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T cell– and B cell–independent adaptive immunity mediated by natural killer cells

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It is commonly believed that only T lymphocytes and B lymphocytes expressing recombination-dependent antigen-specific receptors mediate contact hypersensitivity responses to haptens. Here we found that mice devoid of T cells and B cells demonstrated substantial contact hypersensitivity responses to 2,4-dinitrofluorobenzene and oxazolone. Those responses were adaptive in nature, as they persisted for at least 4 weeks and were elicited only by haptens to which mice were previously sensitized. No contact hypersensitivity was induced in mice lacking all lymphocytes, including natural killer cells. Contact hypersensitivity responses were acquired by such mice after adoptive transfer of natural killer cells from sensitized donors. Transferable hapten-specific memory resided in a Ly49C-I$^+$ natural killer subpopulation localized specifically in donor livers. These observations indicate that natural killer cells can mediate long-lived, antigen-specific adaptive recall responses independent of B cells and T cells.

Immune responses to infectious or damaging agents are commonly categorized as either innate or adaptive. Innate immune responses involve both soluble and cell surface–associated germline-encoded molecules that recognize a finite set of molecular patterns associated with tissue damage and certain pathogens. A hallmark of innate immunity is that repeated exposure to the same challenge does not substantially alter the nature of the ensuing response[1]. In contrast, adaptive immune responses to a previously encountered challenge are qualitatively and/or quantitatively enhanced compared with the first encounter. Moreover, the versatility of adaptive responses is almost unlimited with regard to antigen specificity. A principal mechanism generating this diversity is the random recombination of variable, diversity and joining gene segments during lymphocyte development, which depends on the synergistic activity of proteins encoded by recombination-activating gene 1 (*Rag*1) and *Rag*2 (ref. 2) and gives rise to millions of naive T cells and B cells, each with a unique antigenic specificity[3].

Priming of naive T cells to a pathogen-associated antigen requires the encounter of a T cell with specialized antigen-presenting cells in secondary lymphoid organs and triggers massive T cell proliferation followed by the acquisition of T cell effector functions. Effector T cells give rise to long-lived memory T cells that confer enhanced protection against reinfection. As only T cells and B cells are known to have the machinery required for somatic rearrangement of antigen receptor genes, it is commonly believed that adaptive immunity is strictly dependent on the presence of T cells and B cells. That idea has been challenged based on results obtained with invertebrates[4]. However, there is no evidence so far that any cells other than B cells or T cells can elicit an adaptive immune response in mammals.

A classic example of adaptive immunity is the hapten-induced contact hypersensitivity (CHS) response, in which an epithelial surface is exposed to organic or inorganic molecules that chemically modify proteins. The 'haptenated' molecules are recognized as foreign antigens and trigger the formation of hapten-specific long-lived immune memory. Rechallenge after sensitization precipitates a local recall response associated with tissue swelling caused by the recruitment of inflammatory cells from the blood. A widely used model for studying CHS involves the sensitization of rodents by painting of 2,4-dinitrofluorobenzene (DNFB) or oxazolone (OXA) on the animal's dorsal skin followed several days later by rechallenge at a previously unexposed site, such as the ear[5,6]. The hapten-specific recall response can be measured during subsequent days as increased ear thickness[6]. Both αβ and γδ T cells as well as B-1 B cells have been linked to this phenomenon[7,8]. Indeed, naive mice can be passively sensitized by adoptive transfer of T cells from hapten-sensitized donors, and local injection of a single memory T cell at the site of challenge can be sufficient to achieve that effect[9]. Moreover, the structures of several hapten-specific T cell receptors have been elucidated[10,11]. Given that evidence, it has been accepted that T cells mediate CHS responses, although those findings do not rigorously exclude the possibility of the parallel existence of T cell–independent effectors. Here we demonstrate that hapten-induced CHS responses are not strictly dependent on T cells or B cells but can be

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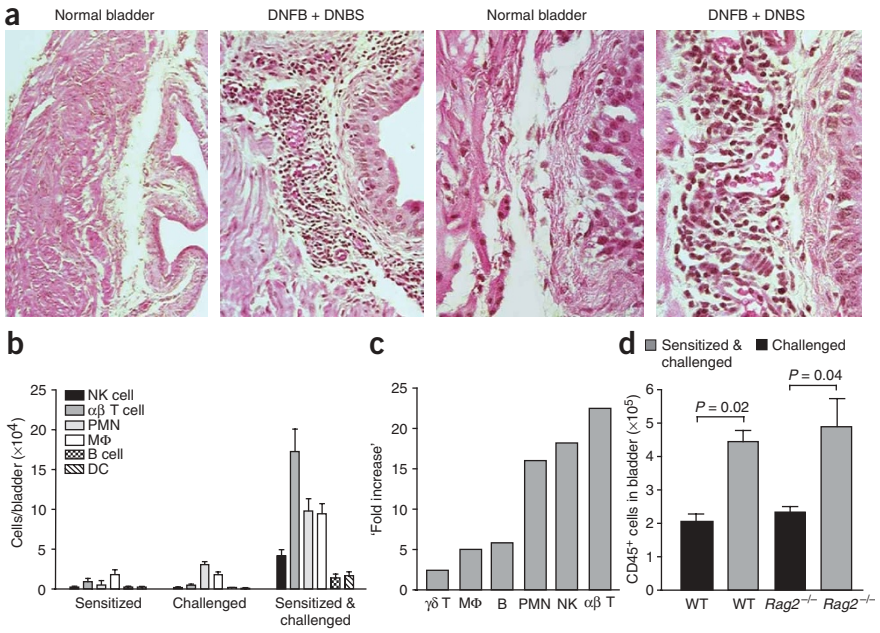

Figure 1 Hapten-induced DTH response in mouse bladder. Mice were sensitized transdermally with DNFB on day 0 and day 1. On day 5, mice were anesthetized and challenged by intravesical DNBS instillation; 48 h after challenge, excised bladders were prepared for histology (**a**) or were digested in collagenase and analyzed by flow cytometry (**b**–**d**). (**a**) Hematoxylin and eosin–stained sections of C57BL/6 bladders (treatment, above images). Original magnification, ×200 (left) and ×400 (right). (**b**) Flow cytometry of bladder-infiltrating leukocytes. Infiltrating CD45⁺ leukocytes were characterized for subset composition with lineage-specific antibodies, and the absolute number of T cells, B cells, NK cells, neutrophils (PMN), macrophages (MΦ) and dendritic cells (DC) in bladders of mice (treatment, horizontal axis) was determined. $n = 4$ mice per group. (**c**) Challenge-induced increase in each subset in **b** relative to that in unchallenged sensitized bladders. (**d**) Total number of CD45⁺ leukocytes in DNBS-challenged bladders of wild-type (WT) and *Rag2*$^{-/-}$ mice with and without prior sensitization (unpaired Student's *t*-test; $n = 3$ mice per group).

independently mediated by a subset of natural killer (NK) cells in the livers of sensitized T cell– and B cell–deficient mice.

RESULTS

CHS in the mouse bladder

The initial objective of our study was to explore CHS responses in hapten-sensitized mice in a tissue other than the skin. We chose mouse bladder as a suitable organ because the 'urothelial' barrier can be challenged relatively easily by intravesical injection of water-soluble reagents. Moreover, the bladder in rodents is accessible to intravital microscopy[12] and the composition of tissue-infiltrating leukocytes can be assessed quantitatively by histology and flow cytometry (M.G. and U.H.v.A., unpublished data).

We sensitized mice on two consecutive days by epicutaneous painting with DNFB. We challenged the urinary tract 5 d later by intravesical injection of 2,4-dinitrobenzene sulfonic acid (DNBS), a water-soluble analog of DNFB that is recognized by DNFB-specific T cells[13]. Histological examination showed considerable inflammatory infiltration in the bladder wall of sensitized (but not naive) mice that was concentrated in the lamina propria (**Fig. 1a**). Collagenase digestion of excised bladders allowed us to generate single-cell suspensions that we analyzed for leukocyte subset content by flow cytometry (**Fig. 1b**). Although challenged bladders in naive mice and unchallenged bladders in sensitized mice contained few leukocytes, challenged bladders in sensitized mice were heavily infiltrated with leukocytes. Compared with baseline leukocyte counts in unchallenged bladders in sensitized mice, the relative increase in leukocyte numbers induced by DNBS challenge was highest for αβ T cells followed by NK cells and granulocytes (**Fig. 1c**). The total number of CD45⁺ leukocytes recovered after DNBS challenge from bladders of sensitized mice was more than twice as high as the number recovered from naive mice (**Fig. 1d**). To determine whether this acquired immune response in the bladder was T cell dependent, we applied the same protocol to *Rag2*$^{-/-}$ mice, which are completely devoid of T cells and B cells[2,14]. Unexpectedly, the sensitization-dependent recruitment of inflammatory cells in challenged bladders of *Rag2*$^{-/-}$ mice was identical in magnitude to our measurements in wild-type mice (**Fig. 1d**).

Intact cutaneous CHS in *Rag2*$^{-/-}$ mice

Having noted in recombination-deficient mice what seemed to be the induction of immunological memory, we sought to determine whether that phenomenon was unique to the bladder. Thus, we sought to determine whether mice lacking T cells and B cells can also mount sensitization-dependent recall responses in the classic ear CHS model. We tested three genetically distinct strains of T cell– and B cell–deficient mice (*Rag2*$^{-/-}$ mice on either the C57BL/6 or C57BL/10 background and severe combined immunodeficiency (SCID) mice on the BALB/c background). In agreement with published observations[15], all three strains developed ear swelling responses to DNFB equivalent to those in wild-type control mice (**Fig. 2a** and data not shown).

After thus confirming that hapten exposure leads to full sensitization in the absence of lymphocytes expressing conventional recombination-dependent antigen receptors, we sought to determine whether that reflected a hapten-specific adaptive immune response or a simple exacerbated innate defense reaction to a recently encountered irritant. We measured the response of mice to another well characterized hapten, OXA, which is chemically distinct from DNFB. *Rag2*$^{-/-}$ C57BL/10 mice showed a significant inflammatory reaction when sensitized and challenged with OXA, but they responded only to haptens to which they had been previously exposed; challenge of DNFB-sensitized *Rag2*$^{-/-}$ mice with OXA had no effect and vice versa (**Fig. 2b**). This finding indicates that immune responses to hapten challenge in *Rag2*$^{-/-}$ mice are inducible and hapten specific.

Next we sought to determine whether hapten sensitization of *Rag2*$^{-/-}$ mice can induce long-lived immunological memory. We allowed DNFB-sensitized *Rag2*$^{-/-}$ mice to 'rest' for 4 weeks before challenge. The resulting CHS response was equivalent to that in wild-type mice (**Fig. 2c**), indicating that mice can mount T cell– and B cell–independent immune responses having all the functional hallmarks of adaptive immunity, given that this CHS response required prior sensitization, was antigen specific and resulted in persistent memory.

NK cell accumulation in hapten-challenged tissues

Which leukocytes might confer hapten-specific memory in the absence of T cells and B cells? We focused on NK cells, because

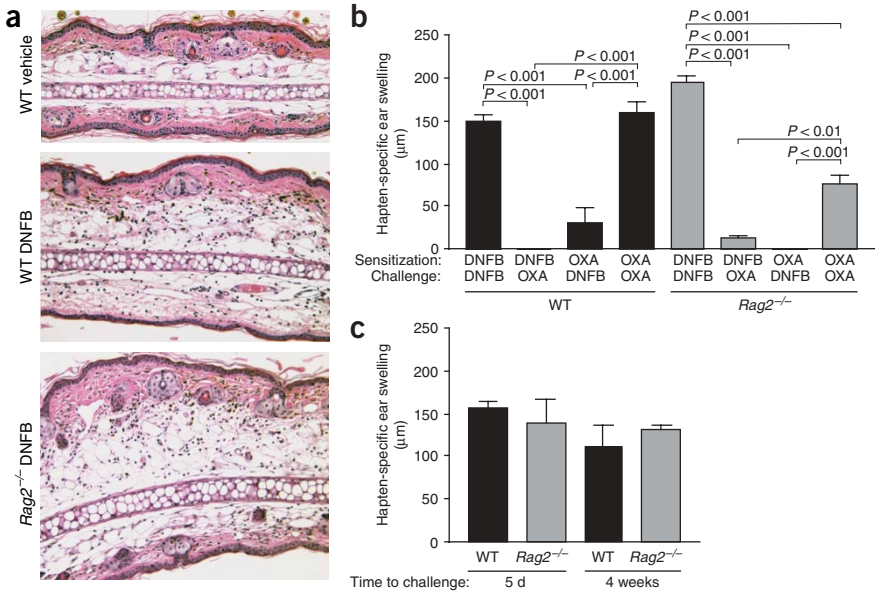

Figure 2 Hapten-induced CHS responses in ears of wild-type and *Rag2*$^{-/-}$ mice. Mice were sensitized on two consecutive days by painting of 0.5% DNFB or 10% OXA on the shaved back; 5 d or 4 weeks later, the right ear was challenged with 0.25% DNFB or 1% OXA and the left ear was painted with vehicle (acetone), and ear thickness was measured 24 h later. To account for acute hapten-induced tissue irritation, background swelling was measured in parallel with that of nonsensitized mice. Hapten-specific ear swelling was calculated as follows: [treated ear thickness – control ear thickness] – background swelling. (**a**) Hematoxylin and eosin–stained tissue sections of ears from DNFB-sensitized, challenged mice (left margins). Original magnification, ×100. (**b**) CHS responses in wild-type and *Rag2*$^{-/-}$ mice are hapten specific. Mice were sensitized with either DNFB or OXA and were challenged with either the same or the other hapten (below graph; $n = 4$ mice per group). (**c**) CHS responses in wild-type and *Rag2*$^{-/-}$ mice are long lived. Mice were sensitized to DNFB and were allowed to 'rest' for 5 d or 4 weeks before challenge. $P = 0.4663$ (ANOVA); $n = 8$–10 mice per group from two independent experiments.

our bladder delayed-type hypersensitivity (DTH) model showed that other than T cells, NK cells had the greatest relative increase at the site of hapten challenge (**Fig. 1c**). By immunofluorescence microscopy, we also detected extravascular cells expressing NK1.1 after sensitization and challenge in sections of inflamed ears from both wild-type and *Rag2*$^{-/-}$ mice, whereas NK1.1^{+} cells were sparse in the contralateral ears that did not receive a challenge (data not shown) and in challenged ears of mice that had not been sensitized previously (**Fig. 3a**).

Given the inherent limitations of immunofluorescence microscopy, it is difficult to detect and accurately count small numbers of tissue-infiltrating leukocytes. Thus, we used an additional approach to investigate the accumulation of NK cells in hapten-challenged skin. We removed ears from sensitized wild-type and *Rag2*$^{-/-}$ mice 24 h after challenge. We then split the ears and transferred them to chemoattractant-containing media. After overnight culture, we recovered large numbers of CD45^{+} leukocytes that included 0.5–1.8% NK cells from hapten-challenged ears, whereas few cells emigrated from

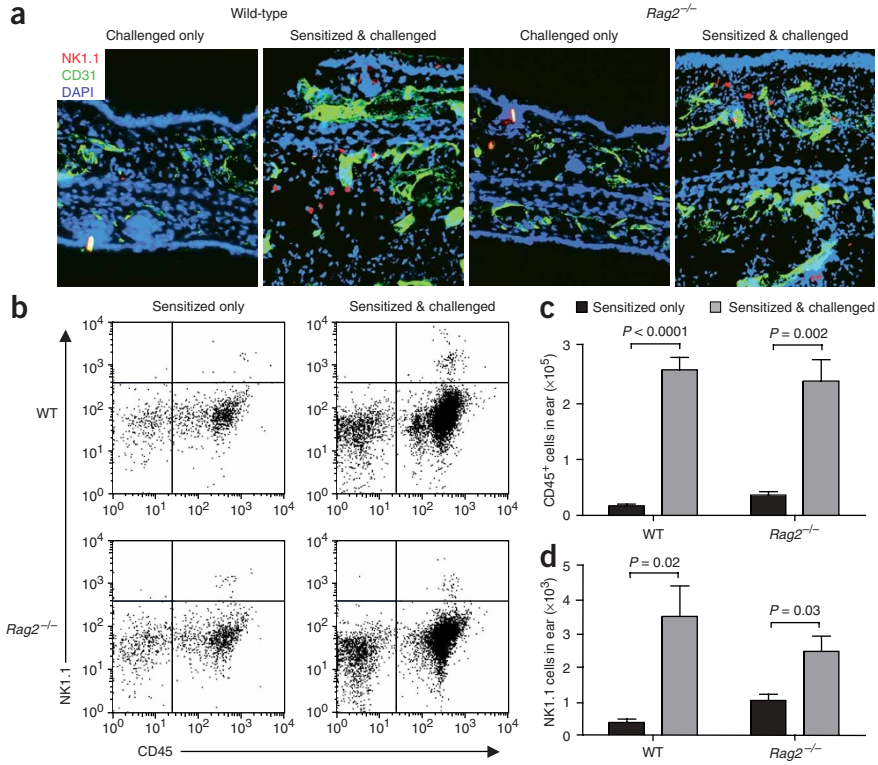

Figure 3 NK cell accumulation in ears of DNFB-sensitized mice versus DNFB-sensitized and challenged mice. (**a**) Immunofluorescence staining of frozen ear sections from DNFB-challenged wild-type and *Rag2*$^{-/-}$ mice with and without DNFB sensitization. Sections are stained for NK1.1 (red) and the 'pan-endothelial' marker CD31 (green) and are counterstained with the nuclear dye DAPI (4,6-diamindino-2-phenylindole; blue). Original magnification, ×100. Micrographs are representative of four experiments. (**b**–**d**) DNFB- and vehicle-challenged ears were removed from sensitized wild-type and *Rag2*$^{-/-}$ mice, were sterilized, were split along the cartilage and were placed in media containing CCL21, leukotriene B_4 and interleukin 2. After 14 h, emigrated cells were counted with a hemocytometer and analyzed by flow cytometry. (**b**) Flow cytometry of NK1.1 expression on CD45^{+} emigrated leukocytes; data are representative of four experiments. (**c**,**d**) Number of CD45^{+} leukocytes (**c**) and CD45^{+} NK1.1^{+} cells (**d**) recovered from cultured ears of wild-type and *Rag2*$^{-/-}$ sensitized-only versus sensitized-and-challenged ears (unpaired Student's *t*-test; $n = 4$ mice per group).

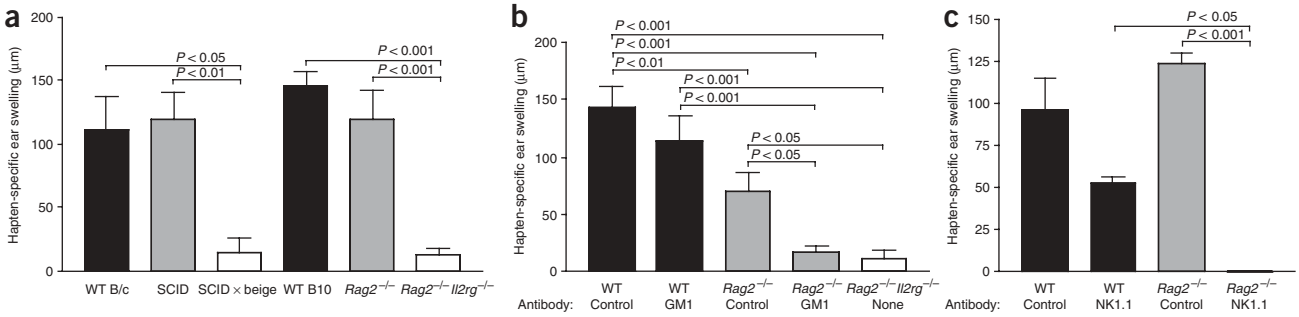

Figure 4 NK cells are needed to mediate CHS responses in T cell– and B cell–deficient mice. (**a**) CHS response to DNFB in wild-type mice (BALB/c (WT B/c) and C57BL/10 (WT B10)); T cell– and B cell–deficient SCID (BALB/c) and *Rag2*$^{-/-}$ (C57BL/10) mice; and T cell–, B cell– and NK cell–deficient SCID × beige (BALB/c) and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (C57BL/10) mice ($n = 6$–10 mice per group). (**b**,**c**) Effect of treatment with NK cell–depleting anibodies on the DNFB-induced CHS response in wild-type and *Rag2*$^{-/-}$ mice. At 24 h before DNFB challenge, sensitized mice were depleted of NK cells with anti-asialo-GM1 (GM1; $n = 5$–6 mice per group; **b**) or with anti-NK1.1 (NK1.1; $n = 3$ mice per group; **c**). Control mice received rabbit IgG (**b**) or mouse IgG2a (**c**), respectively.

vehicle-exposed control ears (**Fig. 3b–d**). Similar to our results in the bladder model, we recovered nearly equivalent numbers of CD45^{+} leukocytes from the hapten-challenged ears of wild-type and *Rag2*$^{-/-}$ mice (**Fig. 3c**). These data demonstrate that hapten challenge after sensitization induces a rapid and substantial influx of NK cells to the inflamed tissue.

NK cells in T cell–independent CHS

The consistent accumulation of NK cells in hapten-challenged tissues prompted us to examine the function of NK cells in DNFB-induced CHS using two separate approaches. First we tested two strains of T cell– and B cell–deficient mice that either contained dysfunctional NK cells (SCID × beige[16]) or completely lacked NK cells (*Rag2*$^{-/-}$ mice lacking the common cytokine receptor γ-chain (*Il2rg*$^{-/-}$)[17,18]). Both double-mutant strains failed to mount a CHS response to DNFB (**Fig. 4a**). Although these observations were suggestive of a function for NK cells, neither the lysosomal storage defect caused by the beige mutation nor deficiency in the common cytokine receptor γ-chain in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice are selective for NK cells, leaving open the possibility that another leukocyte subset could be involved. Therefore, we used a second approach by depleting NK cells from wild-type and *Rag2*$^{-/-}$ mice with antibody to asialo-GM1 (anti-asialo-GM1)[19] or anti-NK1.1 (ref. 20). As expected, NK cell depletion did not abolish CHS responses in wild-type mice, which can develop T cell–dependent memory specific for DNFB. In contrast, *Rag2*$^{-/-}$ mice treated with anti-asialo-GM1 completely lost the ability to respond to DNFB challenge (**Fig. 4b**). We obtained equivalent results when we depleted mice of NK cells using anti-NK1.1 (**Fig. 4c**).

We also compared the ability of wild-type and mutant mice lacking only T cells and B cells (SCID and *Rag2*$^{-/-}$) or additionally defective in NK cells (SCID × beige and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$) to mount CHS responses to two additional haptens (OXA and picryl chloride (2,4,6-trinitrochlorobenzene)). Consistent with our findings using DNFB, *Rag2*$^{-/-}$ mice responded vigorously when sensitized and challenged with OXA, whereas *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice failed to mount a CHS response to OXA (**Fig. 5a**). We were unable to elicit a significant CHS response to OXA in sensitized SCID mice on the BALB/c genetic background (**Fig. 5b**), although this strain developed vigorous CHS after sensitization and challenge with DNFB. This result is in line with published reports on the effects of DNFB and OXA in SCID BALB/c mice[15,21]. Conversely, the third hapten, picryl chloride, induced a mild but statistically significant CHS response in SCID BALB/c mice but not in *Rag2*$^{-/-}$ C57BL/10 mice (**Fig. 5c**,**d**), indicating that there may be strain-specific genetic modifiers that determine whether a given hapten can elicit T cell–independent CHS.

Sensitized NK cells confer CHS

The results reported above demonstrated that NK cells were required for T cell– and B cell–independent CHS responses, but they did not exclude the possibility of involvement of other leukocytes. To address that issue, we isolated and pooled NK cells and non–NK cells from livers and spleens of DNFB-sensitized and naive *Rag2*$^{-/-}$ donors and adoptively transferred the cells into naive *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. Subsequent challenge with DNFB elicited a vigorous CHS response in recipients of sensitized NK cells, whereas recipients of naive NK cells

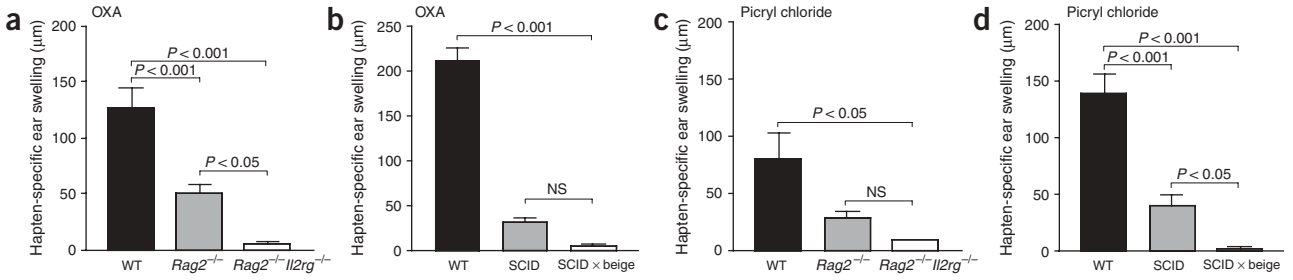

Figure 5 Differential CHS responses of mutant mouse strains to OXA and picryl chloride. The effect of OXA (**a**,**b**) and picryl chloride (**c**,**d**) was determined in various mice (horizontal axes). *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were on a C57BL/10 background; SCID and SCID × beige mice were BALB/c. Results represent four to seven mice per group (ANOVA with Newman-Keuls multiple-comparison test). NS, not significant.

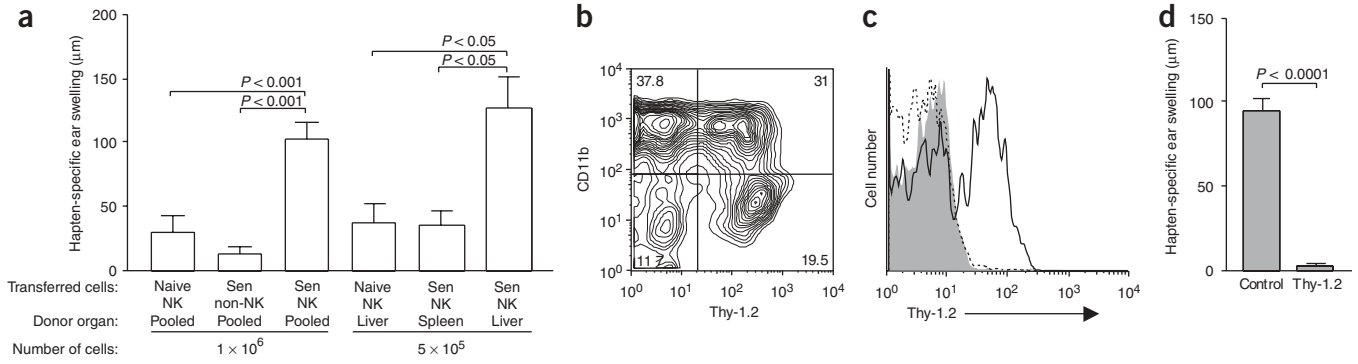

Figure 6 A subset of hepatic NK cells carries DNFB-specific memory in sensitized *Rag2*$^{-/-}$ mice. (**a**) CHS responses after adoptive transfer. Sorted cells pooled from multiple organs (left three bars) or sorted cells from individual organs (below graph; right three bars) were adoptively transferred into naive *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Recipient ears were challenged with DNFB 24 h later ($n = 3$–4 mice/group); CHS responses were measured 24 h after challenge. Sen, sensitized. (**b**–**d**) Anti-Thy-1 abrogates CHS in *Rag2*$^{-/-}$ mice. (**b**) Contour plot of Thy-1.2 and CD11b expression on liver NK1.1$^+$ cells from DNFB-sensitized *Rag2*$^{-/-}$ mice. Numbers in quadrants indicate subset frequency. (**c**) Thy-1.2 staining (detected by secondary phycoerythrin-conjugated goat anti-rat IgG) on NK cells in *Rag2*$^{-/-}$ liver 48 h after treatment with anti-Thy-1.2 (broken line) or isotype control mAb (solid line). Gray histogram, background staining of secondary antibody on NK cells in control mice. Mice were given 100 μg of each mAb 2 d before sensitization and 1 d before challenge. (**d**) Depletion of Thy-1$^+$ NK cells in anti-Thy-1-treated, but not in isotype control mAb–treated (Control), sensitized *Rag2*$^{-/-}$ mice results in complete inhibition of CHS after DNFB challenge (unpaired Student's *t*-test; $n = 6$ mice/group).

or of pooled leukocytes from sensitized donors that had been depleted of NK cells showed no response (**Fig. 6a**). Thus, NK cells are both necessary and sufficient for the transfer of hapten-specific memory from sensitized *Rag2*$^{-/-}$ mice to naive recipients.

Memory NK cells in the liver

To explore the mechanism(s) underlying that unexpected function of NK cells, we sought to determine whether hapten-specific memory is induced by all NK cells in sensitized mice or if only a distinct subpopulation of NK cells is capable of conferring hapten-specific memory. The latter seemed plausible, as published work has demonstrated that mammalian NK cells are composed of multiple subsets based on surface phenotype, immunological activity and anatomic distribution. For example, liver NK cells exert more potent antitumor activity and have distinct transcriptional profiles compared with circulating NK cells[22]. Thus, we purified NK cells from DNFB-sensitized *Rag2*$^{-/-}$ livers and spleens and compared their ability to induce CHS in DNFB-challenged *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. Only recipients of sensitized hepatic NK cells mounted detectable CHS responses (**Fig. 6a**), whereas splenic NK cells from the same donors were devoid of transferable hapten-specific memory activity. Thus, hapten-specific NK memory cells in sensitized *Rag2*$^{-/-}$ mice are localized in the liver.

Hepatic memory NK cell surface phenotype

In an effort to phenotypically define hapten-specific NK cells, we stained *Rag2*$^{-/-}$ hepatic NK cells for CD11b and Thy-1, which are

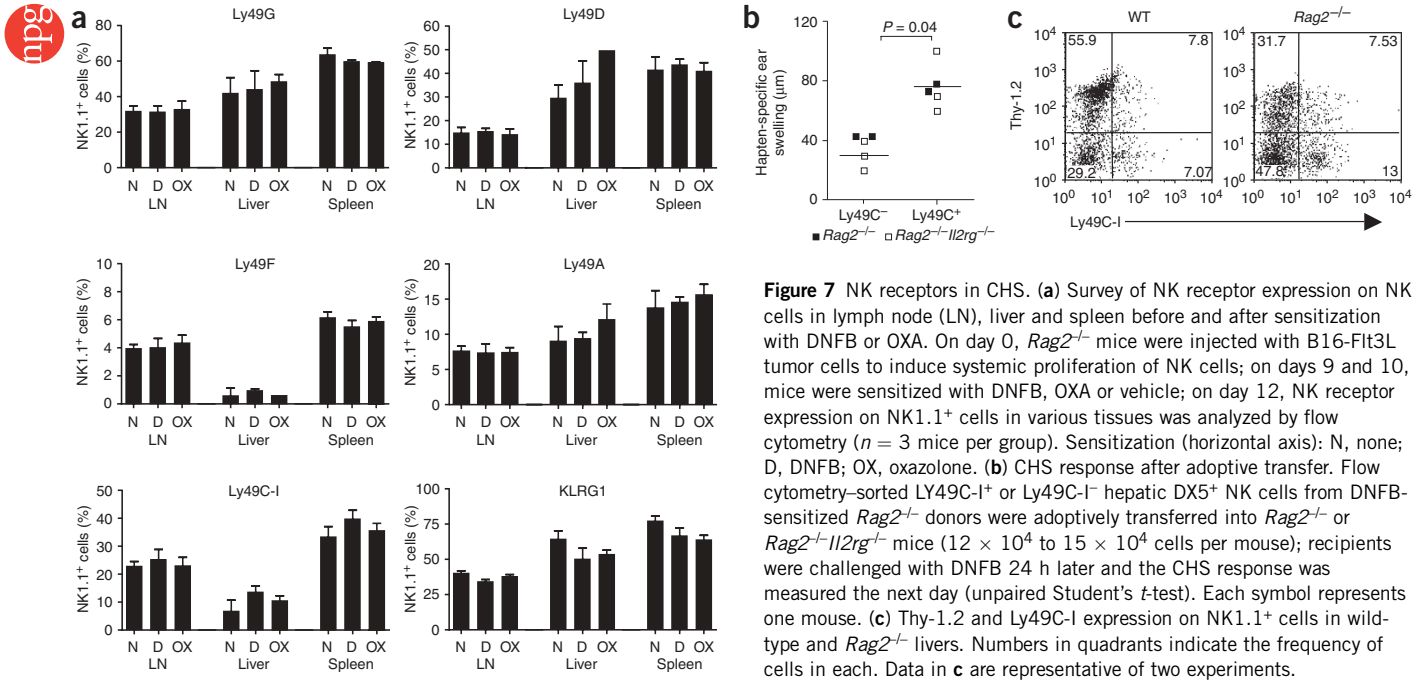

Figure 7 NK receptors in CHS. (**a**) Survey of NK receptor expression on NK cells in lymph node (LN), liver and spleen before and after sensitization with DNFB or OXA. On day 0, *Rag2*$^{-/-}$ mice were injected with B16-Flt3L tumor cells to induce systemic proliferation of NK cells; on days 9 and 10, mice were sensitized with DNFB, OXA or vehicle; on day 12, NK receptor expression on NK1.1$^+$ cells in various tissues was analyzed by flow cytometry ($n = 3$ mice per group). Sensitization (horizontal axis): N, none; D, DNFB; OX, oxazolone. (**b**) CHS response after adoptive transfer. Flow cytometry–sorted LY49C-I$^+$ or Ly49C-I$^-$ hepatic DX5$^+$ NK cells from DNFB-sensitized *Rag2*$^{-/-}$ donors were adoptively transferred into *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (12×10^4 to 15×10^4 cells per mouse); recipients were challenged with DNFB 24 h later and the CHS response was measured the next day (unpaired Student's *t*-test). Each symbol represents one mouse. (**c**) Thy-1.2 and Ly49C-I expression on NK1.1$^+$ cells in wild-type and *Rag2*$^{-/-}$ livers. Numbers in quadrants indicate the frequency of cells in each. Data in **c** are representative of two experiments.

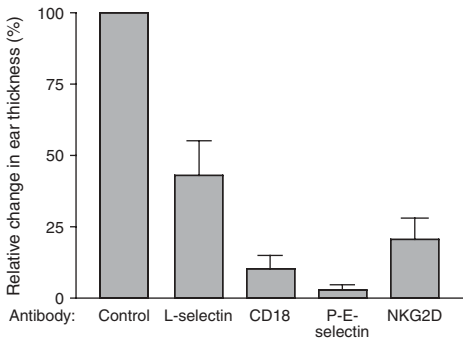

Figure 8 Function of adhesion molecules and NKG2D in CHS responses in *Rag2*$^{-/-}$ mice. *Rag2*$^{-/-}$ mice were treated intravenously with mAbs specific for L-selectin (MEL-14), CD18 (GAME-46), P-selectin (RB40.34), E-selectin (9A9) and NKG2D (C7). All mAbs were given at a dose of 100 μg/mouse; anti-L-selectin was given 24 h before sensitization and all other mAbs were given 2 h before challenge. The hapten-specific ear swelling in each treatment group is expressed as a percentage of that of the isotype control group. $P < 0.001$, inhibition of CHS for control versus all mAb-treated groups ($n = 4$–6 mice per group). P-E-selectin, P- and E-selectin.

upregulated on activated NK subsets[23]. We noted considerable heterogeneity in CD11b and Thy-1 expression, with some NK cell subsets expressing neither, one or both markers (**Fig. 6b**). Systemic treatment with a monoclonal antibody (mAb) specific for Thy-1 has been shown to abrogate CHS responses in wild-type mice[24]. That effect was attributed to antibody-induced depletion of T cells, which are uniformly Thy-1$^+$. However, given our observation that Thy-1 was expressed on about 50% of hepatic NK cells, it seemed plausible that anti-Thy-1 might also remove liver-resident Thy-1$^+$ NK cells. Indeed, treatment with anti-Thy-1 produced depletion of hepatic Thy-1$^+$ NK cells (**Fig. 6c**) and abolished CHS responses to DNFB in sensitized *Rag2*$^{-/-}$ mice (**Fig. 6d**). Thus, it is likely that most or all of the hapten-responsive NK cells in sensitized *Rag2*$^{-/-}$ mice were contained in the Thy-1$^+$ subset.

To further characterize the hepatic NK cells in *Rag2*$^{-/-}$ mice, we analyzed the expression of various activating and inhibitory NK cell receptors. Mouse NK cells express variable combinations of Ly49 receptors, and the absolute number and relative frequency of NK cells expressing each Ly49 receptor combination depends on genetic background[25]. Flow cytometry demonstrated distinct expression patterns of Ly49 receptors in liver, lymph nodes and spleen, but those expression patterns were not altered after hapten sensitization (**Fig. 7a**). A study of C57BL/6 and C57BL/10 mice has shown that NK cells expressing Ly49C, a cognate inhibitory receptor for self major histocompatibility complex (MHC) class Ia molecules, are uniquely able, relative to other NK cells, to exert effector activity in response to a variety of stimuli[26]. To assess whether this self MHC–reactive NK cell subset acquired hapten-specific memory, we sorted hepatic NK cells from DNFB-sensitized *Rag2*$^{-/-}$ C57BL/10 donors based on their reactivity with a mAb specific for Ly49C and another inhibitory receptor, Ly49I. Both receptors recognize MHC class I molecules expressed in C57BL/10 mice[27]. After adoptive transfer into naive *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients, sensitized Ly49C-I$^+$ cells conferred significantly greater CHS responsiveness than did sensitized Ly49C-I$^-$ NK cells (**Fig. 7b**). We next analyzed expression of Thy-1 and Ly49C-I on hepatic NK cells in wild-type and *Rag2*$^{-/-}$ mice (**Fig. 7c**). Only a minority (about 13–20%) of Thy-1$^+$ hepatic NK cells expressed Ly49C-I, and approximately half of the Ly49C-I$^+$ NK cells in *Rag2*$^{-/-}$ and wild-type livers did not express Thy-1, indicating that the hapten-specific Thy-1$^+$ Ly49C-I$^+$ NK cells represent only a small subset (less than 10%) of hepatic NK cells.

Adhesion molecules in NK cell–mediated CHS

Several studies have addressed the function of adhesion molecules during the sensitization and challenge phase of CHS. For example, L-selectin is critical for homing of naive T cells to skin-draining lymph nodes, where T cells are primed by hapten-presenting dendritic cells[28]. When *Rag2*$^{-/-}$ mice were treated with anti-L-selectin before sensitization with DNFB, the hapten-specific CHS response was attenuated (**Fig. 8**), suggesting that NK cells may also be primed in lymph nodes. Subsequently, primed cells must enter the hapten-exposed inflamed skin. Recruitment of effector T cells to inflamed mouse ears requires P-selectin and E-selectin, which mediate slow rolling in dermal venules[29]. Rolling cells must then engage β_2-integrins (CD11–CD18) to arrest and emigrate into inflamed tissues[30]. Those traffic rules also seem to apply to NK cell–mediated CHS responses in DNFB-sensitized *Rag2*$^{-/-}$ mice, as blocking antibodies specific for either the endothelial selectins or CD18 abrogated CHS responses (**Fig. 8**).

Activating receptor NKG2D in NK cell–mediated CHS

Once NK cells have emigrated into the ear, they must detect and respond to the local hapten challenge. As haptens cause tissue irritation, the exposed parenchyma is likely to generate innate 'distress signals'. NK cells have activating receptors that detect markers of cellular injury caused by infection, malignant transformation and other damaging factors[25]. Possibly the best characterized of these sensors is NKG2D, which recognizes a group of MHC class I–related surface proteins that are induced on distressed cells by certain noxious stimuli[31,32]. Ligation of NKG2D triggers functional activation of NK cells, which in the absence of inhibitory signals may then secrete cytokines and exert cytotoxic effects, leading to local exacerbation of inflammation. Treatment with a mAb specific for NKG2D before challenge of sensitized *Rag2*$^{-/-}$ mice suppressed the ensuing CHS response (**Fig. 8**). This effect was not due to depletion of NK cells, as mAb-treated mice had normal numbers of NK cells in their livers and spleens (data not shown). These results suggest that NKG2D might contribute to NK cell–mediated CHS owing to its ability to detect certain stress-induced ligands.

DISCUSSION

The findings presented here have demonstrated a previously unknown function for NK cells: the ability to acquire selective memory of at least three chemically distinct foreign molecular entities (DNFB, OXA and picryl chloride). Using a cross-challenge approach with DNFB and OXA, we have shown that this memory was hapten specific (for example, mice exclusively responded to the hapten to which they were previously sensitized). Further experiments in the DNFB model demonstrated that NK cell memory was long lived and could be passively transferred to naive mice by transfusion of a small hapten-specific subset of primed hepatic NK cells.

The impetus for this work was the unexpected observation that DNFB-immunized *Rag2*$^{-/-}$ mice demonstrated normal hapten-induced inflammation after challenge of either the bladder or the auricular skin. The last observation is consistent with a report that noted normal DNFB-induced ear swelling responses in sensitized nude and SCID mice as well as in mice lacking RAG1 or RAG2 (ref. 15). Our phenotypic analysis of inflammatory cells in challenged bladders and ear explants showed substantial accumulation of NK cells in both wild-type and *Rag2*$^{-/-}$ mice. Those data are in line with studies

in human CHS in which dermal and epidermal inflammatory infiltrates are marked by an influx of NK cells[33]. NK cells are also present, albeit at a low frequency, in normal mouse and human skin (refs. 33,34 and reported here). Moreover, NK cells represent approximately 4% of leukocytes in normal human skin-draining lymph fluid[34]. These data suggest that NK cells participate in the physiological immune surveillance of mammalian skin and that after contact sensitization and renewed exposure to a hapten, NK cells accumulate rapidly in challenged tissues regardless of whether other lymphocytes are present.

Given those observations, we hypothesized that NK cells might be involved in T cell– and B cell–independent CHS. Indeed, double-mutant mice lacking both T cells and B cells as well as functional NK cells were unresponsive to hapten challenge, and NK cell depletion in *Rag2*$^{-/-}$ but not wild-type mice abolished DNFB-induced CHS. Moreover, nonsensitized mice could be passively immunized to DNFB by adoptive transfer of purified NK cells from DNFB-sensitized RAG2-deficient donors. In contrast, adoptive transfer of NK cells from naive mice or of non–NK leukocytes from sensitized donors did not confer hapten sensitivity. These experiments establish that NK cells are both required and sufficient for mediating a potent CHS response in the absence of other lymphocytes.

Where and how might NK cells become first 'educated' about a sensitizing hapten? There is mounting evidence that homing of lymphocytes to secondary lymphoid tissues is critical for immune surveillance and the development of adaptive immunity to regional antigens[35]. To respond to a cutaneous hapten challenge, naive lymphocytes require L-selectin to home to skin-draining lymph nodes, where they are activated by antigen-presenting dendritic cells, particularly Langerhans cells[36]. Consequently, T cell priming by a sensitizing hapten and the ensuing CHS response are compromised in L-selectin-deficient mice owing to a specific defect in homing of lymphocytes to lymph nodes[28]. Moreover, mice devoid of lymph nodes fail to mount CHS responses[37]. Because treatment of *Rag2*$^{-/-}$ mice with anti-L-selectin before sensitization impaired CHS responses, it is likely that lymph nodes are also the main sites of NK cell priming for CHS. Indeed, NK cells are effectively recruited to antigen-challenged lymph nodes, and NK cells have multiple means for communicating with dendritic cells[38,39].

Once the hapten-primed effector cells have returned to the blood, they require a different set of trafficking molecules for entry into hapten-exposed inflamed skin. L-selectin is probably not involved in this migration step, as CHS responses are not affected by the absence of L-selectin after sensitization[28]. Instead, leukocyte recruitment to inflamed skin requires P-selectin and E-selectin, which are constitutively expressed in the lumen of skin venules[29,40]. A hallmark of skin-homing leukocytes is their expression of selectin ligands that mediate slow rolling interactions in selectin-expressing venules. Subsequently, the rolling cells must activate β_2-integrins (CD11–CD18), which bind endothelial intercellular adhesion molecule 1 to allow the cells to arrest and emigrate[30]. NK cells express selectin ligands as well as β_2-integrins[41], and our results have shown that both pathways were required for the induction of a CHS response in DNFB-sensitized *Rag2*$^{-/-}$ mice. Thus, NK cells seem to use the same migratory routes and adhesion pathways that conventional T cells use during the priming and effector phases of CHS[42].

Unexpectedly, hapten-specific memory NK cells and T cells differed in organ distribution in sensitized mice. Although many studies have shown that memory T cells are abundant in the spleen, we were unable to transfer hapten reactivity from sensitized *Rag2*$^{-/-}$ donors with spleen-derived NK cells. In contrast, transfer of liver NK cells was very effective in eliciting contact sensitivity in naive hosts. The mechanisms resulting in the accumulation of intrinsic memory NK cells in the liver are still unclear. Notably, a unique function for the CXCL16-CXCR6 pathway in the retention and survival of NKT cells in the liver has been demonstrated[43]. It will be interesting to explore whether this or other chemokine pathways have a similar function in NK cell migration or homeostasis in the liver.

Experiments of mAb depletion and adoptive transfer using sorted liver NK cell subsets have indicated that the transferable memory activity is concentrated in the Thy-1$^+$Ly49C-I$^+$ fraction, which represents less than 10% of hepatic NK cells. However, this subset is also present in the spleen (D.L.D. and U.H.v.A., unpublished data), indicating that these markers alone are not sufficient to define hapten-specific NK cell memory. Indeed, although sorted Ly49C-I$^+$ NK cells in the liver were more efficient at conferring hapten sensitivity than were their Ly49C-I$^-$ counterparts, there was no substantial difference in the number or frequency of hepatic Ly49C-I$^+$ NK cells before and after hapten sensitization. In fact, the relative frequency of the Ly49C-I$^+$ subset among all tissue-resident NK cells was always substantially lower in the liver than in the spleen or lymph nodes. A similarly biased distribution pattern was also apparent in the Ly49F$^+$ NK population, suggesting the possibility that phenotypically distinct NK cell subsets may be 'preferentially' recruited or retained in certain anatomic compartments based on their individual NK cell receptor repertoire.

It will be necessary to identify the molecular mechanisms by which NK cells discriminate between distinct haptens or hapten-modified macromolecules in the absence of RAG-mediated receptor rearrangement. As NK cells are categorized into many subsets, each characterized by a distinct pattern of activating and inhibitory receptors[25,44], it is possible that hapten-specific NK cell memory is rooted in this population diversity. Moreover, there are substantial differences in the composition of NK cell receptor loci among different strains of mice[45], which might explain the differential hapten effects in BALB/c versus C57BL/10 mice. Ly49C is of particular interest in this context. As mentioned above, the Ly49C-I$^+$ population of hepatic NK cells was more efficient than the Ly49C-I$^-$ hepatic NK cell population in transferring hapten sensitivity to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. In contrast, Ly49C-I$^+$ NK cells were more abundant in the spleen than in the liver, yet splenic NK cells did not confer hapten sensitivity. Thus, it is unlikely that Ly49C-I alone could account for hapten recognition by NK cells. Moreover, hapten sensitization did not detectably alter the composition of other NK subsets as defined by a panel of antibodies specific for Ly49 family members and killer cell lectin-like receptor G1 (KLRG1). A change in subset frequency and/or distribution might be expected if one or more of these receptors were driving hapten-reactive NK cells to undergo clonal proliferation. Similarly, although NK cell–mediated CHS could be blocked with anti-NKG2D, the fact that this activating receptor is expressed on almost all NK cells[46], whereas only a subset of NK cells carry hapten specificity, makes it unlikely that NKG2D is directly responsible for hapten recognition. Its function in NK cell–mediated CHS is perhaps more similar to that of costimulatory molecules on T cells.

Given the considerations discussed above, the possibility that NK cells have other activating receptors that specifically recognize a given hapten and/or hapten-modified macromolecule(s) cannot be ruled out. Lymphocytes in agnathan vertebrates can generate variable lymphocyte receptors through gene rearrangements that do not involve RAG proteins[47]. However, whether a similar system for RAG-independent somatic diversification exists in NK cells or in any other cell type in jawed vertebrates has not been determined.

Finally, given the decades of intense research on hapten-induced CHS, it is surprising that the function of NK cells has been overlooked for so long. This may have been due in part to the fact that T cells and NK cells are by themselves sufficient to maintain normal CHS responses, at least to DNFB. Consequently, NK cell depletion of wild-type mice caused only a mild, statistically nonsignificant reduction in CHS. Similarly, beige mice are known to mount normal CHS responses despite a lysosomal defect in these mice that renders NK cells dysfunctional[48]. A distinct phenotype became apparent only when we examined double-mutant mice carrying both the beige and SCID mutation. Conversely, whereas there is abundant evidence that T cells by themselves can mediate formidable CHS responses[7], there is insufficient published information to rigorously exclude the possibility of a parallel existence of T cell–independent effector populations. Studies spanning more than three decades have shown that treatment of animals with depleting antisera or mAbs specific for antigens expressed on T cells can eliminate hapten-specific immune responses, including CHS[24,49–51]. However, the T cell–associated antigens targeted in those experiments may be shared by other cell types, such as subsets of NK cells (for example, Thy-1) or dendritic cells. For example, depletion with anti-CD8α abolishes CHS responses to DNFB[51]. However, the so-called lymphoid subset of $CD8\alpha^+$ dendritic cells is known to interact with NK cells[52], and depletion of $CD8\alpha^+$ dendritic cells in nude mice using a mAb has been shown to compromise NK cell–mediated anti-tumor immunity[53].

Many studies have used gene-knockout mice to examine the function of immune receptors and leukocyte traffic molecules in CHS responses to several haptens[8]. Many mutant strains show an incomplete reduction (or an increase) in CHS, consistent with a contribution of other effector pathways. However, to identify mechanisms critical for all causative agents of CHS, it is most informative to focus on studies showing a complete absence of CHS in specific mutant mice. A review of the literature has identified three examples in which CHS is abolished in mice in which T cells or antigen-presenting cells are specifically compromised: SCID mice (BALB/c) responding to OXA[15,21]; T cell receptor-α-deficient mice (129/J) responding to picryl chloride[54]; and β_2-microglobulin-deficient mice (C57BL/6) responding to DNFB[51]. Those findings are not necessarily in conflict with our study here. Our observations have indicated that there are strain-dependent differences in the ability of mice to respond to OXA, and the modest T cell–independent response to picryl chloride could be easily missed and is apparently also influenced by strain-dependent genetic modifiers. The failure of β_2-microglobulin-deficient mice to mount a CHS response to DNFB had been attributed to the absence of $CD8^+$ T cells in these mice[51]. However, published work has shown that self MHC class I recognition by developing $Ly49C^+$ cells is necessary to enable these cells to achieve functional competence[26]. Thus, both MHC class I on unidentified 'licensing' cells and Ly49C on NK cells are necessary for normal NK cell function, at least in some settings. However, the issue of whether MHC class I expression is merely necessary to 'license' NK cells during their development[26] or has also a direct function in the priming and/or effector phase of hapten-induced CHS (as is the case for T cell–mediated immunity) will require further 'dissection' of the molecular mechanisms of hapten recognition by NK cells.

In conclusion, our findings have identified a previously unrecognized function of NK cells as mediators of hapten-specific immunity. This unexpected activity of NK cells represents a 'learned' response that persists for several weeks and can exquisitely discriminate between different haptens. Thus, NK cell–mediated CHS features three essential hallmarks of adaptive immunity: acquired activity, long-lived memory and antigen specificity. Although several studies have suggested that the innate immune system can mount adaptive responses in lower animals[4], to our knowledge this is the first observation of a T cell– and B cell–independent acquired immune response in higher vertebrates.

METHODS

Mice. *Rag2*$^{-/-}$ mice (C57BL/6 or C57BL/10 background), *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (C57BL/10), and SCID and SCID × beige mice (both on BALB/c background), as well as age-, background- and sex-matched wild-type mice were purchased from Taconic or were bred 'in-house'. Mice were used at 5–10 weeks of age in accordance with National Institutes of Health guidelines. All procedures were approved by the institutional animal committees at both the CBR Institute for Biomedical Research and Harvard Medical School (Boston, Massachusetts).

Reagents. DNFB, DNBS, picryl chloride and OXA were purchased from Sigma Fine Chemicals. Anti-asialo-GM1 and rabbit IgG were from Wako Pure Chemical Industries; anti-NKG2D was from BioLegend; phycoerythrin-conjugated goat anti-rat IgG was from ImmunoResearch; and anti-E-selectin (9A9) was purified from culture supernatant of hybridoma cells. All other antibodies were from BD Biosciences.

Bladder DTH protocol. On day 0 and day 1, female mice were painted with 25 μl DNFB (0.5% in acetone) on shaved dorsal skin. On day 5, mice were anesthetized, the hair surrounding the genitalia was removed with epilating creme (Nair; Carter Products) and the exposed skin was cleaned with alcohol. With the help of a dissecting microscope, a polyethylene catheter 10 cm in length (0.28-mm internal diameter and 0.61-mm outer diameter; Becton Dickinson) was introduced transurethrally into the bladder and was advanced until resistance was apparent. To minimize dilution and reflux of inocula, urine was drained by application of gentle suprapubic pressure. Mice were then challenged by instillation of freshly prepared DNBS (2 mg in 50 μl sterile 0.9% NaCl) into the bladder. To ensure consistent contact of the compound with the 'urothelium', the bladder was drained 30 min later and the injection was repeated. Controls included sensitized mice treated with intravesical instillation of sterile saline and nonsensitized mice challenged with DNBS. At 48 h after challenge, the bladder was removed and was used either for standard histology or for analysis of leukocyte content. For the latter, bladders were dissected longitudinally and were digested with mild agitation for 30 min at 37 °C with 400 U/ml of collagenase-D (Roche) plus 100 μg/ml of DNAseI (Roche). Bladders were then placed on a 70-μm nylon mesh cell strainer (BD Bioscience) and were dissociated into single-cell suspensions. Washed cells were counted with a hemocytometer and were stained for flow cytometry.

Ear CHS protocol. On day 0 and day 1, shaved dorsal skins of mice were painted with 0.5% DNFB or 10% OXA in 25 μl acetone. Alternatively, mice were immunized on day 0 with 7% picryl chloride (VeZerf Laborsynthesen) in 100 μl acetone. On day 5, the right ear was challenged with 0.25% DNFB or 1% OXA or 1% picryl chloride (10 μl to each side) and the left ear was painted with vehicle (acetone). Ear thickness to the first cartilage ridge was measured 24 h later with a micrometer (Mitutoyo). To account for acute hapten-induced irritation, background swelling was measured in parallel with that of non-sensitized mice. Hapten-specific ear swelling was calculated as follows: (treated ear thickness – control ear thickness) – background swelling. Results were highly reproducible when measurements made by three independent investigators (who in most experiments were 'blinded' to treatment protocol) were compared.

Culture of ear skin sheets. DNFB-challenged and vehicle-exposed ears of DNFB sensitized mice were removed 24 h after challenge, were sterilized with 70% ethanol and were separated along the cartilage with a forceps. The skin sheets were then placed for 14 h on top of 1 ml media (RPMI medium with 10% FCS and standard supplements) containing 100 ng/ml of CCL21 (R&D Systems), 100 nM leukotriene B_4 (Biomol) and 500 U/ml of recombinant human IL-2 (Roche). Emigrated leukocytes in the media were counted with a hemocytometer and their phenotype was analyzed by flow cytometry.

NK cell purification and adoptive transfer. *Rag2*$^{-/-}$ mice were injected subcutaneously with B16 melanoma cells that secrete the ligand for the receptor

tyrosine kinase Flt3 (Flt3L), which caused NK cell proliferation[55,56]. After 9 d, mice were assigned to one of two groups: one group was sensitized with DNFB (0.5% in 50 μl) on day 9 and day 10; the second group remained unsensitized. On day 12, NK cells were purified from splenocyte samples after red blood cell lysis (using 'ACK' lysis buffer) and from the liver with digestion with 400 U/ml of collagenase D (Roche Diagnostics; 37 °C for 30 min) followed by passage through a 40-μm filter, ACK lysis and 30 min of centrifugation over a Percoll gradient (40–60%; Amersham Biosciences). Samples were further enriched for NK cells by negative selection with Dynabeads (Dynal Biotech ASA) with depleting mAbs to Gr-1, I-A^b and Ter119 or to CD4, CD8α and Ter119. The negative selection step was omitted when liver NK cells were sorted separately. NK cells were then stained with phycoerythrin-labeled DX5 mAb and/or anti-Ly49C-I (5E6) and were sorted to more than 95% purity on a FACSVantage DiVa cell sorter (Becton Dickinson). Sorted NK cells were adoptively transferred into recipient mice by tail vein injection.

Immunofluorescence. Ear tissue was collected and was immediately frozen in optimum cutting temperature compound (Tissue-Tek). Sections 7 μm in thickness were cut onto poly-L-lysine-coated glass slides (Sigma Diagnostics), were fixed in 100% cold acetone for 10 min, were air-dried at 25 °C and were stored at –70 °C. For staining, slides were air-dried at 25 °C and were blocked with 5% mouse serum containing 3% BSA in PBS, pH 7.4. Phycoerythrin-conjugated anti-NK1.1 (PK136; Pharmingen) and purified anti-CD31 (MEC13.3; Pharmingen) were diluted in blocking solution and were used at a concentration of 1 μg/ml or 2.5 μg/ml, respectively. Sections were stained for 1 h at 25 °C with primary antibodies. CD31 was detected by incubation with carbocyanine-conjugated goat anti-rat IgG (Jackson ImmunoResearch). As a negative control, species-specific isotype-matched irrelevant antibodies were used. Sections were counterstained with 300 nM 4,6-diamindino-2-phenylindole (Molecular Probes).

Statistical analysis. All data are presented as mean ± s.e.m. and were analyzed by unpaired, one-way analysis of variance (ANOVA) with the Bonferroni multiple-comparison test unless specified otherwise. Significance was set at a P value of less than 0.05.

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AUTHOR CONTRIBUTIONS

J.G.O. designed and did the CHS ear experiments and adoptive transfers of sorted NK cells; M.G. designed the mouse bladder DTH model and did the experiments; and D.L.D. designed and did the analysis of NK cells in CHS ear experiments and adoptive transfers of sorted Ly49C$^+$ and Ly49C$^-$ NK cells.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Janeway, C.A., Jr. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).
2. Jung, D. & Alt, F.W. Unraveling V(D)J recombination; insights into gene regulation. *Cell* **116**, 299–311 (2004).
3. Bassing, C.H., Swat, W. & Alt, F.W. The mechanism and regulation of chromosomal V(D)J recombination. *Cell* **109**, S45–S55 (2002).
4. Kurtz, J. Specific memory within innate immune systems. *Trends Immunol.* **26**, 186–192 (2005).
5. Bloch, B. The role of idiosyncracy and allergy in dermatology. *Arch. Dermat. Syph.* **19**, 175–197 (1929).
6. Asherson, G.L. & Ptak, W. Contact and delayed hypersensitivity in the mouse. (I) Active sensitivity and passive transfer. *Immunology* **15**, 405–416 (1968).
7. Askenase, P.W. Yes T cells, but three different T cells (αβ, γδ and NK T cells), and also B-1 cells mediate contact sensitivity. *Clin. Exp. Immunol.* **125**, 345–350 (2001).
8. Wang, B., Feliciani, C., Freed, I., Cai, Q. & Sauder, D.N. Insights into molecular mechanisms of contact hypersensitivity gained from gene knockout studies. *J. Leukoc. Biol.* **70**, 185–191 (2001).
9. Marchal, G., Seman, M., Milon, G., Truffa-Bachi, P. & Zilberfarb, V. Local adoptive transfer of skin delayed-type hypersensitivity initiated by a single T lymphocyte. *J. Immunol.* **129**, 954–958 (1982).
10. Hochgeschwender, U. *et al.* Dominance of one T-cells receptor in the H-2K^b/TNP response. *Nature* **326**, 307–309 (1987).
11. Iglesias, A., Hansen-Hagge, T., von Bonin, A. & Weltzien, H.U. Increased frequency of 2,4,6-trinitrophenyl (TNP)-specific, H-2^b-restricted cytotoxic T lymphocyte precursors in transgenic mice expressing a T cell receptor chain gene from an H-2^b-restricted, TNP-specific cytolytic T cell clone. *Eur. J. Immunol.* **22**, 335–341 (1992).
12. Bajory, Z., Hutter, J., Krombach, F. & Messmer, K. New method: the intravital videomicroscopic characteristics of the microcirculation of the urinary bladder in rats. *Urol. Res.* **30**, 148–152 (2002).
13. Phanuphak, P., Moorhead, J.W. & Claman, H.N. Tolerance and contact sensitivity to DNFB in mice. I. *In vivo* detection by ear swelling and correlation with in vitro cell stimulation. *J. Immunol.* **112**, 115–123 (1974).
14. Shinkai, Y. *et al.* RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**, 855–867 (1992).
15. Boehncke, W.H. *et al.* Leukocyte extravasation as a target for anti-inflammatory therapy - Which molecule to choose? *Exp. Dermatol.* **14**, 70–80 (2005).
16. MacDougall, J.R., Croy, B.A., Chapeau, C. & Clark, D.A. Demonstration of a splenic cytotoxic effector cell in mice of genotype SCID/SCID.BG/BG. *Cell. Immunol.* **130**, 106–117 (1990).
17. Cao, X. *et al.* Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity* **2**, 223–238 (1995).
18. Goldman, J.P. *et al.* Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor γ chain. *Br. J. Haematol.* **103**, 335–342 (1998).
19. Young, W.W., Jr., Hakomori, S.I., Durdik, J.M. & Henney, C.S. Identification of ganglio-N-tetraosylceramide as a new cell surface marker for murine natural killer (NK) cells. *J. Immunol.* **124**, 199–201 (1980).
20. Schott, E., Bonasio, R. & Ploegh, H.L. Elimination *in vivo* of developing T cells by natural killer cells. *J. Exp. Med.* **198**, 1213–1224 (2003).
21. Herzog, W.R., Meade, R., Pettinicchi, A., Ptak, W. & Askenase, P.W. Nude mice produce a T cell-derived antigen-binding factor that mediates the early component of delayed-type hypersensitivity. *J. Immunol.* **142**, 1803–1812 (1989).
22. Vermijlen, D. *et al.* High-density oligonucleotide array analysis reveals extensive differences between freshly isolated blood and hepatic natural killer cells. *Eur. J. Immunol.* **34**, 2529–2540 (2004).
23. Zhao, Y., Ohdan, H., Manilay, J.O. & Sykes, M. NK cell tolerance in mixed allogeneic chimeras. *J. Immunol.* **170**, 5398–5405 (2003).
24. Biedermann, T. *et al.* Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. *J. Exp. Med.* **192**, 1441–1452 (2000).
25. Lanier, L.L. NK cell recognition. *Annu. Rev. Immunol.* **23**, 225–274 (2005).
26. Kim, S. *et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436**, 709–713 (2005).
27. Hanke, T. *et al.* Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* **11**, 67–77 (1999).
28. Catalina, M.D. *et al.* The route of antigen entry determines the requirement for L-selectin during immune responses. *J. Exp. Med.* **184**, 2341–2351 (1996).
29. Staite, N.D., Justen, J.M., Sly, L.M., Beaudet, A.L. & Bullard, D.C. Inhibition of delayed-type contact hypersensitivity in mice deficient in both E-selectin and P-selectin. *Blood* **88**, 2973–2979 (1996).
30. Grabbe, S. *et al.* β_2 integrins are required for skin homing of primed T cells but not for priming naive T cells. *J. Clin. Invest.* **109**, 183–192 (2002).
31. Raulet, D.H. Roles of the NKG2D immunoreceptor and its ligands. *Nat. Rev. Immunol.* **3**, 781–790 (2003).
32. Gasser, S., Orsulic, S., Brown, E.J. & Raulet, D.H. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* **436**, 1186–1190 (2005).
33. Bangert, C., Friedl, J., Stary, G., Stingl, G. & Kopp, T. Immunopathologic features of allergic contact dermatitis in humans: participation of plasmacytoid dendritic cells in the pathogenesis of the disease? *J. Invest. Dermatol.* **121**, 1409–1418 (2003).
34. Hunger, R.E., Yawalkar, N., Braathen, L.R. & Brand, C.U. The HECA-452 epitope is highly expressed on lymph cells derived from human skin. *Br. J. Dermatol.* **141**, 565–569 (1999).
35. von Andrian, U.H. & Mackay, C.R. T-cell function and migration. Two sides of the same coin. *N. Engl. J. Med.* **343**, 1020–1034 (2000).
36. Cumberbatch, M., Dearman, R.J., Griffiths, C.E. & Kimber, I. Epidermal Langerhans cell migration and sensitisation to chemical allergens. *APMIS* **111**, 797–804 (2003).
37. Rennert, P.D. *et al.* Essential role of lymph nodes in contact hypersensitivity revealed in lymphotoxin-α-deficient mice. *J. Exp. Med.* **193**, 1227–1238 (2001).
38. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-γ for T_H1 priming. *Nat. Immunol.* **5**, 1260–1265 (2004).
39. Degli-Esposti, M.A. & Smyth, M.J. Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nat. Rev. Immunol.* **5**, 112–124 (2005).
40. Weninger, W. *et al.* Specialized contributions by α(1,3)-fucosyltransferase-IV and FucT-VII during leukocyte rolling in dermal microvessels. *Immunity* **12**, 665–676 (2000).

41. Morris, M.A. & Ley, K. Trafficking of natural killer cells. *Curr. Mol. Med.* **4**, 431–438 (2004).
42. Robert, C. & Kupper, T.S. Inflammatory skin diseases, T cells, and immune surveillance. *N. Engl. J. Med.* **341**, 1817–1828 (1999).
43. Geissmann, F. *et al.* Intravascular immune surveillance by CXCR6$^+$ NKT cells patrolling liver sinusoids. *PLoS Biol.* **3**, e113 (2005).
44. Raulet, D.H. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* **5**, 996–1002 (2004).
45. Proteau, M.F., Rousselle, E. & Makrigiannis, A.P. Mapping of the BALB/c Ly49 cluster defines a minimal natural killer cell receptor gene repertoire. *Genomics* **84**, 669–677 (2004).
46. Jamieson, A.M. *et al.* The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17**, 19–29 (2002).
47. Pancer, Z. *et al.* Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* **430**, 174–180 (2004).
48. Galli, S.J. & Hammel, I. Unequivocal delayed hypersensitivity in mast cell-deficient and beige mice. *Science* **226**, 710–713 (1984).
49. Raff, M.C. Role of thymus-derived lymphocytes in the secondary humoral immune response in mice. *Nature* **226**, 1257–1258 (1970).
50. Snippe, H., Willems, P.J., Graven, W.G. & Kamp, E. Delayed hypersensitivity in the mouse induced by hapten-carrier complexes. *Immunology* **28**, 897–907 (1975).
51. Bour, H. *et al.* Major histocompatibility complex class I-restricted CD8$^+$ T cells and class II-restricted CD4$^+$ T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur. J. Immunol.* **25**, 3006–3010 (1995).
52. Andrews, D.M., Scalzo, A.A., Yokoyama, W.M., Smyth, M.J. & Degli-Esposti, M.A. Functional interactions between dendritic cells and NK cells during viral infection. *Nat. Immunol.* **4**, 175–181 (2003).
53. Fernandez, N.C. *et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses *in vivo*. *Nat. Med.* **5**, 405–411 (1999).
54. Szczepanik, M. *et al.* $\gamma\delta$ T cells from tolerized $\alpha\beta$ T cell receptor (TCR)-deficient mice inhibit contact sensitivity-effector T cells *in vivo*, and their interferon-γ production *in vitro*. *J. Exp. Med.* **184**, 2129–2139 (1996).
55. Mora, J.R. *et al.* Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424**, 88–93 (2003).
56. Shaw, S.G., Maung, A.A., Steptoe, R.J., Thomson, A.W. & Vujanovic, N.L. Expansion of functional NK cells in multiple tissue compartments of mice treated with Flt3-ligand: implications for anti-cancer and anti-viral therapy. *J. Immunol.* **161**, 2817–2824 (1998).