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Natural killer cell-mediated contact sensitivity develops rapidly and depends on interferon-α, interferon-γ and interleukin-12

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Summary

Natural killer (NK) cell-mediated contact sensitivity was recently described in mice. Here, we confirm NK cell-mediated contact sensitivity (CS) in SCID and RAG1$^{-/-}$ mice but not in SCID$_{beige}$ mice, which have non-functional NK cells that lack NK cell granules. NK cell-mediated CS was transferred by liver mononuclear cells and the DX5^{+} fraction of liver cells, confirming that NK cells mediate CS in the absence of T and B cells. Participation of NKT cells and B-1 cells was ruled out using Jα18$^{-/-}$ and JH$^{-/-}$ mice, respectively. Remarkably, NK cell-mediated CS was observed just 1 hr after immunization and was detectable as early as 30 min after challenge. Further, we examined cytokine requirements for NK cell-mediated CS, and found that liver mononuclear cells from interleukin-12$^{-/-}$, interferon-γ$^{-/-}$ and interferon-α receptor$^{-/-}$ donors fail to transfer NK cell-mediated CS to naive hosts. Our studies clearly show that dinitrofluorobenzene sensitized NK cells mediate very rapid, antigen-specific cell-mediated immunity, with features of both innate and acquired immune responses.

Keywords: contact sensitivity; cytokines; liver mononuclear cells; natural killer cells.

Introduction

Contact sensitivity (CS) responses to contact sensitizing haptens such as picryl chloride, oxazolone or dinitrofluorobenzene (DNFB) are classical manifestations of T-cell-mediated immunity *in vivo*.[1,2] In CS, the skin is the site of primary immunization induced by contact painting with a concentrated chemically reactive hapten that conjugates to host antigens in the skin. This leads to trafficking of hapten–self-peptide antigen complexes on antigen-presenting cells to the draining lymph nodes where αβ T cells bearing T-cell receptors reactive to the hapten–self complexes are activated and expanded within 4–5 days.[3] Hapten-specific, memory αβ T cells are also generated, and persist for long periods of time in sensitized hosts. Upon secondary exposure to the hapten in dilute solution, usually by challenge on the ear-skin in mice on days 4 or 5, circulating antigen/self-reactive T cells are recruited to these sites of antigen challenge and are stimulated by antigen–self MHC complexes on local antigen-presenting cells.[4] Reactivated hapten-specific T cells then elicit effector functions and inflammation in T helper type 1 (Th1) CS via interferon-γ (IFN-γ) secretion, and in cytotoxic T type 1 (Tc1) CS via local cytotoxicity and IFN-γ release.[5] Interleukin-17 (IL-17) -producing Th17 cells can also be involved in hapten-induced CS.[6]

Apart from αβ T cells, innate-like lymphocytes are also involved in both Th1 and Tc1 CS.[7–10] It was previously shown that γδ T cells play an important role in CS by assisting effector αβ T cells,[10] and that B-1 and natural killer (NK) T lymphocytes are required for elicitation of CS by mediating an early component required for elicitation of the responses.[11]

Recently, NK cell-mediated CS has been described for DNFB, oxazolone, and trinitrophenyl chloride (TNP-Cl),[12] and we have subsequently demonstrated that NK cell-mediated antigen-specific memory responses can be expanded to virally encoded antigens.[13] Altogether, CS responses were observed in immunized mice lacking αβ T cells, γδ T cells and B cells,[12] and were dependent on

CXCR6$^+$ Thy1$^+$ hepatic NK cells.[12,13] This NK cell-mediated CS was elicited in SCID and Rag$^{-/-}$ mice, and could be transferred by NK cells isolated from livers, but not spleens of sensitized donor mice. Remarkably, NK cell-mediated CS had long-lived memory, antigen specificity and featured local accumulation of NK cells into sites of challenge.[12,13] Accumulation of NK cells in the challenged skin was determined by immunofluorescence microscopy and flow cytometry of skin-infiltrating leucocytes.[12] Moreover, NK cell recruitment depends on hepatic CXCR6,[13] and on the ability of NK cells to adhere to P-selectin and E-selectin in dermal microvessels.[12]

Here, we confirm the presence of NK cell-mediated CS elicited in SCID, but not in SCID$_{beige}$ mice, and in RAG$^{-/-}$ mice that are devoid of T or B cells. We further demonstrate that NK cell-mediated CS is transferable with liver mononuclear cells (LMNC), as long as donors were able to produce IFN-α, and capable of IL-12 and IFN-γ production. Remarkably, we show that NK cell-mediated CS is elicited as early as 1 hr following DNFB contact sensitization. Therefore, in normal mice, hapten-specific NK cells are present and ready to respond to sensitization for this CS. Moreover, the inflammatory response mediated by liver NK cells, which begins immediately after challenge, lasts longer than classical Th1 or Tc1 mediated CS.

Materials and methods

Mice

Specific pathogen-free female BALB/c, Rag1$^{-/-}$, SCID and SCID$_{beige}$, IL-4$^{-/-}$, IFN-$\gamma^{-/-}$ and IFN-αR$^{-/-}$ mice (BALB/c background) and CBA/J were obtained from The Jackson Laboratory, Bar Harbor, ME. Breeders of pan-B-cell-deficient JH$^{-/-}$ mice (CB.17, H-2^d) were kindly provided by Mark Shlomchik of Yale University School of Medicine. Breeders of invariant NKT cell-deficient Jα18$^{-/-}$ mice (BALB/c background) were from Masaru Taniguchi, Chiba City, Japan. Additionally, IL-12$^{-/-}$ mice were from Taconic, New York, NY. The IL-13$^{-/-}$ mice were obtained from Dr Andrew McKenzie via Dr Kim Bottomly at Yale. In some experiments, BALB/c mice were from the breeding unit of the Department of Medical Biology, Jagiellonian University College of Medicine. Mice were rested for at least 1 week before use, maintained under specific pathogen-free conditions, and used at 6–10 weeks of age in groups of four to five mice. All experiments were conducted according to guidelines of Jagiellonian University College of Medicine, Yale University School of Medicine and Harvard Medical School.

In some experiments C57BL/6, *Rag1*$^{-/-}$ (C57BL/6), *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (C57BL/6×C57BL/10 F$_1$)[14] and *Itgam*$^{-/-}$ (C57BL/6) mice were used at 6–12 weeks of age (Taconic, Jackson and Charles River Laboratories) according to the institutional animal committees at Harvard Medical School.

Reagents

Dinitroflurobenzene was obtained from Sigma, St Louis, MO; TNP-Cl from Chemica Alta, Edmonton, Canada; Percoll from Amersham Biosciences AB, Uppsala, Sweden; anti-mouse NK cell (DX5) microbeads from Miltenyi Biotec, Auburn, CA and FITC-conjugated DX5 monoclonal antibody was from BD Biosciences, San Jose, CA.

Immunization and elicitation of CS

Mice were contact sensitized with 25 μl 0·5% DNFB in acetone and olive oil (4 : 1) on the shaved skin. Five days later, CS responses were elicited by painting both ears with 5 μl 0·1% DNFB in acetone and olive oil (4 : 1).[15] Ear thickness was measured before challenge using a micrometer (Mitutoyo, Tokyo, Japan) by an observer unaware of the experimental groups, and then 24-hr after challenge. Increased ear thickness was expressed in μm $\pm$ SD.

Adoptive transfer of CS with lymph node and spleen cells

Donors of CS-immune effector cells were contact sensitized with 150 μl 5% TNP-Cl or 25 μl 0·5% DNFB in acetone and olive oil (4 : 1) on the shaved skin. Immune axillary and inguinal lymph nodes and spleens were harvested on day '+4' or '+5', respectively, and 7×10^7 immune cells were injected intravenously into normal syngeneic recipients (positive transfer). Mice were challenged with 10 μl 0·4% TNP-Cl or 5 μl 0·1% DNFB in acetone and olive oil (4 : 1) after cell transfer and tested for CS at various time-points. The mice that did not receive any cell transfer but that were challenged only served as negative controls. Increased ear thickness was expressed in μm $\pm$ SD.

Adoptive transfer of CS with LMNC or purified DX5$^+$ cells

Mice were sensitized with 0·5% DNFB and livers were isolated at different time-points after immunization. Liver mononuclear cells were isolated on Percoll gradient. Then, unseparated LMNC or purified DX5$^+$ NK cells were transferred intravenously into naive syngeneic recipients that were challenged the day after transfer and tested for CS.

Liver mononuclear cell and NK cell isolation

After killing the mouse, the liver was perfused with PBS via the portal vein until opaque, then strained (70 μm;

BD Biosciences), resuspended to 40% isotonic Percoll (Amersham Biosciences) and overlaid onto 60% isotonic Percoll. After centrifugation for 20 min at 900 ***g*** at 25°, the LMNC were isolated at the interface and 40% Percoll combined, and washed with RPMI-1640 (Invitrogen Life Technologies) + 5% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA). Viability was > 90%.

To isolate a pure population of NK cells, LMNC were purified with the use of anti-NK (DX5) microbeads (Miltenyi Biotec) as described by the manufacturers, or were sorted using a BD Bioscience FACSAria cell sorter.

To phenotype NK cells involved in CS, LMNC were stained using NK1.1, CD3, CD11b, CD11c, CD27, CD45, B220, CD90 and Ly49C/I (BD Pharmingen, Biolegend and eBiosciences), and FACS samples were acquired on a BD FACS CANTO and analysed using FlowJo software. Cell sorting was carried out on a BD FACS ARIA using Diva software, and cell purity for all experiments was > 98%.

Intracellular IFN-γ

B cells were left naive or incubated in 20 mg/ml dinitrobenzene sulphonic acid (DNBS) in 1× PBS for 10 min at room temperature in the dark, and washed twice with PBS containing 10% fetal bovine serum. Rag1$^{-/-}$ donor mice were sensitized with 50 μl 0·5% DNFB in acetone, or mock sensitized with 50 μl acetone on days 0 and 1 on the shaved abdomen, and Thy1^{+} CXCR6^{+} NK cells were sorted from livers or spleens at day 4 and co-cultured with DNBS-labelled B cells (100 B:1 NK) for 15 hr in the presence of 10 μg/ml anti-CXCR6 or anti-CXCL16 monoclonal antibody or isotype control. BD GolgiStop containing Monensin was added according to the manufacturer's protocol for the last 10 hr of culture. The NK cells were identified as NK1.1^{+} Thy1^{+} and CXCR6^{+} and FACS analysed for intracellular IFN-γ using flow cytometry. Data are representative of two independent experiments with 10–15 donor mice, three to six wells/group.

Statistics

Data in graphs are shown as mean ± SD. Analysis of variance followed by Student's *t*-test was used for multiple comparisons. Statistical significance was set at $P < 0{\cdot}05$.

Results

Liver NK cells mediate DNFB-induced CS

We tested NK cell-mediated CS in DNFB contact-sensitized wild-type mice. BALB/c mice were compared with SCID versus SCID$_{beige}$ mice on a BALB/c background. On day 5 after DNFB sensitization, ear challenge with DNFB in wild-type BALB/c mice elicited the classical 24-hr CS effector response (Fig. 1a, Group B), compared with naive identically challenged BALB/c controls (Group A).

In comparison, SCID mice, devoid of T and B cells, but with NK cells, also elicited significant 24-hr ear swelling (Group D), compared with naive ear-challenged SCID mice (Group C). Importantly, identically immunized SCID$_{beige}$ mice lacking T, B and NK cells that have no granules elicited no CS response (Groups E and F).

To confirm the data in Fig. 1(a), we performed an adoptive transfer experiment where Rag1$^{-/-}$ mice were used as donors of DNFB immune LMNC. Figure 1(b) shows that DNFB-sensitized Rag$^{-/-}$ LMNC, devoid of T and B cells, transferred CS responses to naive BALB/c mice, and did so as efficiently as wild-type LMNC (Groups C and B). This observation was confirmed by two further experiments showing that both magnetic bead-sorted (Fig. 1c, Group C) and FACS-sorted DX5^{+} liver NK cells (Fig. 1d) transferred CS, whereas NK-depleted DX5^{-} LMNC failed to transfer CS responses to naive hosts. Interestingly, as few as 4500 transferred NK cells sufficed to induce significant ear swelling (Fig. 1d, Group D versus Group A). We concluded that hepatic NK cells are required and sufficient to mediate DNFB-specific CS responses in Rag$^{-/-}$ or SCID mice at quite low numbers of cells.

Rapid activation of liver NK cells

We hypothesized that NK cells might be activated quickly after immunization, as NK cells are a component of the innate immune system. To determine the time required for NK cell activation, LMNC were isolated and transferred 4, 2 or 1 day (Fig. 2a) or 1 hr after DNFB sensitization (Fig. 2b). Interestingly, NK cells from pan-immunoglobulin-deficient mice transferred DNFB 24-hr CS responses as early as 1-day after immunization (Fig. 2a, Group D) and NK cells elicited these adoptive CS responses even only 1-hr after immunization (Fig. 2b, Group C) compared with non-immune mice (Group A). Additionally, employment of JH$^{-/-}$ mice as LMNC donors ruled out any involvement of B cells that are required in classical CS to mediate an initiating phase required to subsequently recruit the CS-effector T cells.[8,9] To prove that indeed NK cells but not NK-depleted LMNC are promptly activated to play their CS effector function, the following experiment with MACS NK-sorted cells was performed. Data presented in Fig. 2(c) show that NK cells isolated from donor mice 2 days or even 1 hr after DNFB sensitization efficiently transfer CS (Groups B and D versus A). The NK-depleted LMNC did not transfer CS (Fig. 2c, Groups C and E versus A).

Rapid elicitation of NK cell-mediated CS

We previously showed that maximal classical CS responses in adoptively sensitized recipients are obtained

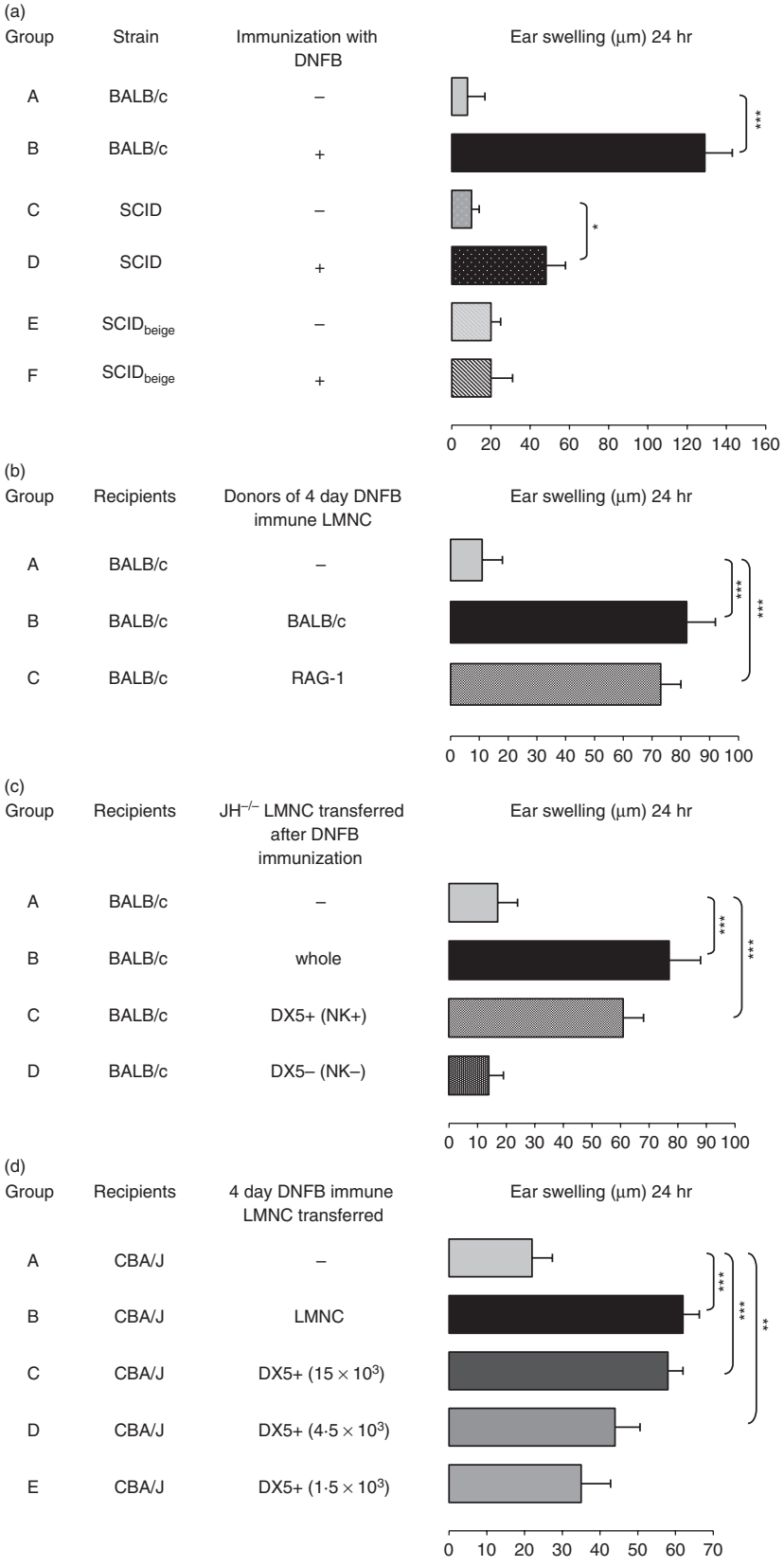


Figure 1. Dinitrofluorobenzene (DNFB) -immune liver natural killer (NK) cells adoptively transfer contact sensitivity (CS). (a) Separate groups of wild-type BALB/c and H-2^d SCID and $SCID_{beige}$ mice were contact sensitized by painting the shaved abdomen with 0·5% DNFB (Groups B, D and F). Five days later CS responses were elicited by challenging the ears with 0·1% DNFB. Naive mice of each type also were similarly challenged (Groups A, C and E). Ear swelling was tested 24 hr after challenge. Results shown as mean ± SD. $n = 8$. $^*P \leq 0{\cdot}05$, $^{***}P \leq 0{\cdot}001$. (b) BALB/c recipients were transferred with 5×10^5 4-day DNFB immune liver mononuclear cells (LMNC) from BALB/c (Group B) or H-2^d $RAG1^{-/-}$ mice (Group C). Mice were challenged next day after cell transfer and tested for CS. Results shown as mean ± SD. $n = 8$. $^{***}P \leq 0{\cdot}001$. (c) BALB/c recipients were transferred with 5×10^5 4-day DNFB immune LMNC from $JH^{-/-}$ mice (Group B) or 1×10^5 $DX5^+$ liver NK or 5×10^5 $DX5^-$ MACS-sorted cells, challenged the next day, and tested for CS. Results shown as mean ± SD. $n = 8$. $^{***}P \leq 0{\cdot}001$. (d) CBA/J recipients were transferred with 5×10^5 4-day DNFB immune LMNC from CBA/J mice (Group B) or decreasing numbers of FACS-sorted $DX5^+$ liver cells (Groups C–E). Next day, animals were challenged and tested for CS. Results shown as mean ± SD. $n = 8$. $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$.

by delaying challenge until 1 day after adoptive transfer to allow manifestations of the early initiating component.[11] Here, we tested whether cell transfer of NK cell-mediated CS with DNFB-immune NK cells was similar. Data presented in Fig. 2(d) showed that challenge need not be delayed and recipients of DNFB-immune NK cells could be challenged immediately to elicit the 24-hr component of NK cell-mediated CS. Hence, NK cells isolated from sensitized donor liver are immediately ready to elicit NK-transferred NK cell-mediated CS as shown in the BALB/c mice (Fig. 2d, Group B) do not require the B cell-mediated initiating phase. In fact, data presented in Fig. 2(e) showed that NK cell-mediated CS is detectable as early as 30 min after challenge, and that ear swelling can be observed for much longer than in classical T-cell-mediated CS experiments (Fig. 2f).

Mature, licensed mouse liver NK cells mediate CS

We and others have previously shown that $NKG2D^+$ $Thy1^+$ $Ly49CI^+$ NK cells isolated from livers, but not from spleens, of sensitized donor mice transfer CS responses to naive hosts.[12,13,16,17] Additionally, we recently showed that NK cell-expressed CXCR6 is required for NK cell-mediated CS.[13] Thy1 is expressed on about 60% of all murine NK cells,[12] and here, we further subdivided the $Thy1^+$ subset of hepatic (Fig. 3a) and splenic (Fig. 3b) NK cells using cell surface expression of Ly49C/I, CD11b, CD27, B220 and CD11c. CD27 is expressed earlier in NK cell development than CD11b, and CD11c and B220 are expressed on mature, activated NK cells.[18,19] It has previously been shown that immunological memory can be transferred using $Thy1^+$ Ly49C/I^+ NK cells, and we now compared the ability of $CD27^+$, immature NK cells to that of mature CD27-$CD11b^+$ NK cells to transfer CS to naive hosts.

To this end, we transferred FACS-sorted hepatic NK cells of the indicated phenotypes and genotypes into lymphocyte-deficient $Rag^{-/-}$ $IL2R\gamma^{-/-}$ recipients, and challenged recipient mice 1 day after transfer to determine their CS responses (Fig. 5c). As published previously, $Thy1^-$ or $Thy1^+$ Ly49C/I^-, unlicensed NK cells, or naive $Thy1^+$ NK cells did not transfer CS to naive hosts[12] (Fig. 3c). Interestingly, immature CD27-expressing $Thy1^+$ NK cells failed to transfer CS to naive hosts, while mature $Thy1^+$ NK cells, which expressed Mac-1 but not CD27, transferred CS to naive recipients (Fig. 3c). It has previously been shown that treatment of $Rag2^{-/-}$ with L-selectin-specific blocking antibody before sensitization attenuates hapten-specific CS responses,[12] suggesting that NK cells may also be primed in lymph nodes. To emigrate into DNFB challenged skin, rolling NK cells may engage integrin β_2 (CD11–CD18) to arrest and emigrate into inflamed tissues, as blocking antibodies specific for either the endothelial selectins or CD18 abrogated CS responses.[12] Therefore, CD11b may not simply be a maturation marker on NK cells, but it may be important for the migration of memory NK cells during CS. Indeed, the transfer of *itgam*-deficient NK cells from DNFB sensitized donor livers into naive host failed to transfer CS (Fig. 3c). Last, we examined the ability of 'natural killer dendritic cells', which have been identified as a subset of mature, activated NK cells,[18,19] to mediate memory responses upon adoptive transfer, by comparing the memory capacity of $Thy1^+$ $B220^+$ $CD11c^+$ NK cells to that of $Thy1^+$ $B220^+$ $CD11c^-$ NK cells, after isolation from livers of sensitized mice. Both subsets transferred CS responses equally well to naive hosts, albeit they did so slightly less than licensed ($Thy1^+$ Ly49C/I^+) or fully mature ($Thy1^+$ Mac-1^+) NK cells. This may be because of the heterogeneity in immature, $CD27^+$ and mature, Mac-1^+ NK cells contained within this population (Fig. 3). Hence, $Thy1^+$ licensed mature NK cells, but not unlicensed, immature NK cells, or $Thy1^-$ NK cells mediate CS responses to DNFB in $H2^b$ mice. Splenic NK cells, which also contain a $Thy1^+$ subset of NK cells that can be further subdivided by the aforementioned markers, never transferred immunological memory into naive hosts.[12,13]

IFN-γ, IL-12 and IFN-α are required for NK cell-mediated CS

In classical CS, local IFN-γ and IL-12 are involved in the mechanism of inflammation.[5] To examine if NK cell-mediated CS likewise depends on these cytokines, we

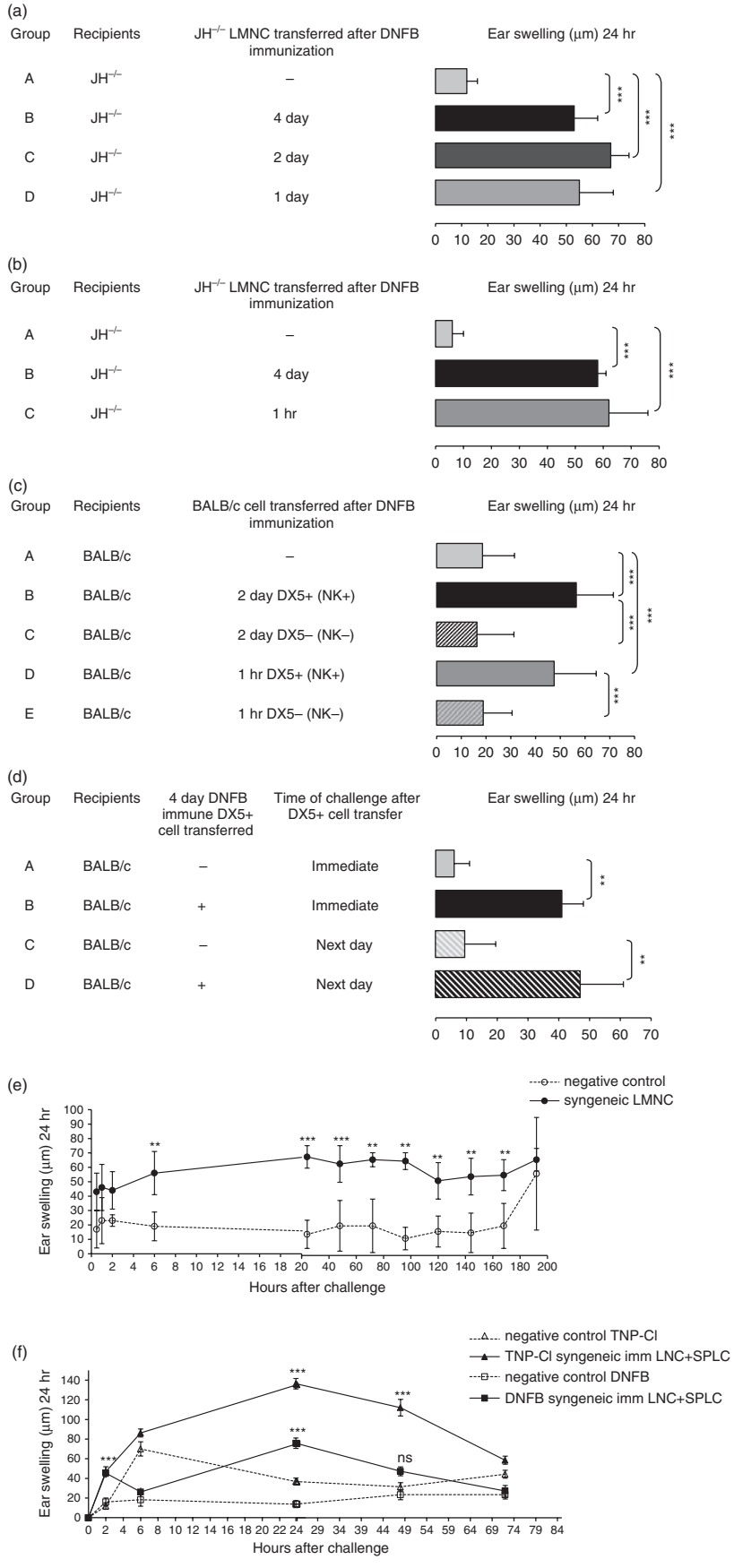


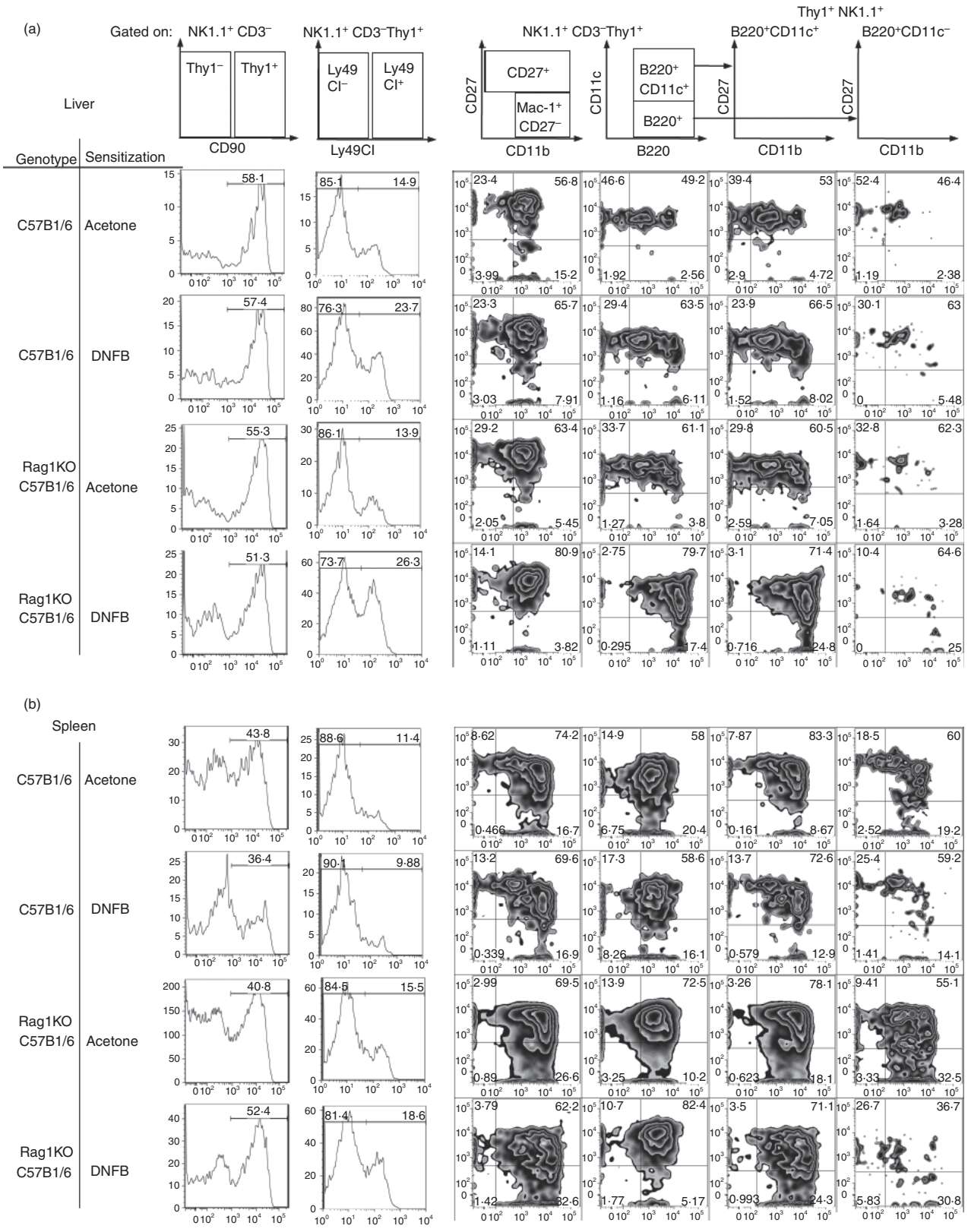
Figure 2. Liver natural killer (NK) cells are ready to transfer contact senstivity (CS) within 1 hr after dinitrofluorobenzene (DNFB) sensitization. (a) 5×10^5 4-day, 2-day or 1-day DNFB immune liver mononuclear cells (LMNC) from $JH^{-/-}$ (Groups B, C and D, respectively) mice were transferred into $JH^{-/-}$, recipients and 1-day later CS responses were elicited by ear challenge. Results shown as mean ± SD. $n = 8$. $^{***}P \leq 0{\cdot}001$. (b) 5×10^5 4-day or 1-hr DNFB immune LMNC from $JH^{-/-}$ (Groups B and C, respectively) mice were transferred into $JH^{-/-}$, recipients and 1-day later CS responses were elicited by ear challenge. Results shown as mean ± SD. $n = 8$. $^{***}P \leq 0{\cdot}001$. (c) 15×10^4 2-day or 5×10^4 1-hr DNFB immune MACS-sorted $DX5^+$ liver cells (Groups B and D, respectively) or 5×10^5 2-day or 5×10^4 1-hr DNFB immune NK-depleted LMNC (Groups C and E, respectively) from BALB/c mice were transferred into BALB/c recipients and 1-day later CS responses were elicited by ear challenge. Results shown as mean ± SD. $n = 14$. $^{***}P \leq 0{\cdot}001$. (d) BALB/c recipients were transferred with 5×10^5 4-day DNFB immune MACS sorted $DX5^+$ liver NK cells (Groups B and D respectively) from BALB/c donors. Recipients and control naive mice were challenged immediately (Groups A and B) or next day after transfer (Groups C and D). Results shown as mean ± SD. $n = 12$. $^{**}P \leq 0{\cdot}01$. (e) BALB/c recipients were transferred with 9×10^5 4-day DNFB immune LMNC from BALB/c donors and then challenged and tested for CS at different time-points. Results shown as mean ± SD. $n = 8$. $^{*}P < 0{\cdot}5$, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$. (f) 7×10^7 4-day TNP-Cl or 5-day DNFB immune lymph node and spleen cells from CBA/J donors were injected intravenously into normal syngeneic recipients (positive controls). Mice were challenged with 10 μl of 0·4% TNP-Cl or 5 μl of 0·1% DNFB in acetone and olive oil (4 : 1) after cell transfer and tested for CS at various time-points. The mice that did not receive any cell transfer but were challenged only served as negative controls. Increased ear thickness was expressed in μm ± SD. $n = 12$. $^{***}P \leq 0{\cdot}001$.

immunized IFN-$\gamma^{-/-}$ and IL-$12^{-/-}$ mice with DNFB and transferred their LMNC to naive BALB/c recipients. Elicited 24-hr responses were reduced significantly in transfers from IFN-$\gamma^{-/-}$ and IL-12p$40^{-/-}$ mice (Fig. 4a, Groups D and E) compared with cell transfer from $JH^{-/-}$ donors (Group B). Additionally, transfer of LMNC from $Ja18^{-/-}$ mice ruled out any involvement of NKT cells in these experiments (Group C).

The requirement for IL-12p40 suggests that either IL-12, or IL-23 is a required cytokine for NK cell-mediated CS responses. To distinguish between these two possibilities, we transferred $DX5^+$ liver NK cells from IL-12p$35^{-/-}$ and IL-12p$40^{-/-}$ DNFB sensitized donors and assessed CS responses upon challenge of recipient mice. As shown in Fig. 4(b), $DX5^+$ liver NK cells from both IL-12p$35^{-/-}$ and IL-12p$40^{-/-}$ DNFB sensitized donor mice fail to transfer CS to naive recipients. Our data clearly show that IL-12 is required for NK cell-mediated CS responses. It has recently been shown that IL-12 is indispensible for mouse cytomegalovirus-specific NK cell expansion and generation of memory NK cells in a signal transducer and activator of transcription 4-dependent mechanism.[20] So, we first provide evidence that IL-12 is also crucial in the development of memory to a non-viral antigen, DNFB, in BALB/c mice. In contrast, IL-$4^{-/-}$ or IL-$13^{-/-}$ DNFB immune LMNC transferred CS responses to naive hosts (Fig. 4c, Groups D and E) at a similar level to LMNC isolated from $JH^{-/-}$ or BALB/c mice (Fig. 4c, Groups B and C), indicating that neither cytokine is required for NK cell-mediated CS responses. Finally, experiments employing IFN-α$R^{-/-}$ mice as NK cell donors showed that IFN-α-mediated signalling is involved in NK cell-mediated CS. We concluded that IFN-γ, IL-12 and IFN-α are required for NK cell-mediated CS. Moreover, IL-4 and IL-13 are not involved in NK cell-mediated CS to DNFB in BALB/c mice.

IFN-γ production by liver NK cells is CXCR6 dependent

Our results are interesting in the light of previously published findings that NK cell-mediated anti-viral activity and IFN-γ production are dependent on IL-12-mediated effects on NK cells[21–24] and that IL-12 is essential for NK cell expansion in the absence of IL-15.[25] We therefore hypothesized that NK cells secrete IFN-γ upon DNFB sensitization, and may continue to do so during CS responses. Indeed, when NK cells were isolated from spleens and livers of DNFB-sensitized mice, and their IFN-γ production was compared with that of naive NK cells, hepatic, but not splenic NK cells produced IFN-γ upon sensitization (Fig. 5a), and continued to do so after challenge (Fig. 5b–d). We then investigated whether IFN-γ production by NK cells is regulated by NK cell-expressed CXCR6. We used naive B cells as antigen-presenting cells to avoid further stimulation by dendritic cells, which are potent activators of NK cells.[26] B cells were labelled on their cell surface with DNBS, a PBS-soluble analogue of DNFB, and used to re-stimulate NK cells isolated from spleens (Fig. 5b) or livers (Fig. 5c,d) of naive or DNFB-sensitized donor mice in the presence of blocking antibody specific to CXCR6, or its ligand CXCL16, or isotype control antibodies (Fig. 5c, d). NK cells isolated from livers, but not from spleens of DNFB-sensitized mice produced IFN-γ *ex vivo* (Fig. 5a), and IFN-γ production was reduced when blocking antibody specific to CXCL16 or CXCR6 was added to the *in vitro* culture (Fig. 5c). Re-stimulation of NK cells with DNBS-loaded B cells *in vitro* did not induce additional IFN-γ-producing NK cells (Fig. 5c,d), demonstrating that, once activated, DNFB-specific NK cells produce IFN-γ and do so for many days. IFN-γ production was again significantly reduced in naive and DNFB-sensitized hepatic NK cells upon addition of blocking antibody specific to CXCR6, or its ligand CXCL16 (Fig. 5c,d). Hence,



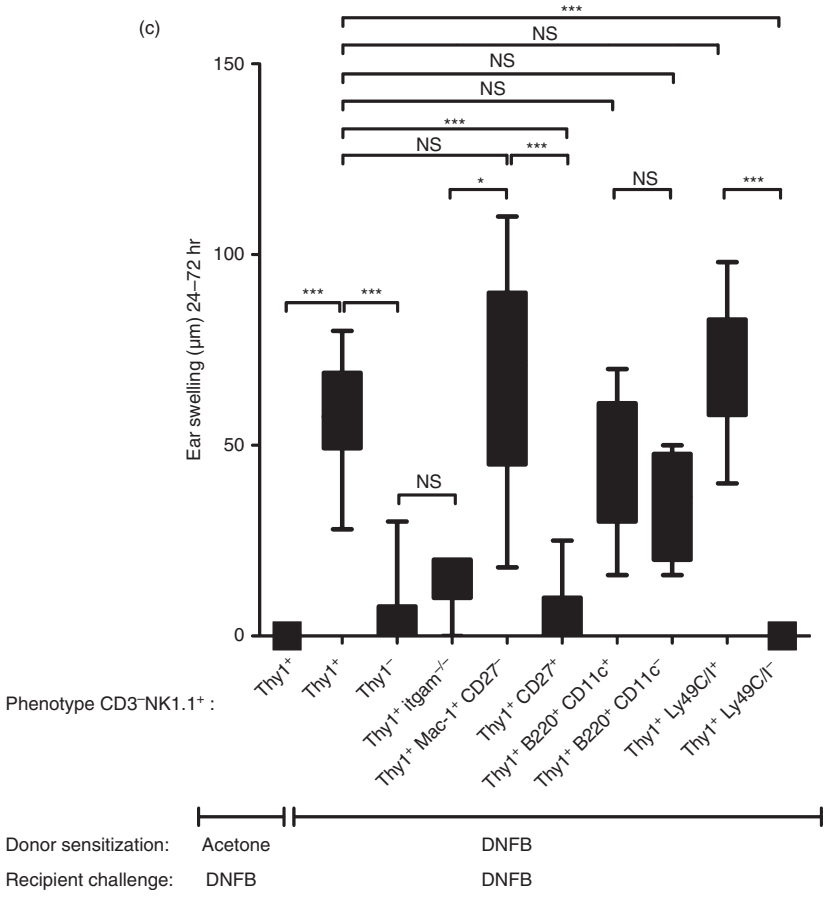


Figure 3. Mature, licensed mouse liver natural killer (NK) cells mediate contact sensitivity (CS) to dinitrofluorobenzene (DNFB). (a,b) Flow cytometric analysis of splenic and hepatic NK cells from naive or dinitrobenzene sulphonic acid (DNBS) -sensitized C57BL/6 or Rag1$^{-/-}$ C57BL/6 mice. Single cell suspensions from livers (a) or spleens (b) of indicated donor animals were stained with antibodies specific to murine CD45, NK1.1, CD3, Thy-1, CD11b, CD11c, CD27, B220, Ly49C/I, and indicated NK cell subsets were either FACS sorted on a BD ARIA, or analysed on a FACSCanto (BD) and analysed using FlowJo software. The expression of CD90 (Thy1) was determined on NK1.1$^+$ CD3$^-$ cells (left histogram), and Ly49C/I expression was determined on NK1.1$^+$ CD3$^-$ Thy1$^+$ cells (right histogram). We also analysed the expression of CD27, CD11b, CD11c and B220 on NK1.1$^+$ CD3$^-$ Thy1$^+$ cells (left two zebra-plot panels), and then analysed the expression of CD27 and CD11c when gating on NK1.1$^+$ CD3$^-$ Thy1$^+$ cells, that are either B220$^+$ CD11c$^+$ or B220$^+$ CD11c$^-$ (right two panels). (c) Mature, licensed mouse liver NK cells mediate CS to DNFB. Ear swelling in naive Rag2$^{-/-}$ Il2rg$^{-/-}$ mice that received adoptively transferred hepatic CD3-NK1.1$^+$ NK cells of indicated phenotype (6×10^4 to 8×10^4 cells per mouse) from Rag1$^{-/-}$ C57BL/6 or *Itgam*$^{-/-}$ C57BL/6 donor mice sensitized with DNFB (0·5% in acetone, 20 μl on shaved abdomen) on days 0 and 1 before transfer on day 4. Sorting gates used are indicated in (a). Recipients were challenged by skin painting with 20 μl DNFB (0·2%) one ear and acetone on the control ear, 24 hr after adoptive transfer of indicated NK cells and ear swelling was determined every 24 hr. Background ear swelling in non-immunized mice was subtracted from ear swelling in the experimental groups. Data are pooled data from three to six independent experiments per NK cell phenotype. Results shown as mean ± SD. n = 7–33 NS, not significant; *$P < 0{\cdot}05$; **$P < 0{\cdot}005$; ***$P < 0{\cdot}0005$.

CXCR6-ligation on NK cells influences IFN-γ production by hepatic NK cells.

In summary, our data show that antigen-primed, mature licensed NK cells mediate rapid CS responses to DNFB, which depend on IFN-α, IL-12 and IFN-γ, but are independent of IL-4 and IL-13 in BALB/c mice. Furthermore, DNFB sensitization elicits IFN-γ production in hepatic, but not splenic NK cells, which continue to produce IFN-γ upon sensitization and challenge. Finally, IFN-γ production by CS-immune NK cells was regulated by interactions between CXCR6 and its ligand, CXCL16.

Discussion

It is commonly accepted that CS can be mediated by either MHC class II-restricted CD4$^+$ Th1 cells, which

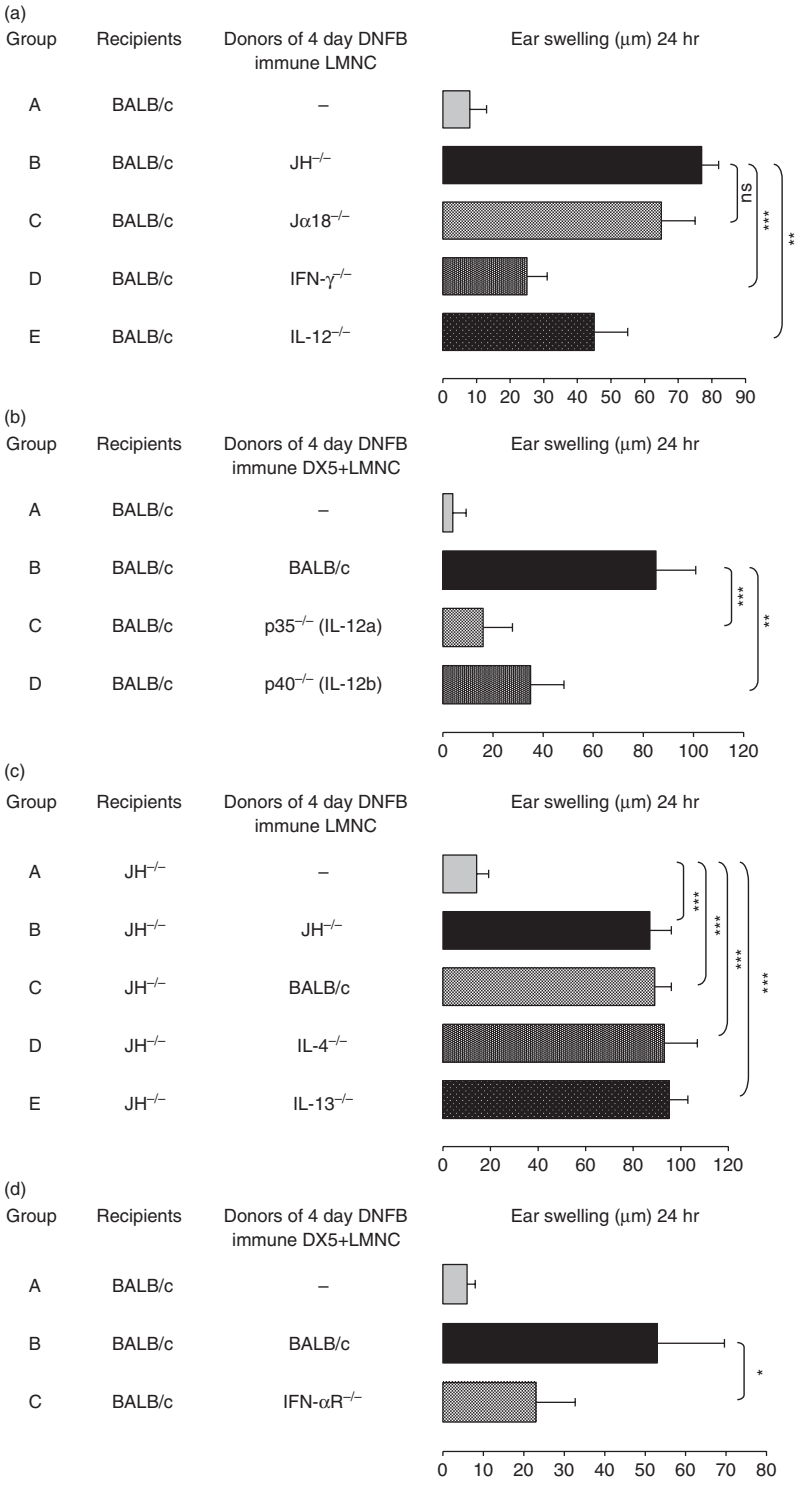


Figure 4. Contact sensitivity (CS) mediated by natural killer (NK) cells is interferon-γ (IFN-γ), interleukin-12 (IL-12) and IFN-α dependent. (a) BALB/c recipients were transferred with 5×10^5 4-day dinitrofluorobenzene (DNFB) immune liver mononuclear cells (LMNC) from $JH^{-/-}$ (Group B), $J\alpha18^{-/-}$ (Group C), IFN-$\gamma^{-/-}$ (Group D) and IL-12p40$^{-/-}$ (Group E) and challenged 1 day later and tested for CS. Results shown as mean $\pm$ SD. $n = 8$. ns = non significant, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$. (b) BALB/c recipients were transferred with 1×10^5 $DX5^+$ 4-day DNFB immune liver $DX5^+$ NK cells isolated from BALB/c (Group B), IL-12p35$^{-/-}$ (Group C) and IL-12p40$^{-/-}$ (Group D) before challenge and test. Results shown as mean $\pm$ SD. $n = 8$. $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$. (c) $JH^{-/-}$ mice were transferred with 5×10^5 4-day DNFB immune LMNC from $JH^{-/-}$ (Group B), BALB/c (Group C), IL-4$^{-/-}$ (Group D) and IL-13$^{-/-}$ (Group E) 1 day before challenge and subsequent CS test. Results shown as mean $\pm$ SD. $n = 8$. $^{***}P \leq 0{\cdot}001$. (d) BALB/c recipients were transferred with 1×10^5 $DX5^+$ 4-day DNFB immune liver $DX5^+$ NK cells isolated from BALB/c (Group B) or IFN-$\alpha R^{-/-}$ (Group C) before challenge and test. Results shown as mean $\pm$ SD. $n = 8$. $^{*}P \leq 0{\cdot}05$.

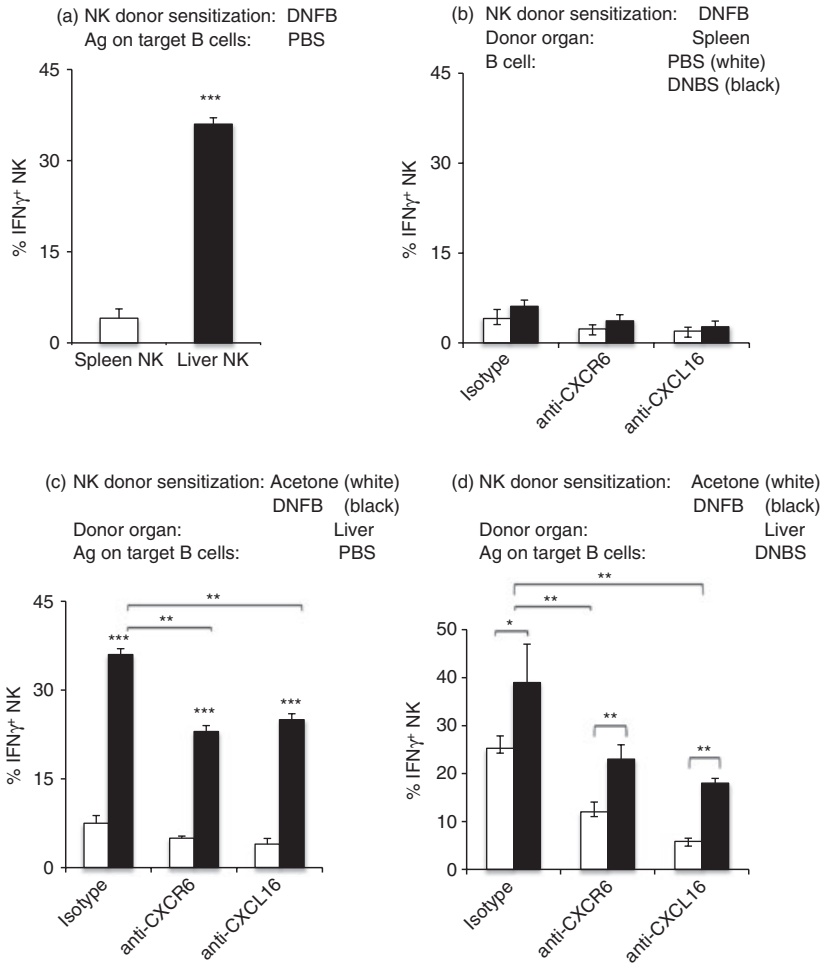


Figure 5. Interferon-γ (IFN-γ) production by liver natural killer (NK) cells is CXCR6-dependent. Rag1$^{-/-}$ donor mice were sensitized with 50 μl 0·5% dinitrofluorobenzene (DNFB) in acetone, or mock sensitized with 50 μl acetone on days 0 and 1 on the shaved abdomen. NK cell donor sensitizations are indicated above each figure. CD45$^+$ Thy1$^+$ CXCR6$^+$ NK cells were sorted from livers or spleens at day 4 and co-cultured with DNBS labelled or control B cells at a 100 : 1 B : NK ratio for 15 hr in the presence 10 μg/ml anti-CXCR6 or anti-CXCL16 monoclonal antibody or isotype control, as indicated in each figure. BD GolgiStop containing Monensin was added according to manufacturer's protocol for the last 10 hr of culture. The mixture of NK cells and B cells was then fixed, permeabilized and stained for intracellular IFN-γ, as well as NK1.1, Thy1 and CXCR6, to distinguish memory NK cells from target B cells. The NK cells were identified as NK1.1$^+$ Thy1$^+$ CXCR6$^+$, and FACS analysed for intracellular IFN-γ using flow cytometry. Data are representative of two independent experiments with 10–15 donor mice. Results shown as mean $\pm$ SD. *$P < 0{\cdot}05$; **$P < 0{\cdot}005$; ***$P < 0{\cdot}0005$.

locally release IFN-γ to recruit a characteristic inflammatory infiltrate,[27] or by MHC class I-restricted CD8$^+$ Tc1 cells, which similarly release IFN-γ but predominately mediate cytotoxic damage to local skin cells such as keratinocytes.[28,29] Moreover, it has also been shown that IL-17-producing Th17 cells can mediate CS responses.[30]

It has recently been shown that liver NK cells mediate CS in mice,[12,13] a finding that has now been confirmed by others.[16,17] The NK cell-mediated CS responses had all the hallmarks of adaptive immunity: sensitization dependence, antigen specificity and long-lived memory, and like CS responses could be elicited months after challenge.[12,13] NK cell-mediated CS also show antigen specificity for different haptens and a variety of protein antigens encoded in anti-viral vaccines.[13]

Our experiments employing SCID and RAG-1 mice (Fig. 1a,b) demonstrate that the CS response can be induced in the absence of T and B lymphocytes, whereas SCID$_{beige}$ mice, which lack functional NK cells, do not develop CS (Fig. 1a). We confirmed these findings by adoptive transfer experiments, which showed that either DX5$^+$ magnetic bead isolated, and NK1.1$^+$ Thy1$^+$ FACS-sorted liver NK cells transfer CS. Furthermore, we demonstrate that DNFB-induced liver NK cells are potent effector cells, and as few as 4500 sorted NK cells suffice to transfer significant CS reactions (Fig. 1d, Group D versus A).

Our previous work showed that in both Th1 and Tc1 mediated CS responses, innate lymphocytes such as B-1 and NKT cells are required for elicitation of CS.[10,11] However, our current experiments employing JH$^{-/-}$ and

Jα18$^{-/-}$ mice as LMNC donors ruled out any involvement of B-1 and NKT lymphocytes in NK cell-mediated CS (Fig. 4a), and demonstrated that the classical B-1 B-cell initiation of CS, as it occurs in T-cell-dependent CS, is not required for NK cell-mediated CS.

Several new aspects of NK cell-mediated CS were described in this study. Most remarkable was the finding that NK cell-mediated CS could be elicited within 1 hr; almost immediately after immunization (Fig. 2b,c). This suggests that hapten-specific effector NK cells are pre-existing in naive hosts, and that they are immediately ready to mediate inflammatory responses upon contact immunization and challenge.

We previously showed that optimal classical CS responses benefit from a 1-day delay between adoptive transfer and ear challenge.[31] During this time, CS-initiating B-1 cells produce 'CS initiating' IgM antibodies. In contrast, NK cell-mediated adoptive CS does not require delayed challenge, as recipients of DNFB immune LMNC could be challenged immediately to elicit NK cell-mediated CS. In fact, NK cell-mediated CS could be detected as early as 30 min post challenge, and persisted for much longer than T-cell-dependent CS, as there was still a significant difference between negative and positive controls 1 week after the challenge. Hence, data presented in Fig. 2 highlight the significantly different kinetics between T-cell-mediated and NK cell-mediated CS responses.

We addressed another novel aspect of NK cell-mediated CS by elucidating its specific cytokine requirements. It is commonly accepted that many cytokines influence NK cell functions, including IFN-α, IL-2, IL-12, IL-15 and IL-18, which can activate NK cells *in vitro* and *in vivo*.[32] Furthermore, NK cell-mediated anti-viral activity and IFN-γ production have been shown to be dependent on IL-12-mediated effects on NK cells,[21–24] and IL-12 is also essential for NK cell expansion in the absence of IL-15.[25] We knew from our data that NK cell-elicited CS responses occur rapidly, and therefore tested the roles of IFN-α, IFN-γ and IL-12 in NK cell-mediated CS responses. Interestingly, DNFB immune LMNC from IFN-αR$^{-/-}$ mice, as well as IFN-$\gamma^{-/-}$ or IL-12$^{-/-}$ mice were not able to transfer NK cell-mediated CS responses compared with wild-type DNFB-immune LMNC, while IL-4 and IL-13 were dispensable for NK cell-mediated CS to DNFB. This clearly demonstrates that NK cell intrinsic IFN-α signalling, and either NK cell detection or production of IFN-γ and IL-12 is required for NK cell-mediated CS. One might speculate that IFN-α may activate adaptive NK, whereas IL-12 may recruit and expand adaptive NK cells and induce IFN-γ production. In fact, hepatic NK cells produced IFN-γ upon DNFB sensitization, and they continued to make IFN-γ throughout DNFB challenge, and IFN-γ production was regulated in part by CXCR6–CXCL16 interactions.

It is noteworthy that *in vitro* re-stimulation of DNFB-sensitized NK cells 4 days after sensitization did not lead to a significant increase in the numbers of NK cells that produced IFN-γ, albeit we did not determine the absolute amount of IFN-γ that was produced by sensitized and re-stimulated NK cells on a per cell basis. Thereby, in contrast to NK cell-mediated CS or delayed type hypersensitivity, Lamp-1-up-regulation, or NK cell-mediated killing, all of which allow for the identification of antigen-specific memory NK cells, IFN-γ production does not allow for a distinction between sensitized and memory NK cells. Indeed, using *in vivo* studies, we have detected IFN-γ production adoptively transferred, antigen-primed NK cells 4 weeks after adoptive transfer into naive hosts (unpublished observation). Further work is needed to precisely define the individual roles of cytokines, chemokines and accessory cells involved in NK cell-mediated CS responses.

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Disclosures

The authors declare that they have no conflicts of interests.

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