch Gladbach, Germany) with auto-MACS and transferred intravenously into a mouse. The ears of these animals were challenged with 20 μ l of 0.5% DNFB 1 hour after the transfer, and the change in ear thickness was measured.

For the reconstitution assay, NKT cells from the liver of BALB/c mice were purified magnetically by positive selection using an Anti-APC Micro Beads (Miltenyi Biotech) after staining NKT cells with APC α -GalCer-loaded CD1d dimer ($>80\%$ expressed APC). 2×10^5 NKT cells were transferred intravenously into a mouse 1 day before sensitization with 25 μ l of 0.5% DNFB.

FITC-induced cutaneous DC migration assay

For FITC-induced cutaneous DC migration, mice were painted on the shaved abdomen with 100 μ l of 2% FITC (Sigma-Aldrich) dissolved in a 1:1 (v/v) acetone/dibutyl phthalate (Sigma-Aldrich) mixture. The number of cutaneous DCs that migrated into the draining LNs was enumerated by flow cytometry.

For the reconstitution assay, 2×10^5 NKT cells were transferred into a mouse 1 day before painting with 100 μ l of 2% FITC.

BMDC culture and preparation of LN-derived DCs

For BMDC induction, 5×10^6 bone marrow cells from B6 mice were cultured in 10 ml of medium supplemented with 10 ng/ml recombinant murine GM-CSF (PeproTech, London, UK) for 5 days using 10-cm tissue culture dishes (Kabashima *et al.*, 2007).

For the preparation of LN-derived DCs, LN cells were prepared from the axillary LNs sensitized with 25 μ l of 0.5% DNFB on abdominal skin 3 days previously. DCs from LNs were purified magnetically by positive selection using CD11c microbeads (Miltenyi Biotech) with auto-MACS ($>70\%$ expressed CD11c).

Coculture of DCs with NKT cells or T cells and detection of DC apoptosis

BMDCs were loaded with 100 ng/ml α -GalCer for 6 hours and cocultured at a density of 5×10^4 DCs in 200 μ l per well in a 96-well microplate at a DC:NKT and DC:T ratio of 1:1 for 48 hours. For the preparation of LN-derived T cells, LN cells were prepared from the axillary LNs sensitized with or without 25 μ l of DNFB on abdominal skin 5 days previously. T cells from LNs were purified using a Pan T-cell isolation kit II as described above. For the purification of NKT cells, liver mononuclear cells were harvested from B6 mice and NKT cells were purified with the auto-MACS cell purification system using the NK1.1 $^+$ iNKT cell isolation kit (Miltenyi Biotech). The purity of NKT cells was more than 80%. For inhibition assays, BMDCs were cocultured with NKT cells, which were stimulated with phorbol-12-myristate-13-acetate (2 ng/ml) and ionomycin (0.5 μ M; Sigma-Aldrich) for 3 hours, in the presence of 10 μ g/ml isotype control antibody (Rat IgG1, eBioscience), 20 μ g/ml anti-CD154 antibody (MR1, eBioscience), 20 μ g/ml anti-CD54 antibody (YN1/1.7.4, eBioscience), 10 μ g/ml anti-IL-2 antibody (JES6-1A12, eBioscience), 10 μ g/ml anti-IL-4 antibody (11B11, eBioscience), 10 μ g/ml anti-IFN- γ antibody (AN-18, eBioscience), or 10 μ g/ml anti-TNF- α antibody (MP6-XT22, eBioscience). DCs were then examined for cell survival and apoptosis by flow cytometry of AV/PI (eBioscience)-stained cells.

Statistical analysis

Unless otherwise indicated, the data are presented as the mean $\pm$ standard error of the mean and are representative of at least three independent experiments. *P* values were calculated with the Wilcoxon signed-rank test. *P* values <0.05 were considered to be significantly different and are marked by an asterisk in the figures.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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