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Critical role for CXCR6 in NK cell-mediated antigen-specific memory to haptens and viruses

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Abstract

Hepatic natural killer (NK) cells mediate antigen (Ag)-specific contact hypersensitivity (CHS) in T-cell and B-cell deficient mice. We now report that hepatic, but not splenic or naïve NK cells also develop specific memory to vaccines containing Ags from influenza, vesicular stomatitis virus (VSV) or human immunodeficiency virus-1 (HIV). Adoptive transfer of virus-sensitized NK cells to naïve recipients enhanced the animals' survival upon lethal challenge with the sensitizing virus, but not a different virus. NK cell memory to haptens and viruses depended upon CXCR6, a chemokine receptor on hepatic NK cells that was required for memory NK cell persistence but not for Ag recognition. Hence, hepatic NK-cells can develop adaptive immunity to structurally diverse Ags, an activity that requires NK-cell-expressed CXCR6.

Introduction

The current textbook view of adaptive immunity is that it is mediated exclusively by T- and B-cells, which rely on recombination-activating gene (Rag)-dependent non-homologous recombination of V(D)J gene segments to generate a diverse repertoire of T- and B-cell receptors[1]. Activation of such receptors by cognate Ag triggers clonal selection, differentiation into short-lived effectors and long-lived memory cells, which, upon Ag re-challenge, mount accelerated and enhanced recall responses. Classic examples of such Ag-

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specific memory, the hallmark of adaptive immunity, include delayed type hypersensitivity (DTH)[2, 3] and hapten-induced CHS[4, 5].

Like T- and B-cells, NK-cells mediate resistance to tumors and infections by a variety of mechanisms that are still incompletely understood[6]. However, NK-cells cannot express Rag-dependent receptors, but use germline encoded receptors that integrate activating and dampening signals[7]. Notable examples of murine NK receptors that detect specific pathogen-associated structures include NKp46 and Ly49H, which recognize influenza A-encoded hemaglutinin (HA)[8, 9] and mouse cytomegalovirus (MCMV)-encoded m157[10], respectively. Mouse NK-cells can express just one or random combinations of several Ly49 receptors, some of which recognize host MHC-I[11, 12]. Ly49H is a member of this family and confers NK-cell-mediated resistance to MCMV infection in C57BL/6 mice. Remarkably, Ly49H$^+$ NK-cells display features of adaptive immunity upon infection with this virus[13]. However, since Ly49H is only found in the C57BL/6 strain[14], it has been unclear whether NK-cells can develop virus-specific memory in other genetic backgrounds or in response to viruses other than MCMV. This question may also be clinically relevant, since certain NK-cell subsets are implicated in the ability of rare HIV-infected individuals to exert long-term control over HIV-1 replication[15, 16].

The idea that NK-cells can mediate adaptive immunity was initially suggested by observations that mouse strains that lacked T- and B-cells developed vigorous CHS responses to different haptens[17, 18]. These responses possessed the hallmarks of adaptive immunity: they were sensitization dependent, persisted for at least four weeks and were only elicited by haptens to which mice had previously been sensitized[17]. Several lines of evidence established that NK-cells were necessary and sufficient for this activity; CHS responses in *Rag2*$^{-/-}$ mice were abolished upon depletion of NK-cells and absent in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, which lack all lymphocytes, including NK-cells[19]. Moreover, following sensitization of donor mice with 2,4-dinitro-1-fluorobenzene (DNFB), adoptive transfer of NK-cells from donor livers, but not from spleens conferred vigorous CHS to naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. Among hepatic NK-cells, DNFB-specific memory was concentrated in a subset expressing Thy1 and Ly49C/I[17]. However, these and all other NK receptors described to date are similarly represented among hapten-reactive (i.e. hepatic) and non-reactive (non-hepatic) NK-cells[17, 20]. Thus, although classic NK receptors contribute to the function of NK-cells, it is unclear whether and to what extent they are involved in the unique adaptive properties of hapten-specific hepatic NK-cells.

It is also unknown why hapten-specific memory NK-cells reside preferentially in the liver. This restricted distribution is similar to NKT cells, which express Rag-dependent invariable T cell receptors[21] and whose intra-hepatic survival and homeostasis requires CXCR6 (CD186), a chemokine receptor that is prominently expressed on NKT cells, a subset of activated T cells and some NK cells[22, 23]. CXCR6 has a single ligand, CXCL16, a chemokine that is constitutively expressed on liver sinusoidal endothelium[24, 25]. Although *Cxcr6*-deficient mice have been described, the function of CXCR6$^+$ NK-cells has not been investigated[22, 26].

We undertook the present study to address several questions. First, we set out to further characterize hapten-specific memory NK-cells and the role of Ag in their maintenance and trafficking Second, we investigated whether NK-cells can develop memory to viruses other then MCMV and whether this ability depended on the host's genetic background or the pathogenicity of the virus. Third, we explored the role of CXCR6 in both hapten- and virus-specific memory NK-cells. Our results obtained in two different mouse strains indicate that a discrete subset of $CXCR6^+$ NK-cells can acquire and retain Ag-specific memory to at least five structurally, chemically and biologically distinct exogenous Ags without requiring Ag persistence. In each case, memory NK-cells were concentrated in the liver and were critically dependent upon CXCR6, which was not required for Ag recognition but appears to regulate effector function and survival of memory NK-cells.

RESULTS

Ag-independent persistence of memory NK cells

Previous experiments in *Rag*$2^{-/-}$mice showed that NK-cells retain memory to DNFB up to four weeks after skin sensitization[17]. However, haptens form covalent adducts with self proteins, which may persist in the body for weeks[27], so it was unclear if NK memory was maintained by residual Ag. To address this, we adoptively transferred sensitized hepatic $Thy1^+$ NK-cells into Ag-free recipients. *Rag*$1^{-/-}$ mice were either left naïve (i.e. treated with solvent) or sensitized with DNFB or oxazolone (OXA) and used as donors for adoptive transfers of FACS-sorted NK-cells into *Rag*$2^{-/-}$*Il2rg*$^{-/-}$ recipients, which were chosen because this strain cannot produce lymphocytes, including NK-cells[19], [28], allowing for easy recovery and analysis of donor NK-cells. One day or four months later, recipients were challenged by painting one ear with hapten, the other with solvent, and ear swelling was measured after 24h (Fig. 1a). Whereas recipients of sensitized splenic NK-cells (not shown) or naïve hepatic NK-cells were unresponsive, the challenged ears of DNFB- or OXA-primed hepatic NK-cell recipients became markedly inflamed at both time points. A response was only elicited when animals were challenged with the sensitizing hapten, but not the other hapten. The difference in recall capacity between transferred NK-cell subsets cannot be explained by differential survival, as all groups harbored similar NK-cell numbers four months after transfer (Fig. 1b). Indeed, consistent with earlier reports[29], the transferred NK-cells expanded ~5-7-fold in the lymphopenic *Rag*$2^{-/-}$*Il2rg*$^{-/-}$recipients irrespective of their organ of origin or immunization history.

While these results indicated that lymphopenia-induced proliferation is not unique to memory NK cells, our findings raised the possibility that NK-cell proliferation was necessary to maintain memory. To address this, we sorted green fluorescent protein (GFP)-tagged NK-cells from naïve and sensitized Rag-sufficient, actin-GFP transgenic donors (C57BL/6) and transferred them into wild-type (WT) mice that were challenged six weeks later. WT recipients of DNFB or OXA primed hepatic NK-cells also mounted vigorous CHS responses when challenged with the sensitizing but not the non-sensitizing hapten, while recipients of sensitized splenic or naïve NK-cells remained unresponsive (Fig. 1c). All recipients harbored similar numbers of GFP^+ NK-cells eight weeks post transfer; the numbers recovered at this time were 24–28% of the original input, suggesting that NK-cells

did not expand (Supplementary Fig. 1a). Thus, NK-cells persist for several months in both lymphocyte sufficient and lymphopenic hosts. Although survival and expansion of adoptively transferred NK-cells are independent of prior sensitization or the source organ, only sensitized hepatic NK-cells acquire transferable Ag-specific memory and do so irrespective of the presence of other lymphocytes.

Accumulation of memory NK-cells at effector sites

While NK-cells are known to require endothelial selectins and β2 integrins to access challenged ears of DNFB-sensitized mice[17], it remained unclear whether this recruitment is Ag-specific. Thus, we sorted NK-cells from naïve CD45.1$^+$ mice and CD45.2$^+$ WT and actin-GFP transgenic donors that had been alternately sensitized with DNFB or OXA. Equal numbers of each population were mixed and injected into naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. One month later, recipient ears were challenged with hapten and ears and livers harvested at various time-points to determine frequency and genotype of tissue-resident NK-cells. While recipient livers contained equal numbers of all three donor cell subsets at every time-point, infiltrating NK-cells in hapten-challenged ears were almost exclusively derived from donors that had been sensitized with the hapten used for challenge (Fig. 1d, Supplementary Fig. 1b). Thus, only DNFB-sensitized NK-cells were recruited to DNFB-challenged ears, while OXA-sensitized NK-cells preferentially accumulated in OXA-challenged ears. We conclude that memory NK-cells not only discriminate between hapten-based Ags to mediate specific CHS responses, but also their recruitment and/or retention at sites of challenge is hapten-specific.

NK-cell memory to viruses

Having confirmed that hepatic memory NK-cells are long-lived and selectively migrate to sites of hapten challenge to mediate Ag-specific recall responses even after a prolonged Ag-free interval, we asked whether NK-cells also develop memory to other exogenous Ags. It was reported recently that NK-cells display features of adaptive immunity in MCMV-infected C57BL/6 mice[13], in which ~50% of NK-cells express Ly49H, which recognizes MCMV-encoded m157 protein. After a primary challenge with MCMV, NK-cells proliferated and gave rise to Ly49H-dependent protective memory. However, unlike hapten-specific memory NK-cells, MCMV-restricted NK-cells are not liver-restricted[13] and have not been found in other mouse strains, most of which do not express Ly49H[14].

To assess more broadly whether NK-cells can develop anti-viral memory, we immunized *Rag1*$^{-/-}$ mice subcutaneously with either noninfectious virus-like particles (VLPs) containing hemagglutinin (HA) and matrix protein (M1) from influenza A/PR/8/34[30], [31] (Supplementary Fig. 2) or UV-inactivated vesicular stomatitis virus (UV-VSV)[32]. Splenic and hepatic naïve and immunized NK-cells were then sorted and adoptively transferred to naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Recipient mice were challenged four weeks later by injection of PR8-VLPs or UV-VSV into one ear and PBS into the contralateral ear. Hepatic NK-cells mediated vigorous and sustained DTH responses to both influenza VLPs and VSV in an immunization-dependent and virus-specific manner, while recipients of sensitized splenic or naïve NK-cells were unresponsive (Fig. 2a).

NK-cell-mediated protection against lethal viral infection

Since these findings indicated that hepatic NK-cells specifically recognize non-infectious viral Ags, we tested if anti-viral NK-cell memory confers protection against lethal viral challenge. Indeed, when *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were given 8×10^4 PR8-VLP-primed hepatic NK-cells and challenged three months later with a lethal dose of influenza A/PR/8/34, they survived longer than naïve animals (median survival time: 26 vs. 23 days, respectively). By contrast, primed splenic or naïve hepatic NK-cells did not confer protection (Fig. 2b).

When recipients of PR8-VLP sensitized hepatic NK-cells were first used in DTH assays using PR8 VLPs and two month later infected with intact virus, the magnitude of the DTH-induced ear swelling correlated with both the Ag dose used for donor sensitization and the degree of protection against subsequent lethal infection (Fig. 2c). Analogous to our findings with haptens, PR8-VLP-sensitized splenic NK-cells conferred very low or absent DTH responses and failed to protect against influenza infection, indicating that memory NK-cells are excluded from the spleen irrespective of their Ag specificity. Of note, recipients of NK-cells purified from lungs of PR8-VLP sensitized donors also displayed a modest DTH response and protection against viral challenge, albeit to a lesser degree than hepatic NK-cell recipients, indicating that some memory NK-cells reside in the lungs (Fig. 2c). Importantly, the protection afforded by NK-cells was virus-specific, since immunization of *Rag1*$^{-/-}$ mice with two different formulations of influenza VLPs protected vaccinated animals against lethal challenge with influenza, but not VSV (Fig. 2d). Analogously, UV-VSV-immunized *Rag1*$^{-/-}$ mice survived longer than PBS-treated controls upon challenge with VSV, but not influenza.

While the experiments above suggest that NK-cells may be attractive vaccine targets, all recipients of memory NK-cells eventually succumbed to VSV infection (Fig. 2d). This could either reflect an inability of memory NK-cells to confer sterilizing immunity or the number of memory NK-cells may have been insufficient for the relatively high load and systemic dissemination of VSV in our protocol (500 pfu IV). To distinguish between these possibilities, we monitored the survival of UV-VSV immunized and naïve *Rag2*$^{-/-}$ Balb/c mice in response to localized infection with the LD_{50} for this strain (250 pfu VSV IM). Upon challenge with this protocol nearly all immunized animals survived, while ~half of the naïve and control immunized mice died, as expected (Fig. 2f). Thus, in the absence of T and B-cells, memory NK-cells can provide effective and specific protection against certain viruses, at least when the infectious agent is administered locally and at a moderate dose.

NK sensitization to influenza A does not require hemagglutinin

Influenza A-encoded HA is a ligand for Nkp46, an activating receptor on murine (and human) NK-cells[8, 9, 33]. However, Nkp46 is found on all NK-cells irrespective of anatomic localization, which seemed at odds with the finding that only hepatic (and pulmonary) but not splenic NK-cells developed influenza-specific memory. Therefore, we tested whether HA recognition is required for influenza-specific memory NK-cells by immunizing *Rag1*$^{-/-}$ mice with HA-free M1-VLPs (Supplementary Fig. 2) and comparing the ensuing DTH response to that elicited by HA$^+$ PR8-VLPs. Irrespective of their HA content, both VLPs efficiently cross-sensitized mice, indicating that NK-cells do not require HA to recognize

influenza (Fig. 2f). Indeed, immunization of *Rag1*$^{-/-}$ mice with either HA^{+} PR8-VLPs or HA^{-} M1-VLPs significantly and equivalently prolonged the animals' survival upon lethal challenge with influenza, but not VSV (Fig. 2d). While the above findings do not rule out a role for Nkp46–HA interactions, they indicate that this pathway is neither required nor sufficient for protection against influenza. However, it is possible that mice have evolved other pattern receptors for influenza and/or VSV, since mice are susceptible to these viruses[14]. Thus, we wondered if murine NK-cells could recognize and remember a virus that could not have imposed evolutionary pressure on mice.

Murine NK-cells develop memory to HIV-1

We addressed this question by sensitizing *Rag1*$^{-/-}$ mice with VLPs containing HIV-1-derived group antigen and envelope (gag/env)[34]. HIV-1 is a lentivirus that causes acquired immunodeficiency syndrome (AIDS) in humans, whereas mice are resistant to HIV-1 infection[35]. One month after donor sensitization with HIV-1-VLPs, we transferred splenic or hepatic NK-cells to naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients and challenged recipient ears one month later by injecting either HIV-1-VLPs or PR8-VLPs. Primed hepatic, but not splenic or naïve NK-cells mounted a vigorous recall response to HIV-1, but not influenza A (Fig. 3a). Moreover, Rag-independent memory to HIV-1 and influenza was inducible in both C57BL/6 and BALB/c mice and was always specific for the virus used during sensitization (Fig. 3b), indicating that hepatic NK-cells can develop specific memory to diverse viral Ags irrespective of viral host restrictions or genetic background

CXCR6 in hepatic NK cell-mediated CHS

Next, we set out to explore why memory NK-cells are apparently restricted to the liver. This organ provides a specialized milieu for NKT cells, which require CXCL16, a chemokine ligand for CXCR6 that is constitutively expressed on hepatic sinusoidal endothelium[22, 36]. CXCR6 is also expressed on hepatic NK cells[22], but its significance for NK-cell biology had been unclear. Using *Cxcr6*$^{+/gfp}$ mice in which one allele of *Cxcr6* was replaced with eGFP[22], we determined that 35–55% of hepatic NK-cells, but only 3–5% of splenic NK-cells express CXCR6. This frequency was unchanged in Rag1$^{-/-}$CXCR6$^{+/gfp}$ mice (Fig. 4a) in which NK-cells were the only GFP^{+} hepatic leukocytes (not shown). To assess the role of CXCR6, we explored its contribution to hapten-induced CHS using three complementary approaches: adoptive transfer of sensitized *Cxcr6*$^{+/gfp}$ NK-cells that were sorted into CXCR6-expressing (GFP^{+}) and non-expressing (GFP^{-}) subsets (Fig. 4b, Supplementary Fig. 3a–c); direct sensitization and challenge of WT or *Rag1*$^{-/-}$*Cxcr6*$^{gfp/gfp}$ and *Rag1*$^{-/-}$*Cxcr6*$^{+/gfp}$ littermates (Fig. 4c, Supplementary Fig. 4a); and treatment of sensitized WT or *Rag1*$^{-/-}$ mice with anti-CXCR6 (Fig. 4d, Supplementary Fig. 4b).

Following adoptive transfer, all NK-cell subsets proliferated equivalently in naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts (Supplementary Fig. 3b), but only primed GFP^{+} hepatic NK-cells elicited CHS responses, indicating that memory NK-cells are concentrated within the CXCR6^{+} subset (Fig. 4b, Supplementary Fig. 3a). On the other hand, adoptive transfer of GFP^{+} splenic NK-cells failed to mediate CHS, suggesting that CXCR6 expression is insufficient to develop memory. Following transfer, GFP^{+} hepatic NK-cells persisted unchanged for at least six weeks (Supplementary Fig. 3c), while some loss of GFP was seen

among GFP$^+$ splenic NK-cells, and ~20% of liver-derived (but not splenic) GFP$^-$ NK-cells became GFP$^+$. It is unlear whether this conversion reflects differentiation of mature NK-cells or *de novo* development from GFP$^-$ precursors.

While CXCR6 alone is apparently insufficient to enable NK-cell memory, our adoptive transfer experiments are consistent with a role in NK-cell-mediated adaptive immunity. Thus, we compared the responsiveness of *Cxcr6*$^{+/gfp}$ and *Cxcr6*$^{gfp/gfp}$ mice to DNFB and OXA. CHS responses were markedly decreased, but not abolished in *Cxcr6*$^{gfp/gfp}$ animals that contained T and B-cells (Fig. 4c, **left**), whereas *Rag1*$^{-/-}$*Cxcr6*$^{gfp/gfp}$ mice were unresponsive to both DNFB (Fig. 4c, **right**) and OXA (Supplementary Fig. 4a). Hence, in the absence of T and B-cells, NK cell-expressed CXCR6 is absolutely required for NK-cell-mediated CHS, while WT mice depend only partially on CXCR6. Because NK-cell depletion in WT mice does not reduce CHS responses[17], the compromised response in *Cxcr6*$^{gfp/gfp}$ mice probably reflects a combined effect on NK and NKT cells[37].

The above results are consistent with three non-exclusive roles for CXCR6 in 1. development or survival, 2, trafficking or 3. effector function of memory NK-cells. To address the latter possibility, sensitized mice were given neutralizing anti-CXCR6 (Supplementary Fig. 4b) or isotype-matched control mAb 12h before hapten-challenge and CHS responses were compared. Anti-CXCR6 reduced CHS responses of WT mice to DNFB (Fig. 4d, **left**) and OXA (Supplementary Fig. 4c, **left**), and abolished such responses in sensitized *Rag1*$^{-/-}$ animals (Fig. 4d, **right**, Supplementary Fig.4c, **right**). This effect of anti-CXCR6 was not due to NK-cell depletion, nor did short-term mAb treatment result in relocalization of *Cxcr6*$^{+/gfp}$ NK-cells to organs other than the liver (Supplementary Fig. 4b). Following treatment of WT or *Rag1*$^{-/-}$ mice with anti-CXCR6 or anti-CXCL16, the number of *Cxcr6*$^{+/gfp}$ NK-cells that were recovered from multiple organs was similar to control mAb-treated animals.

Having established that memory NK-cells require CXCR6 for CHS, we asked if this pathway plays a role in anti-viral NK-cell memory. *Rag1*$^{-/-}$ mice were sensitized to UV-VSV or influenza- or HIV-1-VLPs and treated with anti-CXCR6 or isotype 12h before challenge by injection of viral recall Ag into one ear. The ensuing DTH response was abolished after CXCR6 blockade irrespective of the type of viral Ag or the animals' MHC I haplotype (Fig. 5a). Furthermore, anti-CXCR6 abolished the protective effect of memory NK-cells upon lethal challenge with influenza or VSV (Fig. 5b). Thus, for each of the five antigenic entities tested, CXCR6 was essential for NK-cell-mediated adaptive immunity irrespective of the genetic background.

Role of CXCR6 in memory NK-cell homeostasis

CXCR6 may exert this critical activity, at least in part, by regulating hepatic NK-cell homeostasis, similar to its role in hepatic NKT cell survival[22]. Compared to *Cxcr6*$^{+/-}$ mice, steady-state livers of *Cxcr6*$^{-/-}$ mice contained normal number of GFP$^-$ NK-cells, but significantly reduced numbers of GFP$^+$ NK-cells (Fig. 6a,b **and** Supplementary Fig. 5a), while the small fraction of GFP$^+$ NK-cells in other organs remained unchanged (Fig. 6a). Thus, CXCR6 is required for the development and/or survival of CXCR6$^+$ NK-cells exclusively in the liver where CXCL16 is constitutively expressed. Concomitantly, four

weeks after adoptive transfer of equal numbers of GFP^+ and GFP^- hapten-sensitized NK-cells from *Cxcr6*$^{+/-}$ donors, the GFP^- NK-cells were equally distributed between spleens and livers, while the distribution of the GFP^+ subset was strongly biased toward the liver (Fig. 6c). Moreover, when we transferred equal numbers of GFP^+ and GFP^- hepatic NK-cells sorted from *Cxcr6*$^{-/-}$ or *Cxcr6*$^{-/-}$ donors, the CXCR6-heterozygous NK-cells expanded in the liver of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients irrespective of their GFP expression, while in recipients of CXCR6-deficient NK-cells only the GFP^- subset expanded, whereas GFP^+ NK-cells were very rare (Fig. 6d). Furthermore, adoptive transfer of DNFB primed GFP^+ hepatic NK-cells to naïve hosts only conferred hapten responsiveness when NK-cells were from CXCR6$^{+/gfp}$ donors, whereas the same number of *Cxcr6*$^{-/-}$ NK-cells did not transfer hapten sensitivity (Fig. 6e) and survived poorly even upon rechallenge with DNFB (Supplementary Fig. 5b).

NK cell-mediated hapten-specific cytotoxicity

Given that a relatively short (12h) mAb-induced inhibition of CXCR6 efficiently blocked NK cell-mediated responses to haptens and viruses *in vivo*, we tested if CXCR6 regulates acute NK effector functions such as degranulation and killing. Naïve or primed hepatic or splenic NK-cells were cocultured with target B-cells that had either been surface-modified with DNBS, a water-soluble analog of DNFB, or mock treated. Ratios of control:haptenated B-cells were determined in the presence or absence of NK-cells 12 h later. Only DNFB-sensitized hepatic NK-cells killed DNBS haptenated targets, whereas no other subset showed hapten-specific killing activity (Fig. 7a). Furthermore, only the $CXCR6^+$ hepatic NK-cell subset was capable of killing DNBS-modified targets (Fig. 7b), which is in excellent agreement with our CHS-DTH experiments. However, all NK-cell samples killed MHC-I deficient target cells with similar efficiency, indicating that no subset was compromised in its ability to kill appropriate targets *per se*.

Addition of CXCR6 mAb to cytotoxicity assays boosted killing of haptenated B-cells by *Cxcr6*$^{+/-}$ NK cells, especially at suboptimal target:effector (T:E) ratios (Fig. 7b,c). We therefore compared NK-cell mediated hapten-specific killing in the presence of blocking mAb s to CXCR6 or CXCL16 or of recombinant CXCL16 at a T:E ratio of 1:25. Blockade of the CXCR6-CXCL16 pathway significantly enhanced the cytotoxicity of sensitized hepatic NK cells, while addition of CXCL16 significantly dampened the response (Fig. 7c). We then measured the frequency of actively degranulating NK cells, using transient surface expression of lysosomal Lamp-1a (CD107a)[38], about 10% of hepatic, but not splenic DNFB-sensitized NK-cells degranulated upon exposure to DNBS-modified target cells, whereas naïve and OXA-sensitized hepatic NK-cells did not respond (Fig. 7d,e **and** Supplementary Fig. 6). Interestingly, addition of anti-CXCR6 during the 3h *in vitro* coculture did not affect degranulation, whereas DNFB-sensitized NK-cells obtained from animals 12h after *in vivo* administration of anti-CXCR6 failed to upregulate Lamp-1a. In conclusion, besides the wide-spread capacity of NK-cells to respond to `missing self'[39], the hepatic memory NK-cell subset has the unique ability to exert cytotoxic activity also upon encounter of MHC-I sufficient target cells that are decorated with an Ag to which they were previously sensitized. This adaptive capacity appears to be rapidly lost *in vivo* when CXCR6 signaling is disrupted, even though CXCR6 is apparently not required for hapten

recognition, but rather attenuates the cytotoxic effector activity of memory NK-cells during the *in vitro* assay.

DISCUSSION

1594

This study identifies a subset of hepatic NK-cells that could acquire Ag-specific memory to at least five structurally, chemically and biologically distinct Ags. Adaptive immune responses mediated by this population depend upon CXCR6, which is expressed on ~half of helpatic NK-cells, suggesting that the liver harbors two different NK-cell populations. $CXCR6^{-}$ NK-cells share features with splenic NK-cells; their survival and homeostasis is CXCR6-independent and they cannot develop memory to haptens or viruses (except perhaps MCMV). By contrast, the second hepatic NK-cell subset constitutively transcribes the *cxcr6* locus and contains cells that can acquire long-lived specific memory to diverse Ags, including haptens and complex assemblies of viral glycoproteins. The paucity of GFP^{+} NK-cells in livers of $CXCR6^{gfp/gfp}$ mice indicates that the $CXCR6^{+}$ subset, unlike $CXCR6^{-}$ NK-cells, requires sustenance from sinusoidal endothelium, which constitutively presents CXCL16, a chemokine that exists as both a secreted polypeptide and a luminal surface transmembrane protein.

The restricted steady-state distribution of CXCl16, the only known ligand of CXCR6, may explain why memory NK-cells are undetectable in blood[40] or spleen[17]. On the other hand, the few $CXCR6^{+}$ splenic NK-cells were incapable of mediating recall responses, indicating that CXCR6 is required but not sufficient to induce and/or maintain memory. Indeed, the role of CXCR6 appears complex. Anti-CXCR6 injection 24h before Ag challenge inhibited NK-cell-mediated recall responses *in vivo* without depleting $CXCR6^{+}$ NK-cells. Since Ag recognition by NK-cells remained intact after anti-CXCR6 exposure *in vitro*, the *in vivo* effects most likely reflect a role for CXCR6–CXCL16 in maintaining memory NK-cell differentiation and/or function. Moreover, anti-CXCR6 enhanced the Ag-specific cytotoxicity of memory NK-cells *in vitro*, whereas CXCL16 attenuated cytotoxicity. By contrast, killing of MHC-I deficient targets remained unaffected, suggesting that CXCR6 modulates a proximal signalling step downstream of Ag recognition but upstream of the terminal pathway that enables cytotoxicity triggered by activating NK-receptors. Of note, anti-CXCR6 did not alter the frequency of the ~10% hepatic NK-cells that degranulated upon Ag exposure, suggesting that anti-CXCR6 boosted killing by a finite pool of hapten-specific NK-cells without increasing the size of that pool.

The hepatic milieu might have two effects on memory NK-cells: on one hand, continuous CXCR6 signaling, presumably triggered by transmembrane and/or secreted CXCL16 in sinusoids, promotes long-term survival and effector potential. On the other hand, tonic CXCR6 engagement might safeguard against NK-cell-mediated hepatotoxicity by preventing full-fledged killing activity. Once memory NK-cells are released from the liver and access peripheral sites of Ag challenge, the absence of CXCR6 ligands may unleash their full effector potential but might also limit their persistence. However, it should be noted that CXCL16 can be upregulated on activated DCs[25] and in inflamed lung[23] and

skin[41]. This might provide a `home away from home' to sustain memory NK-cells at extra-hepatic effector sites.

Although CXCR6 is apparently not directly involved in Ag recognition by NK-cells, it will be important to assess whether and how it regulates the underlying molecular mechanism(s) of Ag recognition, which remain(s) elusive. None of the known markers associated with memory NK-cells[17, 20] are sufficient to confer hapten specificity, as they are also found on splenic NK cells. Furthermore, hapten sensitization does not change the expression of Ly49 receptors or NKG2D or mRNAs for any other known NK-cell expressed activating receptors (our unpublished data). In this regard, hapten-specific NK-cells differ from MCMV-specific memory NK-cells, which upregulate KLRG1,CD43, Ly6C, and Ly49H and are found in both liver and spleen[13].

While engagement of inhibitory receptors by self-MHC-I usually blocks NK-cell activation, DNFB-sensitized hepatic NK-cells killed DNBS-haptenated B cells, which expressed MHC-I. Conceivably, covalent DNBS modification of target B-cell proteins could have included MHC-I molecules. However, this effect could not have masked MHC-I recognition by NK-cells because DNBS-modified B cells were not lysed by splenic or OXA-sensitized hepatic NK-cells, which killed MHC-I-deficient targets efficiently. Thus, the pathway(s) triggered in memory NK cells by cognate Ag overrides inhibitory signals from MHC-I. Moreover, NK-cell memory to haptens and viruses developed in both Balb/c and C57BL/6 mice, which express distinct MHC and Ly49 haplotypes, suggesting that Ag recognition by hepatic memory NK-cells differs from the way by which conventional NK-cells detect pathological cells with altered or absent MHC-I.

Whatever the mechanism, the finding that T and B cell-deficient mice can develop protective memory against lethal infections with influenza A and VSV has intriguing implications for vaccine development and anti-viral immunity. These results are consistent with recent investigations on NK cell memory to MCMV[13], although there are differences between the tissue distribution of the ensuing memory NK-cells, genetic background restriction and possibly also the viral recognition mechanism. NK-cells in C57BL/6 mice express LY49H, a germline-encoded activating receptor that is necessary for MCMV recognition and memory[13]. Similarly, murine and human NK-cells use NKp46 to detect influenza-derived HA[8, 9]. However, hepatic NK-cells developed influenza-specific memory without exposure to HA, while splenic $NKp46^{+}$ NK-cells did not develop memory. Thus, NKp46 is neither required nor sufficient to generate influenza-specific memory NK-cells.

We cannot rule out that NK-cells express other, as yet unidentified, germline-encoded receptors that recognize influenza or VSV Ags. Such receptors could conceivably have evolved since mice are susceptible to these viruses, albeit neither is endemic in wild mice[14]. However, murine NK cells also recognized and remembered HIV-1, which does not infect mice and, therefore, is unlikely to have driven the evolution of specific recognition mechanisms.

Of note, specific NK-cell haplotypes are over-represented in HIV-infected individuals who exert long-term control over HIV-1 replication and progression to AIDS[15, 42]. These

patients harbor a subset of NK-cells expressing KIR3DS1, which inhibited HIV-1 replication *in vitro*[16]. Intriguingly, a recent multiple-cohort genome-wide association study identified the rs2234358 polymorphism in the *CXCR6* gene as another marker that was strongly associated with long-term non-progression to AIDS, independent of other genetic polymorphisms[43]. CXCR6 is a minor coreceptor in HIV-1 infection, but it is unclear if this function is responsible for its role in HIV progression. Although CXCR6 is expressed on some human NK-cells[44], it is uknown whether this or other human NK populations possess distinct functional properties, such as the capacity to carry Ag-specific memory. If such functions exist, they could potentially be exploited to develop novel vaccines or to treat primary or acquired immunodeficiencies.

Materials and Methods

Mice

Rag1$^{-/-}$ (C57BL/6), *Rag2*$^{-/-}$(BALB/c), *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (C57BL/6xC57BL/10 F1)[45], and *Cxcr6*$^{-/-}$ (C57BL/6), and 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 (Boston, Massachusetts). Actin-gfp (C57BL/6), *Cxcr6*$^{+/-}$ and *Cxcr6*$^{-/-}$ mice on the C57BL/6 *Rag1*$^{-/-}$ background were generated and bred in-house.

Cell isolations and cell sorting

Spleens, lungs, lymph nodes, bone marrow, and livers were cut into small pieces with a sterile scalpel, and passed through 40μm mesh filters. For analysis of skin-infiltrating lymphocytes, mouse ears harvested and ear sheets mechanically pulled apart, cut into small pieces with a sterile scalpel, digested using collagenase D (5mg/ml in PBS 2% bovine serum), and passed through 40μm mesh filters. Lymphocytes were enriched by density gradient centrifugation using Nycodenz (Cederlane Labs) according to manufacturers protocol. For FACS analysis or cell sorting, cells were stained using NK1.1, TCRβ and TCRδ or CD3, Thy1, CD45, Lamp-1, and CXCR6 (BD Pharmingen, Bioledgend, and R&D Biosciences), and FACS samples acquired on a BD FACS CANTO and analyzed using FlowJo software. NK-cells were identified as CD45^{+} NK1.1^{+} CD3^{-} or TCR^{-}, and sometimes also Thy1^{+} and/or gfp^{+}. Cell sorting was carried out on a BD FACS ARIA using Diva software, and cell purity for all experiments was > 98%.

CHS and DTH

Mice were sensitized days 0 and 1 by skin painting on the shaved abdomen with 50μl 0.5% DNFB in acetone, or 50μl 5% OXA in acetone/methanol (1:1). Control mice received solvent only (Sigma-Aldrich). On day 4, mice were either used as donors for cell sorting, or their right ear challenged with 20μl of 0.2% DNFB in acetone, or 20μl of 1% OXA in acetone/methanol, and the left ear painted with vehicle. Viral DTH was induced by subcutaneous immunization of Rag$^{-/-}$ mice with PBS (control) or 5μg of viral Ag days 0 and 7. One month later, mice were either used as donors for adoptive transfer experiments, or challenged subcutaneously with 25μl PBS containing 2μg viral antigen into one ear, and PBS into the control ear. Ear thickness was measured every 24 hrs by micrometer (Mitutoyo, Japan (No. 193-101)). To account for acute hapten- or viral-induced irritation,

background swelling was measured using naïve mice. Sensitization dependent, antigen specific ear swelling was calculated as: [treated ear thickness – control ear thickness] – background swelling.

Generation of VLPs and influenza virus for live virus challenge

Generation of VLPs has previously been described[30, 34]. Briefly, Spodoptera frugiperda (Sf9) cells were coinfected with recombinant baculovirus (rBVs) expressing HA and/or M1, or gag and env. Culture supernatants were harvested at 3 days post infection, cleared by low-speed centrifugation (2000×g for 20min at 4°C), VLPs were concentrated using cross flow filtration, and purified through a 20–30–60% discontinuous sucrose gradient at 100,000×g for 1h at 4°C. The VLP bands were collected, dialyzed against phosphate buffered saline and analyzed by western blot. Mouse adapted influenza A/PR/8/34 virus was grown once in 10-day old embryonated hen's eggs and was used for live virus challenges. Anesthetized mice were infected intranasally by instillation of 50μl containing indicated pfu of Influenza A PR8 in PBS. Alternatively, 500 pfu of VSV was injected intravenously, or 250pfu intramuscularly. Control mice received PBS only.

In vitro killing and Lamp-1 upregulation

NK-cells were sorted from hapten sensitized donors at day 4 and cocultured in indicated ratios with a 1:1 mixture of autologous DNBS labeled or MHC class-$I^{-/-}$ B-cells and unmodified WT control B-cells. Target and control cells were distinguished by CFSE or congenic markers. 12 hrs after coincubation with NK cells, ratios of target cells:control cells were determined using FACS analysis. Specific lysis was calculated as: (1 - [control]/[target]$_{input}$ / [control]/[target]$_{NK\text{-}exposed}$]) x100%. To determine Lamp-1a upregulation on NK cells, NK-cells were cocultured with DNBS labeled B-cells and 10μg/ml FITC-conjugated mAb specific for Lamp-1a, in the presence of anti-CXCR6 clone 221002 mAb 10 μg/ml or isotype control, or CXCL16 protein, and FACS analyzed for anti-Lamp-1a incorporation after 3 hrs.

DNBS labeling of B cell targets

naïve B-cells were isolated by negative selection using CD43 magnetic beads (Miltenyi Biotech), and suspended at 10^8cells/ml in PBS. DNBS in PBS was added to a final concentration of 20mg/ml, cells incubated 10 min at RT and washed twice with PBS containing 10% bovine serum.

Numbers of experiments and Statistical analysis

3–5 independent experiments were done for all figures shown, and data from all experiments were pooled and shown. Total numbers of mice are indicated in the figure legends. Data are presented as mean plus minus standard deviation. Statistical significance was determined using Students t-test, ANOVA, or log rank (Mantel-Cox) test. Significance was set at a P-value of less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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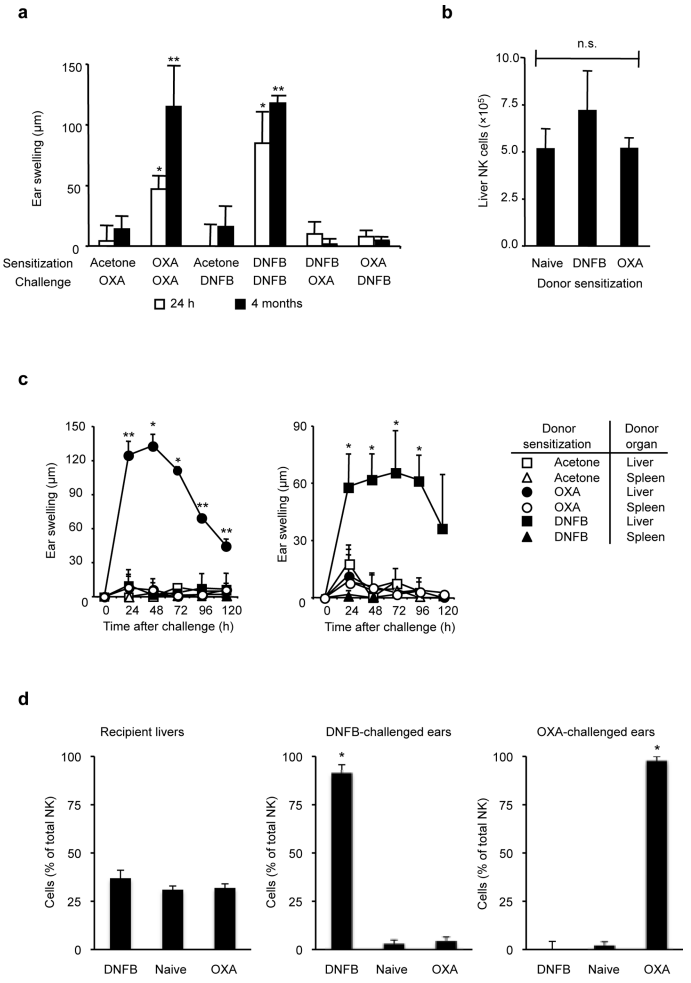

Fig. 1.

Liver NK-cells develop specific memory to haptens. (**a**) Hapten-specific CHS responses in naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients of hepatic CD45^{+}NK1.1^{+}Thy1^{+} NK-cells (1×10^{5}) from naïve (vehicle-exposed), DNFB or OXA sensitized Rag1$^{-/-}$ donors. Recipients were challenged 24h or four months post transfer. Hapten-specific ear swelling was determined after 24hrs by subtracting background swelling in naïve mice from that in NK recipients. *n*=10–15 recipients/group. *p<10^{-2}; **p<10^{-3} (**b**) Survival and expansion of adoptively transferred NK-cells is not altered by prior sensitization. Two weeks after the 4 month challenge, recipient mice shown in **a** were analyzed by FACS and the number of liver-resident CD45^{+} NK1.1^{+} cells was determined. Results were similar for DNFB or OXA challenged mice, so data were pooled. No NK1.1^{+} cells were detected in mock recipient *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (not shown). (**c**) Liver-restricted memory NK-cells arise in the presence of T and B-cells. Sorted CD45^{+}NK1.1^{+}CD3^{-}Thy1^{+} NK-cells (10^{5}) from actin-GFP transgenic donors were transferred to naïve C57BL/6 mice; recipients were challenged six weeks later and analyzed as in **a**. *n*=10–15 recipients/group. *p<10^{-3}; **p<10^{-4} (**d**) Recruitment of memory NK-cells to sites of challenge is Ag-specific. Hepatic CD45^{+}NK1.1^{+}Thy1^{+} NK-cells from naïve CD45.1^{+} WT donors (C57BL/6) and from CD45.2^{+} DNFB or OXA sensitized WT or actin-GFP transgenic donors were mixed (10^{5}

each) and adoptively transferred into naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. One month post adoptive transfer, recipient ears were challenged with either DNFB or OXA. Livers and ears were harvested at 24, 48 and 72 h, and analyzed for the presence of NK-cells whose origin was distinguished by congenic/fluorescent markers using FACS. No NK-cells were found in acetone challenged control ears (not shown). The mean of all mice analyzed at 24, 48 and 72 h is shown (*n*=6–7 recipients/group). *$p<10^{-11}$.

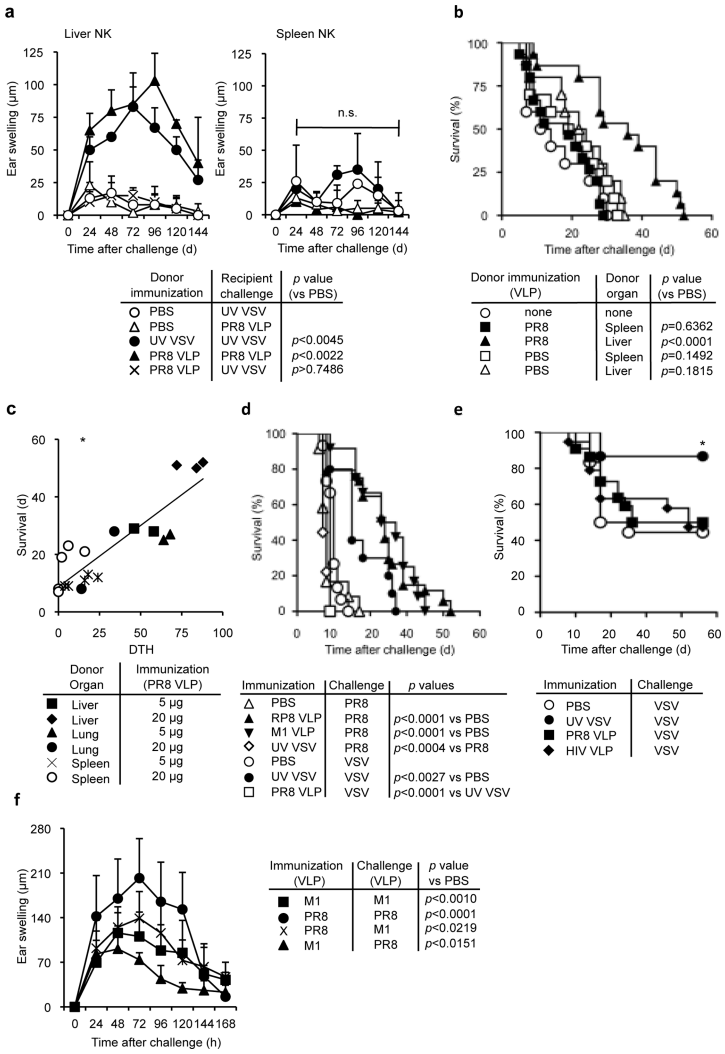

Figure 2.

Liver NK-cells develop specific memory to viral Ags. (**a**) One month after immunization of Rag1$^{-/-}$ mice with viral Ags, 8×10^4 splenic or hepatic CD45$^+$ NK1.1$^+$ NK-cells were adoptively transferred to naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Recipient ears were challenged by sq injection two months later. Virus-specific DTH was determined after 24h by subtracting background swelling in naïve mice from that in NK-cell recipients. *n*=8–10 recipients/group. (**b**) Influenza-sensitized hepatic NK-cells prolong survival of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients upon lethal influenza infection. Using the sensitization and transfer protocol in **a**, recipients were intranasally infected with influenza A PR8 (500pfu) three months after NK-cell transfer. *n*=15–19 recipients/group. (**c**) Two month after assessment of DTH, the NK-cell recipients used in **a** were infected with influenza A PR8 (500pfu), and survival was determined and correlated to DTH. *n*=19. **p=0.0001* (**d**) Rag1$^{-/-}$ mice develop virus-specific immunity to influenza and VSV. One month after immunization with influenza VLPs or UV-VSV, Rag1$^{-/-}$ mice were challenged with live virus (2,500 pfu PR8 i.n. or 500 pfu VSV i.v.) and survival was monitored. *n*=8–12 mice/group. (**e**) Rag2$^{-/-}$ mice develop long-term protective, virus-specific immunity to VSV. One month after immunization with

influenza VLPs or UV-VSV, Rag1$^{-/-}$ mice were challenged by i.m. injection of VSV at LD50 (250pfu) and monitored for survival. *n*=15–22 mice/group. **p=0.0116* (**f**) NK-cell memory to influenza A does not require HA. Rag1$^{-/-}$ mice were immunized with HA-containing (PR8) or HA-free (M1) VLPs, challenged one month later and analyzed as in **a**. *n*=10–15 mice/group.

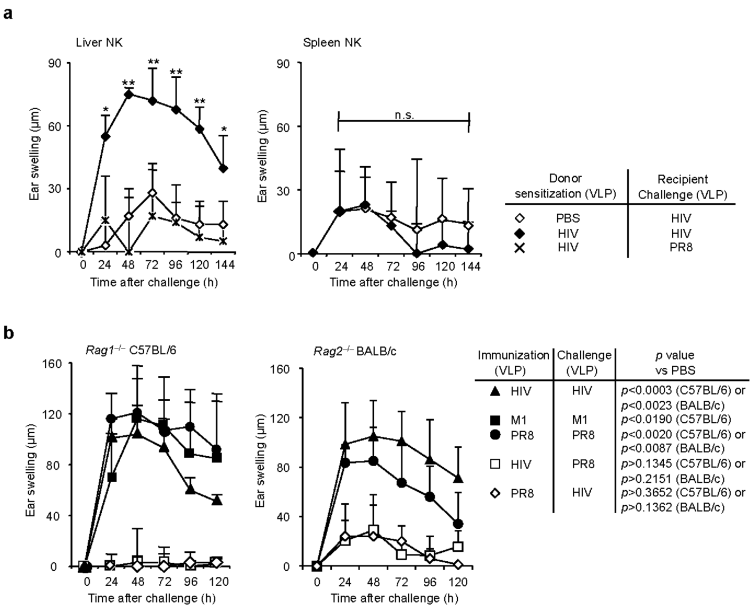

Figure 3.

Mouse liver NK-cells recognize and discriminate between HIV-1 and influenza A. (**a**) One month after subcutaneous immunization of Rag1$^{-/-}$ donor mice with influenza or HIV-1 VLPs, hepatic (left) or splenic (right) CD45^{+} NK1.1^{+} NK-cells were adoptively transferred to naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients (8×10^{4} cells/mouse). Two months later, recipients were challenged by subcutaneous VLP injection into one ear. n=12–15 recipients/group. *$p<10^{-2}$; **$p<10^{-3}$. (**b**) NK-cell recognition of HIV-1 derived gag/env containing VLPs and influenza PR8 derived HA and/or M1 containing VLPs occurs independent of genetic background. C57BL/6 Rag1$^{-/-}$ (left) and BALB/c Rag2$^{-/-}$ mice (right) were immunized with VLPs and challenged one month later. In all experiments, background ear swelling in non-immunized mice was determined in parallel and subtracted from measurements in experimental groups. n=10–15 mice/group.

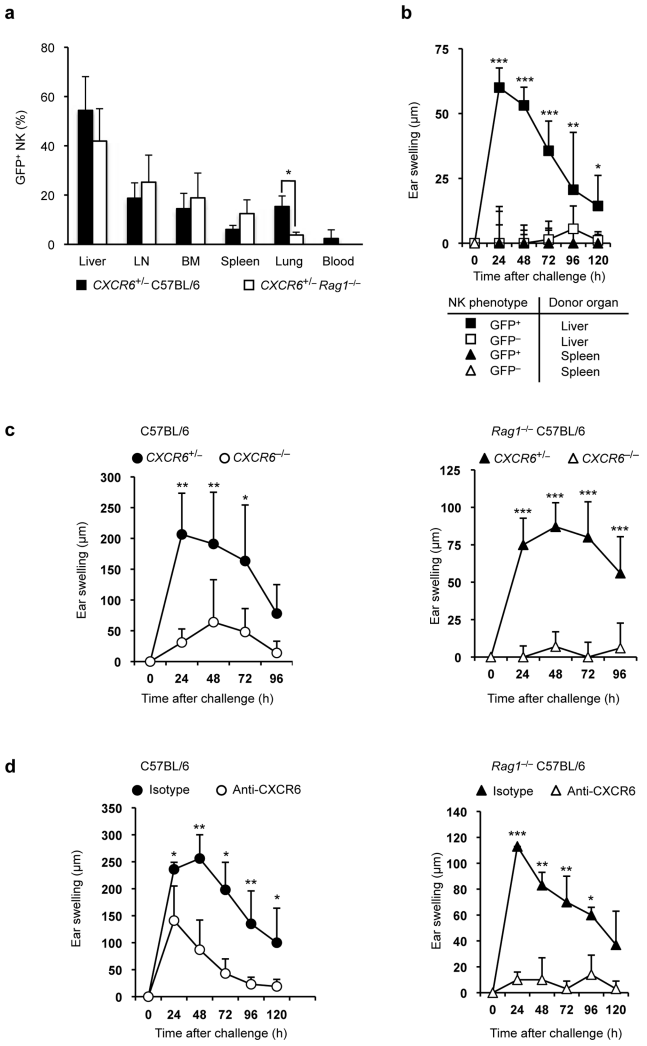

Figure 4.

NK cell-expressed CXCR6 is required for NK cell-mediated adaptive immunity to haptens. (**a**) The percentage of CXCR6-expressing CD45$^+$ NK1.1$^+$ NK-cells from *Cxcr6*$^{+/-}$ mice on *Rag1*-sufficient (C57BL/6) and *Rag1*$^{-/-}$ background were analyzed in different tissues by FACS. LN, lymph node; BM, bone marrow. *p<10^{-2}; **p<10^{-3}; ***p<10^{-4} (**b**) 10^5 NK-cells from DNFB sensitized Rag1$^{-/-}$ *Cxcr6*$^{+/-}$ donor spleens or livers were sorted for NK1.1 and GFP expression and transferred to naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. Animals were challenged with DNFB on one ear, and solvent on the other one month later and ear swelling was determined. n=10–12 recipients/group. *p<10^{-2}; **p<10^{-3}; ***p<10^{-4} (**c**) Effect of CXCR6 deficiency on DNFB-induced CHS in lympho-competent (C57BL/6, left panel) and Rag1$^{-/-}$ mice (right panel). n=10–12mice/group. (**d**) Effect of anti-CXCR6 mAb (100 µg/mouse administered i.v. 24h prior to challenge) on DNFB-induced CHS in C57BL/6 (left panel) and Rag1$^{-/-}$ mice (right panel). Hapten sensitized animals were challenged five days post sensitization. n=10–15 mice/group. *p<10^{-2}; **p<10^{-3}; ***p<10^{-4}.

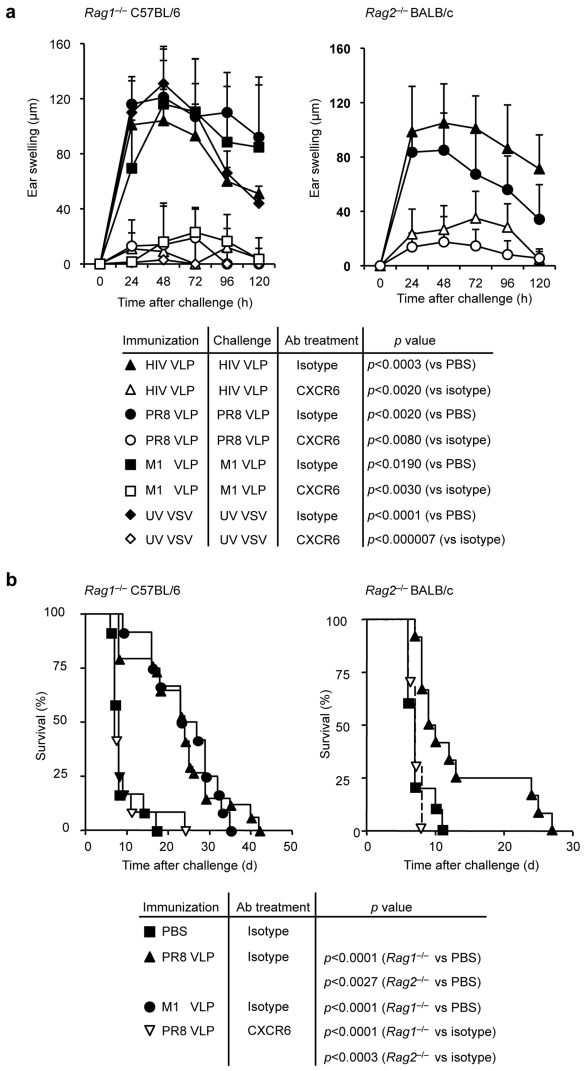

Figure 5.

NK cell-expressed CXCR6 is required for NK cell-mediated adaptive immunity to viruses. (**a**) Effect of anti-CXCR6 mAb (100 μg/mouse 24h prior to challenge) on anti-viral DTH responses in Rag deficient mice on C57BL/6 (left panel) or Balb/c background (right panel). (**b**) NK cell-mediated protection against lethal influenza A infection depends on CXCR6. $Rag^{-/-}$ mice were immunized and infected one month later with Influenza A PR8 (2,500 pfu for $Rag1^{-/-}$ (left panel) and 10,000 pfu for $Rag2^{-/-}$ (right panel)). Anti-CXCR6 or isotype control mAb (100 μg) was injected on days 1 and 5. n = 8–12 mice/group.

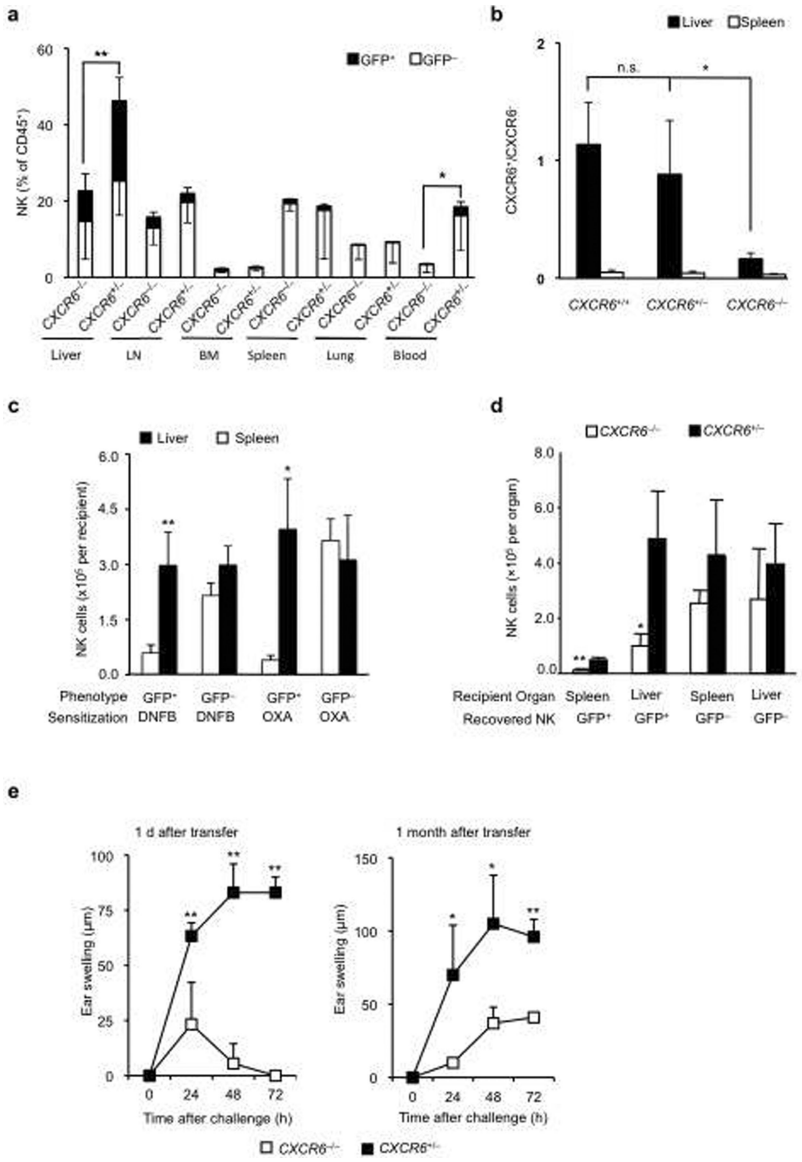

Figure 6.

CXCR6 regulates hepatic NK-cell homeostasis. (**a**) Frequencies of GFP$^+$ and GFP$^-$ NK-cell subsets were determined in different organs of *Cxcr6*$^{+/-}$ and *Cxcr6*$^{-/-}$ mice. NK-cells were identified as CD45$^+$NK1.1$^+$ cells. LN, lymph node; BM, bone marrow. *, $p<10^{-2}$ **; $p<10^{-3}$ (**b**) NK-cell subset ratios in liver and spleen of WT (+/+; C57BL/6), *Cxcr6*$^{+/-}$ and *Cxcr6*$^{-/-}$ mice. n=12–15 mice/group. *, $p=2\times10^{-5}$. (**c**) Differential distribution of NK-cells recovered from liver or spleen one month after adoptive transfer of sorted subsets (1×10^5) to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. n=10–15 mice/group. *, $p<10^{-3}$; **, $p<10^{-4}$ (**d**) GFP$^+$ but not GFP$^-$ *Cxcr6*$^{+/-}$ NK-cells outcompete their *Cxcr6*$^{-/-}$ counterparts upon adoptive transfer. 1×10^5 GFP$^+$ or GFP$^-$ CD45$^+$ NK1.1$^+$ NK-cells were sorted from *Cxcr6*$^{+/-}$ or *Cxcr6*$^{-/-}$ donors, mixed, transferred to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients and counted by FACS in liver and spleen two weeks later. n=8–10 recipients/group. *, $p<10^{-3}$; **, $p<10^{-4}$ (**e**) *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice received 8×10^4 DNFB primed CD45$^+$ NK1.1$^+$ GFP$^+$ NK-cells from *Cxcr6*$^{+/-}$ or

Cxcr6$^{-/-}$ donors and were challenged 24h or 1 month later (n=8 mice/group). * $p<10^{-2}$; **, $p<10^{-3}$.

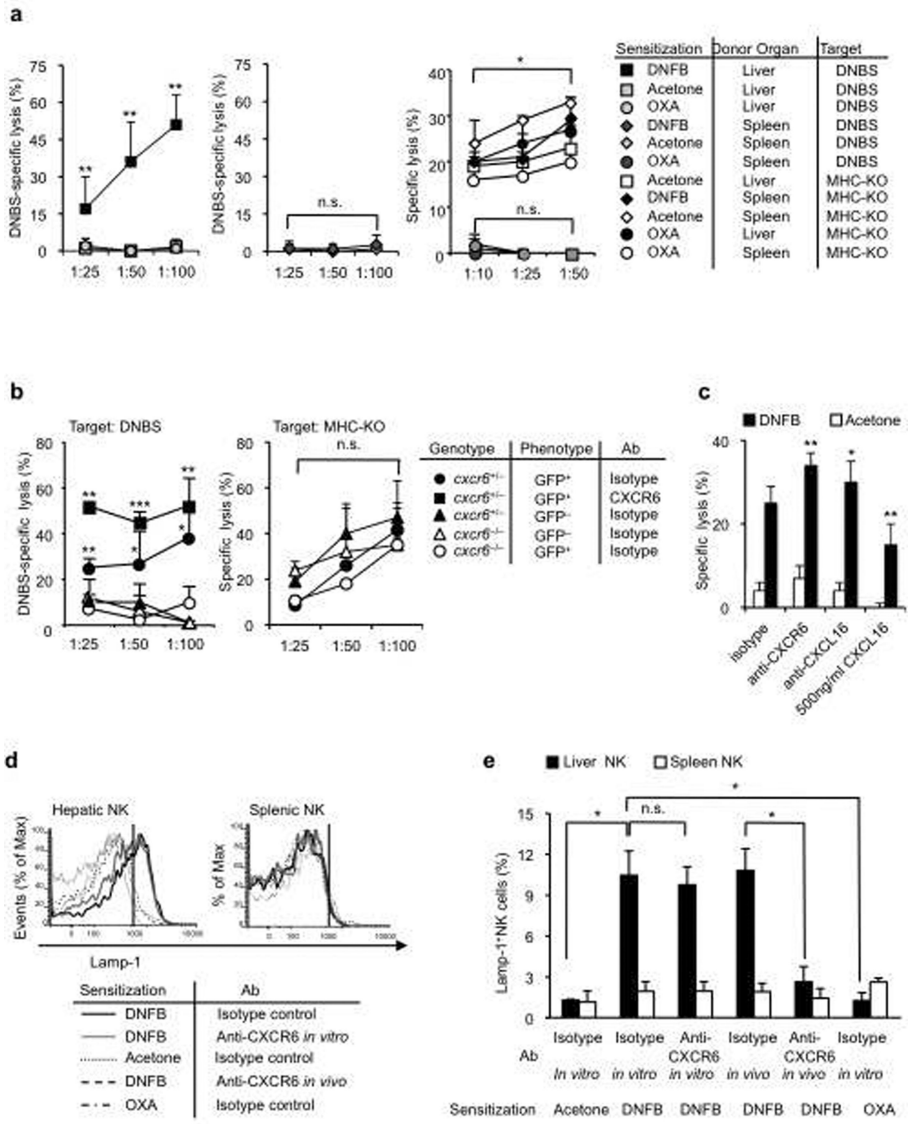

Figure 7.

Hepatic memory NK-cells mediate hapten-specific killing in vitro. (**a**) Naive and hapten-sensitized CD45$^+$ NK1.1$^+$ NK-cells were cocultured for 12h with a mixture of two populations of B-cells that were differentially labeled with high or low amounts of CFSE at indicated target to effector (T:E) ratios. CFSE^{lo} B-cells served as control, whereas CFSE^{hi} B-cells were either from WT donors and haptenated with DNBS (left and middle panels) or from MHC-I$^{-/-}$ donors (right panel). Hapten-specific killing was determined from the ratio of CFSE^{lo}:CFSE^{hi} cells corrected for input. n=10–20 donor mice/group. *, $p<10^{-8}$; **, $p<10^{-12}$ (**b**) Killing capacity of DNFB-primed hepatic CD45$^+$ NK1.1$^+$ NK-cells from *Cxcr6*$^{+/-}$ or *Cxcr6*$^{-/-}$ donor mice was determined as in **a** in the presence of anti-CXCR6 or idotype control mAb. n=12 donor mice/group. *, $p<10^{-2}$; **, $p<10^{-3}$; ***, $p<10^{-5}$. (**c**) Killing capacity of DNFB primed hepatic CD45$^+$ NK1.1$^+$ NK-cells from Rag1$^{-/-}$ donors at 1:25 T:E ratio was determined in the presence of 10μg/ml anti-CXCR6 or anti-CXCL16 mAb or 500ng/ml CXCL16 and compared to cultures treated with 10μg/ml isotype control.

n=15 donor mice/group. *, $p<10^{-2}$; **, $p<10^{-3}$. (**d,e**) NK1.1^{+} cells were analyzed for binding of anti-Lamp-1 by FACS. Rag$^{-/-}$ donor mice were sensitized with acetone, DNFB or OXA on days 0 and 1, and NK1.1^{+} NK-cells were sorted from livers or spleens. Some donor mice were injected with 100μg anti-CXCR6 or control mAb 12hrs prior to NK-cell isolation. NK-cells were cocultured with DNBS labeled B-cells in the presence of 10μg/ml FITC-conjugated anti-Lamp-1 with and without addition of 10μg/ml anti-CXCR6 or isotype control. (**d**) NK-cells were FACS analyzed for anti-Lamp-1 incorporation after three hours. (**e**) Bars represent means and SEM of pooled data from 3–5 independent experiments; 10–18 donor mice total; 12–20 individual wells per group. *, $p<10^{-9}$; **.