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Natural killer cell memory in infection, inflammation and cancer

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Abstract | Immunological memory can be defined as a quantitatively and qualitatively enhanced immune response upon rechallenge. For natural killer (NK) cells, two main types of memory exist. First, similarly to T cells and B cells, NK cells can exert immunological memory after encounters with stimuli such as haptens or viruses, resulting in the generation of antigen-specific memory NK cells. Second, NK cells can remember inflammatory cytokine milieus that imprint long-lasting non-antigen-specific NK cell effector function. The basic concepts derived from studying NK cell memory provide new insights about innate immunity and could lead to novel strategies to improve treatments for infectious diseases and cancer.

The ability to form immunological memory is traditionally considered as a hallmark of adaptive immunity. However, increasing evidence suggests that innate immune cells can also 'remember' prior exposures to certain stimuli. Immunological memory, defined as responding qualitatively or quantitatively at a higher magnitude upon a secondary immune stimulation, has been demonstrated in invertebrates, as well as in innate immune cells in mammals. In invertebrates (from shellfish to worms and insects) recall responses against pathogens have been described (see REF. 1). In mammals, the ability to mount recall responses has been shown for innate immune cells of the myeloid lineage, which has been referred to as 'trained immunity' (REF. 2) (BOX 1), as well as for natural killer (NK) cells[3]. So far, concepts emerging from heterologous recall responses of 'trained' myeloid cells have been only incompletely evaluated in other immune cell types and might be highly relevant for certain aspects of secondary immune responses mounted by NK cells.

NK cells are innate lymphocytes that are activated on encounter with infected cells, allogeneic cells or transformed cells[4–7]. Although NK cells are generally poised for rapid cytolytic activity[8], in many cases they need to be functionally differentiated[9] or primed by cytokines or other immune cells, such as by dendritic cells (DCs)[10], to exert optimal effector responses. The activation of NK cells is determined by a delicate balance of signals delivered by activating receptors, which recognize stress-induced ligands on tumour cells or virus-infected cells, and inhibitory receptors, which predominantly engage MHC class I molecules[11] (BOX 2). In this review, we discuss emerging data that provide evidence that NK cells can acquire immunological memory, an activity that has traditionally been associated with T cells and B cells. We focus on the roles of memory NK cells in contact hypersensitivity (CHS) responses, in viral infection and in cancer, and we discuss the therapeutic potential of targeting NK cells for improved treatments of infectious diseases and cancer.

Identification of memory NK cells

In 2006, the observation that mice lacking T cells and B cells could develop CHS responses to various distinct haptens introduced the concept that NK cells can mediate antigen-specific memory responses[12,13]. Several lines of evidence support the notion that NK cells can contribute to immunological memory. First, in hapten-sensitized recombination-activating gene 2 (*Rag2*)-knockout mice, the depletion of NK cells by treatment with an NK1.1 (also known as KLRB1)-specific antibody abolished CHS responses on hapten rechallenge[13]. Second, CHS responses did not develop in hapten-sensitized mice lacking both *Rag2* and *Il2rg* (interleukin-2 receptor subunit-γ), which lack mature NK cells as well as T cells and B cells[13]. The recall responses observed in the *Rag2*$^{-/-}$ mice were sensitization-dependent, persisted for more than 4 months after priming and were transferable upon adoptive transfer of hepatic NK cells to naive mice[14]. Importantly, the recall responses were specific for the hapten used for sensitization and not for an unrelated hapten[14]. Moreover, NK cells from mice sensitized with 2,4-dinitro-1-fluorobenzene (DNFB) were recruited to DNFB-challenged ears, whereas NK cells from oxazolone (OXA)-primed mice preferentially accumulated in OXA-challenged ears[14]. Of note, another study did not observe the development of a CHS response after DNFB-sensitized RAG2-deficient mice were challenged with this hapten[15]. Nonetheless, in this study, the adoptive transfer of hepatic NK cells from DNFB-sensitized

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Box 1 | **Memory in myeloid cells in mammals**

A paradigm shift in our understanding of host defence has been triggered by the mounting evidence for innate immune memory, leading to increased responses to secondary infections. It provides protection against reinfection in a T cell- and B cell-independent manner. Exposure of monocytes to bacillus Calmette–Guérin (BCG), for example, can elicit an enhanced response to *Candida albicans* or *Schistosoma mansoni*. Protection appeared to be at least in part independent from T cells[80] and involves activated tissue macrophages[81]. Similarly, challenge of mice with CpG-DNA, which engages Toll-like receptor 9 (TLR9), can confer long-term protection against *Listeria monocytogenes* infection[82], which could be transferred by a subpopulation of dendritic cells to naive recipient mice[83]. Recent studies have unravelled the cellular processes and dissected genetic and epigenetic differences of naive monocytes and monocytes briefly stimulated with β-glycan (also known as TGFR3) and then rested for an additional 5 days[84,85]. These β-glycan-pretreated monocytes, which were referred to as 'trained' monocytes, exhibited a distinct epigenetic programme that did not require DNA replication. Instead, aerobic glycolysis involving an AKT–mTOR– hypoxia-inducible factor 1α (HIF1α) pathway was identified as the basis of trained immunity. Similarly, an enhanced response of macrophages to bacterial infection in mice previously stimulated with lipopolysaccharide is mediated by epigenetic changes caused by the ATF7 transcription factor[86]. These epigenetic and metabolic changes in myeloid cells coincided with their improved responsiveness to subsequent challenges regardless of the nature of restimulation.

mice to naive recipients resulted in the development of hapten-specific recall responses in the recipient mice, confirming the results of the previous studies.

After the initial identification of memory NK cells residing in the liver in the CHS model, the concept of NK cell memory was extended to other organs and other diseases. Sun *et al.* demonstrated that NK cell memory responses against mouse cytomegalovirus (MCMV) exist and that memory NK cells can reside in organs other than the liver, with these cells being identified in the spleen, blood circulation, lung, kidney and other lymphoid tissues[16,17]. Paust *et al.*[14] reported that after subcutaneous immunization of mice with non-infectious virus-like particles (VLPs) containing proteins from influenza virus or HIV-1 or with ultraviolet light-inactivated vesicular stomatitis virus (VSV), the transfer of hepatic NK cells resulted in prolonged survival of the mice after a lethal challenge with the sensitizing virus, but not with an unrelated virus. In addition, in the influenza model, lung NK cells from sensitized mice were effective in mediating protection against viral challenge[14]. A protective effect for memory NK cells in the absence of T cells and B cells was also shown in models of vaccinia virus[18] and herpes simplex virus 2 (HSV-2)[19] infection. More recently, antigen-specific NK cell memory was observed in rhesus macaques after immunization with simian immunodeficiency virus (SIV)[20]. Therefore, there is mounting evidence for NK cell memory responses in different models of viral disease in both mice and primates.

Intriguingly, NK cell memory responses have also been observed in the absence of a defined antigen. A brief *in vitro* exposure of mouse NK cells to the cytokine combination of IL-12, IL-15 and IL-18 results in the development of NK cells that show a sustained ability to produce high levels of interferon-γ (IFNγ) on stimulation with IL-12 and/or IL-15 for at least 4 months after adoptive transfer into RAG2-deficient mice[21,22]. These data indicate that NK cells can remember an exposure to an inflammatory cytokine milieu. Similar cytokine-induced memory NK cells can be generated from human NK cells[23].

Generation of memory NK cells

In the following section, we address the receptors, cytokines and signalling pathways that have been implicated in the development of memory NK cells. Recent literature reveals certain similarities, but also profound differences, in the prerequisites for the generation of hapten-specific, virus-specific or cytokine-induced memory NK cells (summarized in FIG. 1).

Receptors driving the generation of memory NK cells. The receptors expressed by NK cells that convey hapten specificity have remained elusive, although several features of these hapten-specific NK cells have been described. LY49C$^+$ (also known as KLRA3) or LY49I$^+$ NK cells isolated from the liver transferred hapten sensitivity more efficiently than hepatic LY49C$^-$ or LY49I$^-$ NK cells[13]. As LY49C$^+$ or LY49I$^+$ NK cells isolated from the spleen of these same hapten-sensitized mice failed to transfer CHS responses to naive recipients, it is unlikely that the LY49C or LY49I receptors are directly responsible for hapten recognition. Furthermore, as the receptor responsible for hapten recognition has not been identified, whether the hepatic LY49C$^-$ or LY49I$^-$ NK cells fail to respond owing to lack of licensing or simply because they lack the putative hapten-specific receptor is unresolved. NK cell-mediated hapten-specific CHS responses were partially blocked by treating mice with a neutralizing natural killer group 2, member D (NKG2D)-specific antibody upon hapten challenge; however, as NKG2D is an invariant, non-polymorphic receptor that is expressed on essentially all NK cells, as well as on T cells, it is unlikely to be directly responsible for hapten recognition. Instead, NKG2D might function as a co-stimulatory receptor during the effector phase of the memory response owing to its ability to detect stress-induced ligands, which might be induced by the irritants used to elicit the CHS response.

Treatment with blocking antibodies specific for the endothelial selectins (P- and E-selectin) or for CD18 (also known as β2 integrin) before challenge with hapten also abrogated CHS[13], probably by blocking the migration of NK cells to the ear. Subsequent studies revealed an important role for CXC-chemokine receptor 6 (CXCR6) in the induction or maintenance of the NK cell memory response[14]. Again, CXCR6 is unlikely to mediate direct hapten recognition but instead might regulate NK cell effector function, trafficking or the survival of memory NK cells. Indeed, it was shown that IFNγ production by hapten-sensitized liver NK cells *in vitro* in response to DNFB-labelled B cells was impaired by the addition of a CXCR6-specific monoclonal antibody or the addition of a monoclonal antibody targeting the CXCR6 ligand CXC-chemokine ligand 16 (CXCL16)[24]. Curiously, NK cells isolated from the liver within one hour after sensitization with DNFB could transfer hapten-specific CHS responses to naive

recipients[24], implying that a high precursor frequency of NK cells must possess a receptor for DNFB as this time period is too short for clonal expansion of the responding cells.

Most of our mechanistic knowledge of the signals that drive the generation of virus-specific memory NK cells originates from the model of MCMV infection, in which the MCMV-specific activating NK cell receptor LY49H (also known as KLRA8) and its cognate viral ligand m157 have been identified. The importance of the LY49H receptor in NK cell-mediated resistance against MCMV was first revealed by classic genetic studies[25,26] and by experiments using a neutralizing LY49H-specific antibody[27]. The mechanism by which this receptor prevented disease had remained unclear. However, in 2007, it was reported that the MCMV-encoded MHC class I-like glycoprotein m157 engages LY49H to activate NK cells and confers host protection in MCMV-resistant C57BL/6 mice[28,29]. The existence of a MCMV-specific receptor, LY49H, and a specific viral antigen, m157, facilitated subsequent studies tracing the expansion, contraction and differentiation of MCMV-specific NK cells into a long-lived, self-renewing memory subset[16]. In the absence of LY49H or m157, this activation programme is not induced, indicating a non-redundant role of the LY49H–m157 axis in this process. Moreover, the co-stimulatory molecule DNAX accessory molecule 1 (DNAM1; also known as CD226) is required for optimal differentiation of MCMV-specific effector and memory NK cells[30]. Accordingly, the DNAM1 ligand CD155 (also known as PVR) is upregulated after MCMV infection *in vivo,* predominantly on infected monocytes and DCs. Inhibitory NK cell receptors might function as checkpoints to control different steps in the generation of memory cells. LY49H$^+$ NK cells that lack inhibitory LY49 receptors and recognize self-MHC class I ligands preferentially respond during the expansion phase following MCMV infection[31]. Whether CD96 (also known as TACTILE) and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT)[32], two inhibitory receptors that bind to the DNAM1 ligand CD155 and counteract NK cell activation, control the formation of memory NK cell populations has not been determined. The receptors that drive the generation of memory NK cells in other viral infections, including influenza virus, HSV-2 and vaccinia virus infection, also remain to be defined. Of note, influenza virus haemagglutinin was not required for NK cell sensitization against influenza virus[14], excluding a role for the interaction of haemagglutinin with the activating receptor NKp46 (also known as NCR1) in this model.

Receptors involved in the generation of NK cell memory against HCMV. In 2004, Guma *et al.* presented the first evidence that healthy individuals who have previously encountered human CMV (HCMV) and are serologically positive for HCMV, but not for other herpes viruses, have higher frequencies of a subset of NK cells expressing the activating CD94–NKG2C receptor compared with individuals that are serologically negative for HCMV[33]. This study suggested that, in analogy to the MCMV model, interactions between the CD94–NKG2C receptor and ligands on HCMV-infected cells might drive the generation of a memory NK cell subset. Subsequent studies of patients who were infected by HCMV or in whom the virus was reactivated owing to immunosuppression after solid organ or haematopoietic stem cell transplantation confirmed that the CD94–NKG2C$^+$ NK cell population preferentially expands during acute HCMV infection, subsequently persist as memory cells and can constitute up to 70% of the total NK cell population in some individuals[34–36]. Moreover, after haematopoietic stem cell transplantation, CD94–NKG2C$^+$ NK cells from HCMV-seropositive donors demonstrated enhanced function in response to re-exposure to HCMV in HCMV-seropositive recipients whereas CD94–NKG2C$^+$ NK cells from HCMV-seronegative donors did not, suggesting the existence of a memory response against HCMV infection[37]. In *in vitro* experiments in which HCMV-infected fibroblasts were co-cultured with NK cells either knocked down for HLA-E by short hairpin RNA or treated with CD94- or NKG2C-specific blocking antibodies[38], the interaction of CD94–NKG2C with its ligand HLA-E[39] was shown to drive the expansion of the CD94–NKG2C$^+$ NK cell population (FIG. 2a). In addition to direct interaction with virus-infected fibroblasts, both cell–cell contact with CD14$^+$ monocytes and soluble factors produced by CD14$^+$ monocytes were required for expansion of the CD94–NKG2C$^+$ NK cell population in this system[39].

Box 2 | **Regulation of NK cells by activating and inhibitory receptors**

Natural cytotoxicity receptors (NCRs) on natural killer (NK) cells include the NKp30 (also known as NCR3) and NKp46 receptors that signal through the immunoreceptor tyrosine-based activation motif (ITAM)-bearing CD3ζ or high-affinity IgE receptor subunit-γ (FcεRIγ) adaptor proteins and NKp44 (also known as NCR2) that signals using the ITAM-bearing DAP12 adaptor protein (also known as TYROBP)[87]. The activating CD94–NKG2C (natural killer group 2, member C) receptor that recognizes HLA-E and many of the activating killer cell immunoglobulin-like receptors (KIRs) also use DAP12 (REF. 88). Additional activating NK receptors include the CD2 family (for example, 2B4), DNAX accessory molecule 1 (DNAM1), which recognizes CD155 and CD112 (also known as nectin 2) as ligands, and NKG2D, which recognizes numerous ligands expressed on stressed cells including MHC class I polypeptide-related sequence A (MICA), MICB and UL16-binding proteins (ULBPs) in humans as well as retinoic acid early inducible 1 (RAE1), murine ULBP-like transcript 1 (MULT1) and H60 ligands in mice. Certain NK cell receptors, such as mouse LY49H, LY49D and NKG2D, can bind to both DAP12, mediating signals through ITAMs, and DAP10 (also known as HCST), delivering PI3K-activating signals, which result in extremely potent activation through these receptors[89–91]. The activation of NK cells is counteracted by inhibitory receptors, many of which possess immunoreceptor tyrosine-based inhibition motifs (ITIMs). Such inhibitory receptors include members of the LY49 family in rodents and the KIR family in humans, which recognize polymorphic epitopes on MHC class I proteins. The inhibitory CD94–NKG2A receptor recognizes the non-classical MHC class I molecules HLA-E, in humans, and Qa1, in mice. More recently, additional inhibitory receptors on NK cells have been identified that recognize non-MHC ligands. For example, the inhibitory ITIM-bearing TIGIT (T cell immunoreceptor with immunoglobulin and ITIM domains) and CD96 receptors compete with their activating counterpart DNAM1 for binding to the ligand CD155 (REF. 32). Additionally, TIM3 (also known as HAVCR2)[92,93], which inhibits by a so far undefined mechanism, and the E3 ubiquitin ligase CBL-B, which inactivates the TAM family of receptors (TYRO3, AXL and MER)[94], have also been shown to inhibit the activation of NK cells.

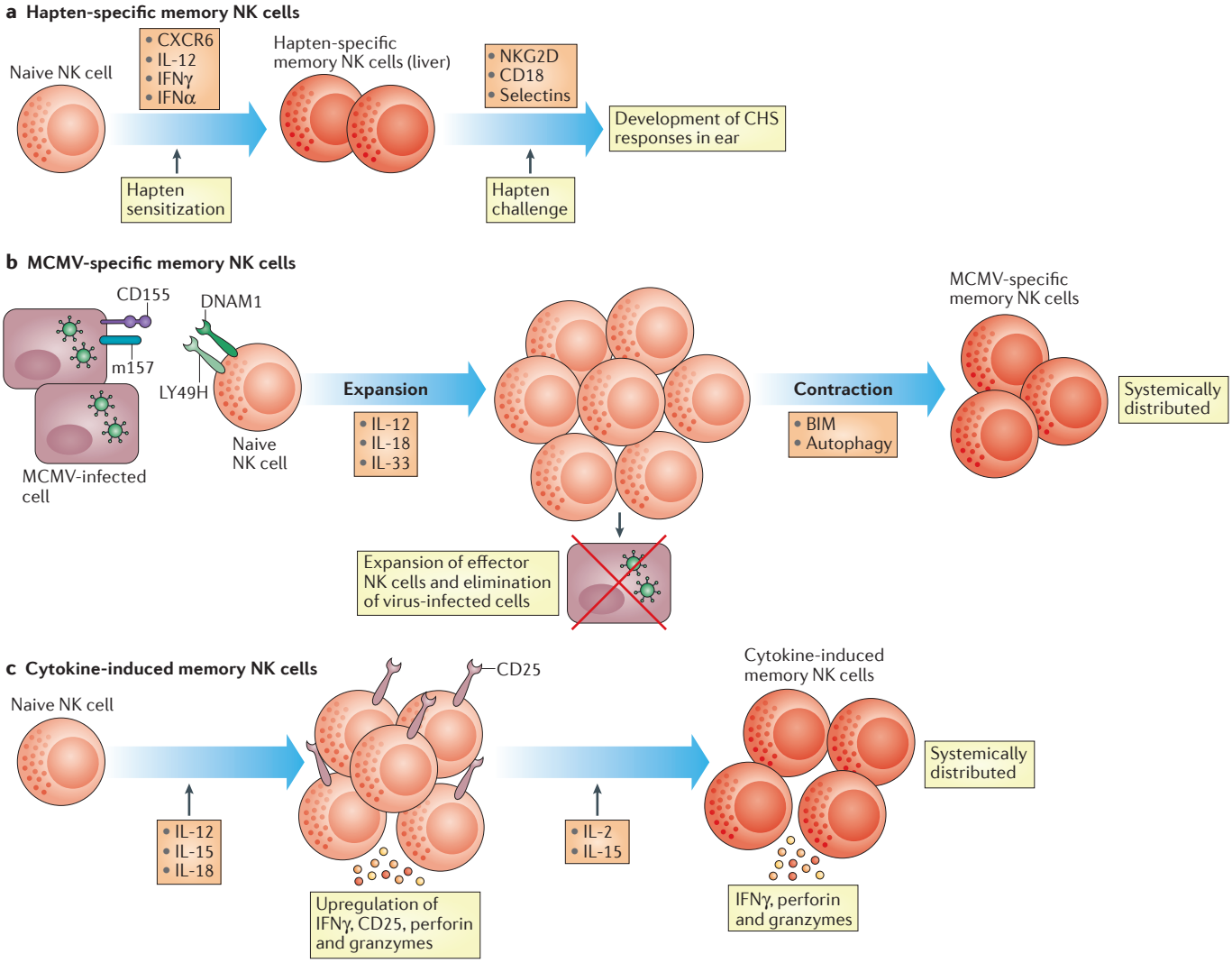

Figure 1 | **Pathways for the generation of memory NK cells. a** | Following sensitization of mice with haptens, hapten-specific memory natural killer (NK) cells are detected in the liver. The generation of these memory NK cells is dependent on the cytokines interleukin-12 (IL-12), interferon-γ (IFNγ) and IFNα. Antibody-mediated blockade of the NK cell receptor natural killer group 2, member D (NKG2D) or CXC-chemokine receptor 6 (CXCR6), or molecules that are involved in NK cell trafficking (such as CD18 and P- and E-selectin) prevents the development of contact hypersensitivity (CHS) responses in the ear after hapten challenge. **b** | During mouse cytomegalovirus (MCMV)-infection, naive NK cells expressing the LY49H receptor expand with contribution of signalling mediated by the DNAX accessory molecule 1 (DNAM1) receptor and the inflammatory cytokines IL-12, IL-18 and IL-33. Inflammatory cytokines drive the expression of zinc finger and BTB domain-containing 32 (ZBTB32) and the microRNA miR-155, which are involved in the expansion of 'effector' NK cells. Following the elimination of the virus, the BIM and autophagy pathways regulate the contraction of the expanded populations of NK cells, giving rise to a population of MCMV-specific memory NK cells. Although MCMV-specific memory NK cells distribute systemically in mice, memory NK cells specific for vaccinia virus reside in the liver, and influenza virus-specific memory NK cells are found in the liver and lungs (not shown). In non-human primates, simian immunodeficiency virus-specific memory NK cells reside in the spleen and liver (not shown). **c** | *In vitro*, the brief exposure of NK cells to the cytokines IL-12, IL-15 and IL-18 results in the upregulation of IFNγ, perforin and granzymes, and the production of high levels of CD25, the high-affinity α-chain of the IL-2 receptor, is also induced. After adoptive transfer, these cytokine-induced memory NK cells persist long term, and their ability to produce abundant cytokines and express perforin and granzymes is maintained. The presence of IL-2 (or IL-15) further increases NK cell numbers and their ability to express IFNγ, perforin and granzymes after adoptive transfer.

There is emerging evidence that the formation of NK cell memory for HCMV can also occur in individuals carrying a homozygous null allele of *KLRC2*, which encodes NKG2C[41]. So far, the receptors driving the NK cell response against HCMV in the absence of CD94–NKG2C are poorly understood. In this context, intriguingly, expansion of NK cells expressing the activating killer cell immunoglobulin-like receptors (KIRs) KIR2DS2, KIR2DS4 and KIR3DS1 (REF. 42) has been noted, potentially implicating these KIRs in this process.

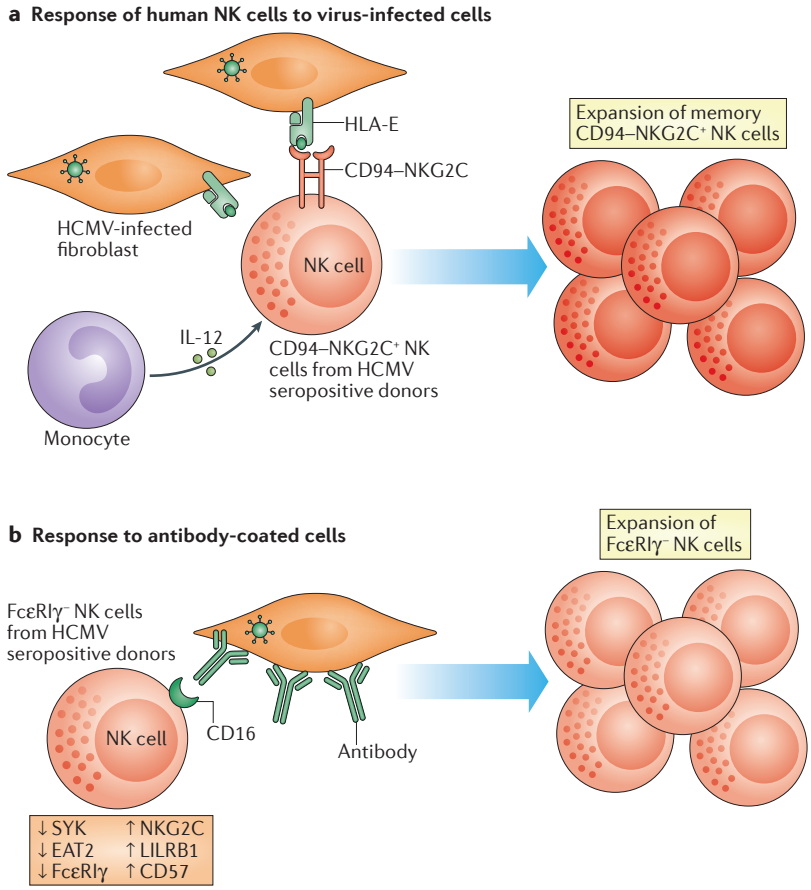

Figure 2 | **NK cell memory in HCMV-seropositive donors. a** | In human cytomegalovirus (HCMV)-seropositive donors, a population of CD94–NKG2C (natural killer group 2, member C)-positive NK cells expands on interaction of CD94–NKG2C with HLA-E that is upregulated on fibroblasts during HCMV infection. Monocytes producing interleukin-12 (IL-12) have an essential role in supporting the expansion of these NK cell populations. NK cell expansion in response to virus-infected fibroblasts can also occur in NKG2C-deficient individuals, but the relevant receptors and ligands that are involved in this setting are poorly understood. **b** | A population of CD94–NKG2C+ high-affinity IgE receptor subunit-γ (FcεRIγ)-negative NK cells has been identified in the sera of HCMV-seropositive individuals that express low amounts of SYK and/or EWS/FLI1-activated transcript 2 (EAT2) and can be expanded by co-culture with HCMV-infected fibroblasts coated with HCMV-specific antibodies. LILRB1, leukocyte immunoglobulin-like receptor subfamily B member 1.

In *KLRC2*-null children that are deficient in NKG2C protein, anti-HCMV IgG titers were significantly elevated, suggesting that the lack of expression of NKG2C may be associated with altered control of HCMV in childhood[43]. Accordingly, recent studies have identified a high-affinity IgE receptor subunit-γ (FcεRIγ)-deficient NK cell subset[44], referred to as 'adaptive NK cells', which is associated with HCMV infection and exhibits elevated CD16-mediated responses against HCMV-infected target cells coated with HCMV-specific antibodies[45] (FIG. 2b). Together, these studies indicate that redundant mechanisms for the generation of memory NK cells in HCMV-seropositive individuals exist.

Expansion of the CD94–NKG2C+ NK cell population has also been observed after infection with other viruses such as hantavirus[46], HIV[47] and hepatitis C virus (HCV)[40], although only in individuals who are persistently infected with HCMV. Furthermore, a recent report described durable antigen-specific memory NK cell responses in rhesus macaques after SIV infection and after vaccination[20]. In this study, an important role for the NKG2 receptors (NKG2C or NKG2A) in the NK cell response to SIV Gag- or envelope glycoprotein gp160 (Env)-pulsed DCs was demonstrated by experiments using a blocking monoclonal antibody that crossreacts with both NKG2A and NKG2C[20].

Cytokines and pathways contributing to NK cell memory generation. In addition to signals mediated by activating NK receptors, pro-inflammatory cytokines are instrumental for NK cell memory generation in response to haptens or MCMV. In the context of CHS, hepatic NK cells in hapten-sensitized *Il12*$^{-/-}$, *Ifng*$^{-/-}$ or *Ifnar1*$^{-/-}$ mice failed to induce CHS after adoptive transfer to challenged hosts, indicating an important role for IL-12, IFNγ and type I IFNs in the generation of NK cell effector function or NK cell memory[24]. Similarly, in the MCMV model, NK cells deficient in the IL-12 receptor or the signalling molecule STAT4 (signal transducer and activator of transcription 4) exhibited defective proliferation and failed to become long-lived memory cells that mediated protection against MCMV infection[48]. In a co-culture system of human PBMCs and HCMV-infected fibroblasts, a pivotal role for IL-12 in the expansion of the CD94–NKG2C+ NK cell subset was also observed[39]. In this study, neutralization of IL-12, but not IL-15 or IL-18, or blockade of type I IFN receptor in the PBMC and HCMV-infected fibroblast co-cultures significantly decreased expansion of the CD94–NKG2C+ NK cell subset. Accordingly, in the MCMV model, IL-15 was dispensable for the expansion and memory formation of LY49H+ NK cells[49], but both IL-18 and IL-33 contributed to the expansion of MCMV-specific NK cells, but not to the generation of memory[50,51]. Of note, humans with genetic defects in IL-12 production were not reported to have impaired resistance to HCMV[52], indicating the existence of redundant pathways for HCMV immunity. IL-12 and IL-18 produced during MCMV infection lead to upregulation of CD25, the IL-2Rα chain forming the high-affinity IL-2 receptor, in NK cells[53,54]. Expression of CD25 enables NK cells to respond to low amounts of IL-2 (REF. 55). Thus, inflammatory cytokines not only directly, but also indirectly, enhance NK cell expansion by facilitating NK cell responses to IL-2 produced by CD4+ T cells. The contribution of CD4+ T cell help to LY49H+ NK cell expansion and memory formation during MCMV infection has not yet been studied extensively, although CD25-deficient NK cells appear to expand normally but may undergo contraction with faster kinetics than wild-type NK cells (L.L.L. and Y. Kamimura, unpublished observations). Nevertheless, T cells were shown to compete with NK cells for IL-15, regulating the NK cell memory progenitor pool[56].

Recently, the Immunological Genome Project (www.ImmGen.org) generated a kinetic portrait of mouse NK cell gene expression by profiling transcription of naive LY49H+ NK cells before MCMV infection

and as early effectors (day 1.5), late effectors (day 7) and memory cells (day 27) after MCMV infection[8]. Many genes were specifically regulated in memory NK cells, identifying a unique signature of memory NK cells distinct from effectors and naive NK cells, but with similarities to memory CD8$^+$ T cells. The transcription factor *Zbtb32* (zinc finger and BTB domain-containing 32) was among the highest upregulated genes early after infection. Using ZBTB32-deficient mice, it was demonstrated that ZBTB32 facilitated proliferation by counteracting the anti-proliferative factor BLIMP1 (also known as PRDM1)[57]. In addition, NK cells lacking the microRNA miR-155 exhibited severely reduced effector and memory cell numbers after MCMV infection[58]. Although the genes encoding the known receptors expressed by NK cells do not require rearrangement, NK cells lacking RAG showed impaired expansion and persistence after MCMV infection, suggesting an important role for RAG in the optimal generation of MCMV-specific memory NK cells[59]. In a *Rag1* reporter system, it was observed that approximately one third of the NK cell population had transiently expressed *Rag1* early in their development and that these NK cells generated a more robust memory response to MCMV infection than NK cells in the same mouse that had not transiently expressed *Rag1* (REF. 59). So far, it is unknown whether hapten-specific[13,14], influenza-specific[14], vaccinia virus-specific[18] and HSV-2-specific[19] memory NK cells are affected by the expression of Rag genes during their development. NK cells deficient in the anti-apoptotic factor BIM (encoded by *Bcl2l11*) expand normally in response to MCMV infection, but contraction of the LY49H$^+$ NK cell pool and functional NK cell memory formation are impaired[60]. More recently, similarly to the mechanisms driving CD8$^+$ T cell memory formation, autophagy was shown to be required for survival of effector NK cells during the contraction phase by an *Atg3*-dependent mechanism[61]. In future studies, it will be important to determine whether and to what extent unique pathways driving NK cell memory generation exist that differ from CD8$^+$ T cell memory formation.

Cytokine-induced memory NK cells. In 2009, Cooper *et al.* reported that even in the absence of a specific antigen, a brief *in vitro* incubation of mouse NK cells with the cytokines IL-12, IL-15 and IL-18 resulted in the generation of NK cells that possessed the ability to secrete high amounts of IFNγ for up to 4 months after adoptive transfer into *Rag1*$^{-/-}$ mice compared with NK cells pretreated only with IL-15 (REFS 21,22). This long-term competence for IFNγ production was cell intrinsic and maintained after homeostatic proliferation[21]. Human NK cells that are briefly exposed to IL-12, IL-15 and IL-18, washed and then cultured in IL-2 or IL-15 for additional days also produce highly elevated amounts of IFNγ upon restimulation with tumour cell lines or with IL-12 and IL-15 (REFS 23,54). Similar to observations with human HCMV-driven CD94–NKG2C$^+$ NK cells, the brief exposure of human NK cells to IL-12, IL-15 and IL-18 resulted in stable demethylation of the *IFNG* conserved non-coding sequence 1 (CNS1)[62]. Therefore, even in the absence of a specific antigenic stimulus, NK cells can remember prior exposure to polarizing cytokines.

Whether all cytokine-induced NK cells or only subsets of these are endowed with longevity and the ability to produce large amounts of cytokines upon restimulation is currently not clear. As these NK cell remember their prior activation by cytokines, they have been referred to as memory or memory-like NK cells, although whether they provide enhanced host defence against infection has not been investigated. IL-12 and IL-18 contribute to the generation of NK cell memory in the MCMV model that is driven by the LY49H–m157 interaction. Whether these cytokines also have a physiological role in the generation of memory NK cells during viral infection in the absence of a cognate interaction between the NK cells and pathogens in other models needs to be determined. Of note, cytokine-induced memory cells could be of high therapeutic value as illustrated by the observation that antitumour immune responses were greatly improved upon adoptive transfer of cytokine-induced memory NK cells into tumour-bearing mice[54].

Characteristics of memory NK cells

The phenotypic characteristics of memory NK cells in different experimental systems are summarized in TABLE 1. Importantly, hapten-specific, virus-specific and cytokine-induced memory NK cells show distinct and overlapping functional properties and tissue localization patterns as discussed below.

Phenotypical characteristics of memory NK cells. Initially, the liver-resident memory NK cells that drive CHS responses to DNFB or OXA upon transfer into naive recipients were described to express NK1.1, LY49C or LY49I and THY1 (also known as CD90)[13,14]. Using CXCR6-deficient mice, CXCR6 expressed by liver NK cells was shown to be involved in hapten-specific memory generation and function[14]. Different laboratories have subsequently further phenotypically characterized liver NK cells in the context of CHS. In a model of DNFB-induced CHS, adoptive transfer of DX5$^+$ (DX5 is also known as CD49b and α2 integrin) NK cells isolated from livers of DNFB-sensitized BALB/c or C57BL/6 mice into syngeneic mice conferred CHS responses after DNFB-challenge[24,63]. In a follow-up study by the same group, the liver NK population that most potently conferred CHS was further defined as mature CD11b$^+$ (also known as αM integrin), CD27$^-$ and LY49C$^+$ and/or LY49I$^+$ cells[24]. CD49a (also known as α1 integrin) and CXCR6 expression was not assessed in this study.

By contrast, in an OXA-induced CHS model in C57BL/6 mice, the liver NK cell population mediating hapten-specific memory was characterized as CD49a$^+$ cells lacking DX5 expression[64,65]. The CD49a$^+$DX5$^-$ population that conferred the hapten-specific memory response also expressed NKp46 (also known as NCR1), CXCR6, THY1.2, TRAIL (also known as TNFSF10), CD27 and CD51 (also known as αV integrin), but not

Table 1 | **Phenotypical markers of memory NK cells**

Species	Model of NK memory generation	Phenotype of memory NK cells	Refs
Mouse	Hapten-specific memory NK cells in the liver	Expression of THY1, LY49C and/or LY49I and CXCR6	13,14
		Expression of NKp46, CD51, THY1, CD49a, CXCR6 and TRAIL, CD27^{low} and lack of DX5 and CD127 (also known as IL-7R)	64,66
		Expression of DX5, THY1, LY49C and/or LY49I and CD11b, and lack of CD27 expression	24,63
	Vaccinia virus-specific memory NK cells in liver	Expression of THY1 and DX5	18
		After activation, expression of KLRG1, CD27^{low}, CD11b^{hi} and LY6C^{hi}	
	MCMV-specific memory NK cells	Expression of LY49H, CD11b^{hi}, KLRG1^{hi}, LY6C^{hi} and CD27^{low}	16
	Cytokine-induced memory NK cells	CD11b^{hi}, KLRG1^{hi}, LY6C^{hi}, CD43^{hi} and CD27^{low}	54
Human	Expanded populations of NK cells from HCMV-seropositive donors	CD94–NKG2C^{hi} and CD57^{hi}	35
		Lack of NKG2A expression	
	Expanded populations of NK cells from HCMV-seropositive donors	Lack of FcεRIγ, SYK and EAT2 expression	44,62
		Low levels of PLZF expression	
	Expanded populations of NK cells from HCMV-seropositive donors	Expression of inhibitory KIR2DL for self HLA class I ligands	42
		Expression of activating KIR2DS2, KIR2DS4, or KIR3DS1 in some individuals	
	Cytokine-induced memory NK cells	Expression of CD94, NKG2A, NKG2C and CD69	23,54
Rhesus macaque	SIV-induced memory NK cells	Expression of NKG2 (NKG2A or NKG2C)	20

CXCR6, CXC-chemokine receptor 6; EAT2, EWS/FLI1-activated transcript 2; FcεRIγ, high-affinity IgE receptor subunit-γ; HCMV, human cytomegalovirus; IL-7R, interleukin-7 receptor; KIR, killer cell immunoglobulin-like receptor; KLRG1, killer cell lectin-like receptor subfamily G member 1; MCMV, mouse cytomegalovirus; NK, natural killer; NKG2, natural killer cell protein group 2; PLZF, pro-myelocytic leukaemia zinc finger; SIV, simian immunodeficiency virus; TRAIL, TNF-related apoptosis-inducing ligand.

the IL-7 receptor (also known as CD127). Parabiosis experiments indicated that the CD49a$^+$DX5$^-$ NK cells reside in the liver, whereas CD49a$^-$DX5$^+$ NK cells circulated systemically[64,66]. Moreover, CD49a$^+$DX5$^-$ liver-resident NK cells show a unique transcriptomic and functional profile, suggesting that these cells might be part of a cell lineage distinct from circulating conventional NK cells[66], and they exhibit certain features of group 1 innate lymphoid cells (ILC1s)[65]. Importantly, adoptive transfer studies revealed that CD45a$^+$DX5$^-$ NK cells remained DX5$^-$ and did not differentiate into DX5$^+$ cells[64]. Taken together, there is consensus that hepatic NK cells confer hapten-specific NK cell memory responses, but the exact nature of the liver NK cell subpopulation (DX5$^+$ versus DX5$^-$) conferring CHS responses remains controversial. So far, the reasons for the divergent observations are not explained.

Vaccinia virus-specific memory NK cells were contained within the DX5$^+$ sorted population and expressed THY1. Within the first week of vaccinia virus challenge, these cells upregulated killer cell lectin-like receptor subfamily G member 1 (KLRG1) and were CD27^{low}, CD11b^{hi} and lymphocyte antigen 6C (LY6C)^{hi} (REF. 18). Although there are no unique markers to identify MCMV-specific LY49H$^+$ memory NK cells, they are characterized by the expression of high levels of CD11b, KLRG1 and LY6C and the loss of CD27 (REF. 16). A similar marker profile was observed on cytokine-induced mouse memory NK cells, regardless of whether they expressed LY49H[54]. In humans, the memory NK cells that develop in response to HCMV infection express high levels of the CD94–NKG2C receptor, and many of these cells co-express CD57 and lack NKG2A[35]. These HCMV-driven CD94–NKG2C$^+$ NK cells may also lack expression of FcεRIγ, SYK and EWS/FLI1-activated transcript 2 (EAT2; also known as SH2D1B)[45,67]. In addition to NKG2C, NK cells expanding in response to HCMV infection express the activating KIRs KIR2DS2, KIR2DS4 or KIR3DS1 (REF. 42).

Functional characteristics of NK memory cells. Functionally, memory immune responses are characterized by a quantitatively and qualitatively increased effector response upon restimulation. Whether NK cell memory responses develop faster than responses mediated by naive NK cells has not been analysed in detail. As noted above, the NK cell-mediated CHS responses in the DNFB model develop rapidly, within one hour after NK cell adoptive transfer into challenged mice[24]. In the MCMV infection model, LY49H$^+$ memory NK cells produce higher amounts of IFNγ as compared with naive NK cells, demonstrate more robust cytotoxic responses and provide enhanced host protection following reinfection with MCMV, but not following infection with heterologous pathogens[16,68]. In human studies, Romagnani and colleagues demonstrated that the CD94–NKG2C$^+$ NK cell populations that expanded in HCMV-seropositive individuals exhibited a demethylated CNS1 region in the *IFNG* locus[62]. Transfection experiments using NK cell lines illustrated that demethylation of CNS1 facilitated *IFNG* transcription. In addition, the global methylation profile of these expanded human CD94–NKG2C$^+$ NK cells greatly differed from naive NK cells, suggesting that, during HCMV infection, epigenetic remodelling of these NK cells affected their future behaviour.

Global genetic and epigenetic changes in NK cells from HCMV-seropositive individuals were further dissected in two recent studies[45,67]. The reports identified a unique subset of NK cells in HCMV-seropositive donors that is characterized by the lack of the immunoreceptor tyrosine-based activation motif (ITAM)-bearing FcεRIγ adaptor protein, variable loss of SYK and EAT2, and low amounts of the transcription factor pro-myelocytic leukaemia zinc finger (PLZF; also known as ZBTB16). This FcεRIγ-deficient subset contains both CD94–NKG2C$^-$ and CD94–NKG2C$^+$ NK cells, indicating that signals from receptors other than NKG2C may shape this NK cell subset. The FcεRIγ-deficient NK cells were designated adaptive NK cells, and they showed population expansion after activation through the Fc receptor CD16 (which signals by its association with

CD3ζ chain in the absence of FcεRIγ chain) or when co-cultured with antibody-coated target cells infected with influenza virus or HCMV[45]. These observations further highlight that alternative pathways for NK cell subset expansion during HCMV infection might exist that do not necessarily require the interaction of CD94–NKG2C with HLA-E. Intriguingly, unlike in humans, MCMV-specific mouse memory NK cells do not lose expression of SYK or FcεRIγ. It will be important to further explore genetic and epigenetic alterations upon NK cell subset expansion and memory generation in different types of viral infection.

Factors leading to the differentiation of adaptive NK cells in humans have not yet been elucidated. Intriguingly, the expanded population of CD94–NKG2C$^+$ NK cell that are detected in HCMV-seropositive individuals demonstrate a diminished response to inflammatory cytokines such as IL-12 and IL-18 (REF. 42). Moreover, HCMV-expanded CD94–NKG2C$^+$ NK cells display lower levels of IFNγ production and degranulation in response to re-stimulation with pertussis or H1N1 influenza vaccine antigens[69]. The observation that HCMV-expanded CD94–NKG2C$^+$ cells have lower responses to heterologous challenges could be highly relevant for designing vaccines to viruses other than HCMV. In future studies, it will be important to dissect stimulus-dependent functional properties of HCMV-expanded NK cells in more detail.

Tissue localization patterns of memory NK cells. Distinct patterns of tissue localization have been observed for hapten-specific, virus-specific or cytokine-induced memory NK cells. Hapten-[13,14,24,63,64], VSV-[14] and vaccinia virus[18]-specific memory NK cells reside in the liver. In influenza VLP-sensitized mice, memory NK cells isolated from the lungs also provided protection against influenza challenge after adoptive transfer[14]. Both splenic and hepatic NK cells from rhesus macaques that were infected with SIV mediated antigen-specific memory responses towards SIV Gag or Env proteins[20]. By contrast, MCMV-specific NK cells are distributed systemically in all organs[16], in a similar manner to cytokine-induced NK cells[54].

It is tempting to speculate that different types of memory NK cells might be nurtured by different organ environments. Accordingly, hapten-specific memory NK cells in the liver apparently rely on the chemokine receptor CXCR6 (REF. 14), which binds to CXCL16, a chemokine that is highly abundant in the liver. The factors that make memory NK cells exit the liver and rapidly migrate to the ear upon challenge are currently unknown. The distinct tissue localization of different types of memory NK cells might also enable them to quickly respond to organ-specific infections and interact with other tissue-resident cell types such as ILCs and myeloid cell populations. In response to systemic viral infection, memory NK cells distributed through the whole body might efficiently mediate viral control. Using parabiosis experiments, it was recently reported that not only ILCs but also subsets of NK cells carrying markers of conventional NK cells are tissue resident in different organs including the small intestine, lung and salivary gland[70]. It will be important to further dissect and characterize NK cell subsets in different organs, to understand their relationship to conventional NK cells and ILCs and to unravel their unique functionality and memory potential during disease.

Therapeutic implications

As discussed above, there is evidence from a number of different experimental settings that NK cells endowed with characteristics of memory cells can be generated. It is less clear whether these memory NK cells are physiologically relevant in different disease settings. It is possible that memory NK cells are physiologically relevant for some, but not all diseases, with the clearest evidence of their utility coming from the setting of viral infections, such as CMV. So far, no evidence for a physiological role of NK cell memory in cancer or in bacterial infections has been reported, but these are areas that need further investigation. Below, we outline how NK cell memory activities could be harnessed for therapeutic purposes.

Exploiting NK cell memory for antiviral therapy. Recent discoveries of NK cell memory generation in viral infection could potentially have a major effect on novel vaccination strategies against different types of viruses. Strategies activating NK cell antiviral immunity could support and improve current T cell-based vaccination strategies. So far, the relative contribution of NK cell memory to protection against viral infection in hosts with an intact adaptive immune system is unclear. Because the number of effector cells that are engaged ultimately determines elimination of pathogens, memory NK cells with enhanced effector functions may cooperate with effector T cells and antibodies to provide more rapid and effective control of infection. It is also possible that memory NK cells can support T cell memory responses by rapidly providing inflammatory cytokines and chemokines that further orchestrate the migration and function of adaptive immune cells.

Reciprocally, effector and memory T cells might support NK cell memory responses by providing IL-2, which further drives NK cell expansion and sustains memory. Whether memory NK cells limit T cell responses by directly killing activated T cells has not been assessed but should be considered[71]. It is tempting to speculate that, in situations in which T cell memory is impaired, memory NK cells might in part provide protection against disease. In this respect, patients infected with HIV, which specifically impairs T cell responses, might be able to mount efficient NK cell memory responses. Accordingly, antigen-specific NK cell memory responses in rhesus macaques were observed after SIV infection[20], supporting the potential for boosting NK cell memory responses also against HIV-1. Similarly, after haematopoietic cell transplantation, recall responses of NK cells, which are the first lymphocyte population to recover, may contribute to host defence against infection, in

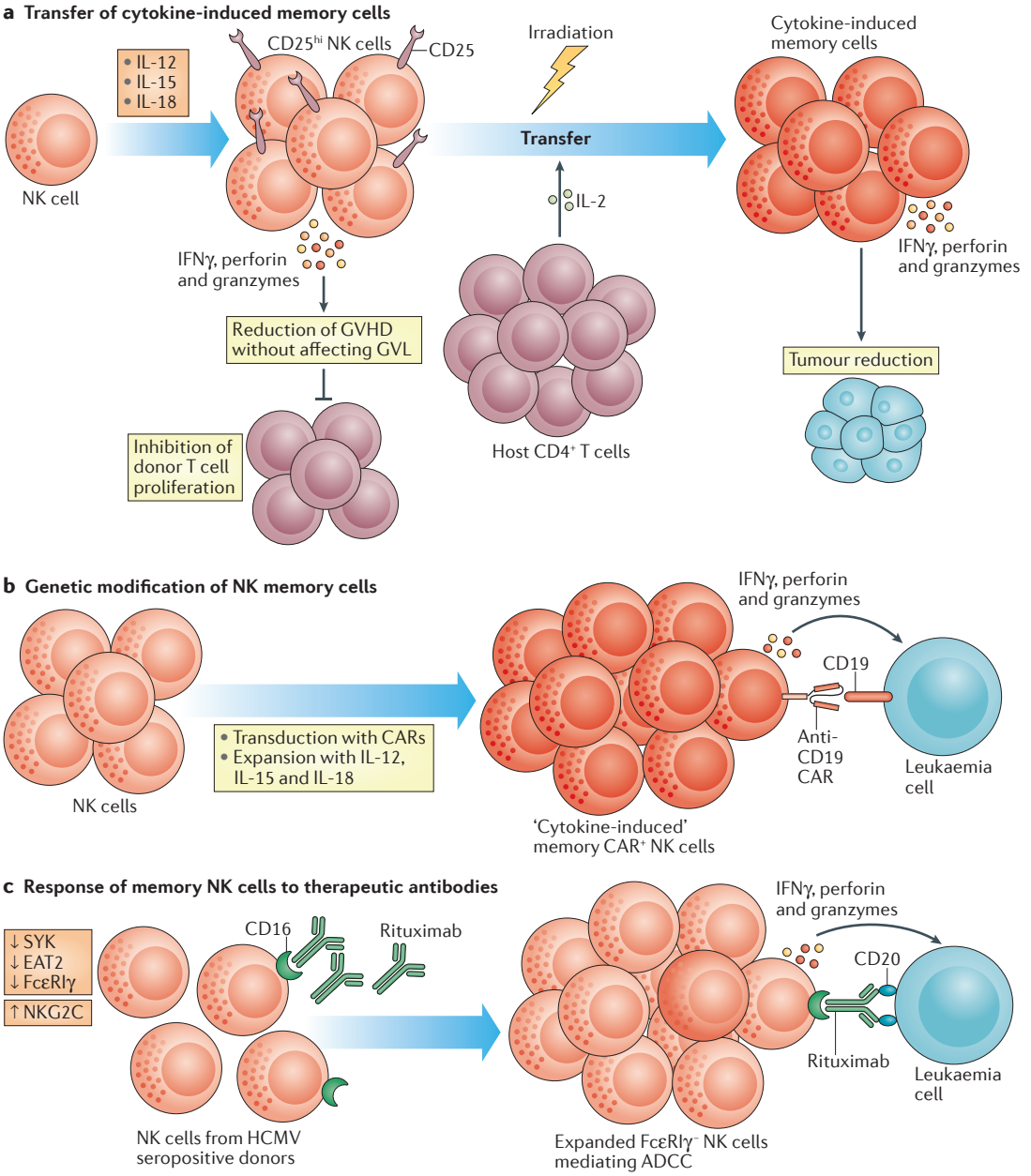

Figure 3 | **Potential applications of memory NK cells for tumour therapy. a** | A brief exposure of natural killer (NK) cells to the cytokines interleukin-12 (IL-12), IL-15 and IL-18 *in vitro* before adoptive transfer into recipient mice leads to the generation of long-lived memory NK cells with a sustained ability to exert antitumour effector function that can efficiently control tumour growth. NK cells that have been pre-activated by IL-12, IL-15 and IL-18 can suppress graft-versus-host disease (GVHD) in mice without affecting graft-versus-leukaemia (GVL) activity, and these cells impair the proliferation of donor alloantigen-specific T cells. **b** | Proliferating NK cells can be transduced with chimeric antigen receptors (CARs) for immunotherapy of cancer. Depending on the stimulation conditions and the cytokines that are present during the culture, it is possible to generate NK cells with the potential to exert long-term effector memory functions. **c** | Populations of high-affinity IgE receptor subunit-γ (FcεRIγ)-negative NK cells that expresses low amount of SYK and/or EWS/FLI1-activated transcript 2 (EAT2), might be expanded by treatment with therapeutic monoclonal antibodies, such as rituximab, and might persist long term as memory cells with high antitumour activity. These NK cells show increased antibody-dependent cellular cytotoxicity (ADCC) activity. HCMV, human cytomegalovirus; IFNγ, interferon-γ; NKG2C, natural killer protein group 2, member C.

particular HCMV. Thus, vaccine formulations that enhance NK cell memory generation could greatly improve current vaccination protocols in particular for viruses that are recognized by NK cells.

Exploiting NK cell memory in cancer therapy. The novel discoveries of NK cell memory in viral infection have stimulated emerging interest in exploiting NK cell memory for clinical application in cancer (FIG. 3). Of note,

repeated exposure of mouse NK cells to NK receptor ligand-expressing tumour cells results in NK cell dysfunction and not to enhanced effector responses[72]. A similar NK cell dysfunction was observed with NK cells chronically exposed to myeloid cells expressing retinoic acid early transcript 1 (RAE1; also known as mRNA export factor and a ligand of the activating NKG2D receptor) in the tumour microenvironment[73]. So far, NK cell memory against tumours has not been observed under physiological conditions. These findings imply that the receptors, such as NKG2D, that are involved in the recognition of tumour cells by NK cells may not be endowed with the capacity to efficiently generate memory. Alternatively, because the generation of memory NK cells during viral infection requires the production of certain cytokines (for example IL-12) during the acute infection, the generation of memory NK cells against tumours may also require host-derived factors, in addition to specific ligands for activating NK receptors that might be lacking in the tumour microenvironment.

However, the concepts formulated from studying NK cell memory generation in the setting of viral infection might help to improve protocols for generating long-lived tumour-reactive NK cells for cancer therapy. The adoptive transfer of *ex vivo* activated NK cells is a promising treatment option for patients suffering from different types of cancer, in particular haematological neoplasia. Infusions of IL-2-activated NK cells, for example, induced remission of acute myeloid leukaemia (AML) in a subset of patients[74]. However, adoptive transfer of autologous IL-2-expanded NK cell populations into patients suffering from solid tumours did not result in clinical benefits, and the adoptively transferred NK cells became rapidly functionally impaired in the patients[75].

A challenge for the field is to design optimal strategies for NK cell activation before infusion and to optimize clinical protocols to achieve persistence and expansion of NK cells with potent antitumour activity in the patients after transfer. In this regard, following adoptive transfer to tumour-bearing mice, cytokine-induced memory NK cells pre-activated with IL-12, IL-15 and IL-18 displayed potent antitumour activity and significantly increased the survival of the recipient mice[54] (FIG. 3a). Large numbers of these cytokine-induced memory NK cells were observed in the tumour and spleen, and these NK cells produced high amounts of IFNγ, perforin and granzymes upon re-stimulation[54]. Furthermore, transferred cytokine-induced memory NK cells were still detected 3 months after adoptive transfer[54]. Based on the concepts of cytokine-induced NK memory cells, a Phase I clinical trial using IL-12, IL-15 and IL-18-pre-activated NK cells for treatment of patients with AML recently opened at Washington University School of Medicine, St Louis, Missouri, USA (NCT01898793). The intratumoural application of the cytokines IL-12 and IL-18 was also shown to revert dysfunction of NK cells in the tumour bed of MHC class I-deficient tumour cells[76]. Whether NK cells with a sustained ability for long-term effector function were generated in these studies has not been evaluated. Accordingly, cytokine-producing DCs or vectors endowing NK cells with the capacity to produce IL-12 and IL-18 could be implemented.

A recent study revealed that NK cells pre-activated with IL-12, IL-15 and IL-18 suppress graft-versus-host disease in a mouse model of mismatched haematopoietic cell transplantation without affecting graft-versus-leukaemia activity[77]. These NK cells inhibited proliferation of donor T cells, probably by competing for IL-2 with the proliferating T cells. Thus, cytokine pre-activated NK cells may hold great promise for improving current therapies against leukaemia. Moreover, *in vitro* expansion protocols for NK cell populations before infusion into patients could benefit from the knowledge gained from NK cell memory studies. Genetic modification of immune cells by chimeric antigen receptors (CARs) targeting the immune cell directly to the tumour cells is a promising novel therapeutic strategy in cancer therapy. In this respect, CAR T cells yielded promising results but raised safety issues. NK cell expansion protocols for subsequent genetic modification by CARs could use the cytokines identified to drive their expansion during viral infections[78] (FIG. 3b). Moreover, the presence of inflammatory cytokines during NK cell expansion might also enable subsequent competence for long-term survival and effector function in patients. Thus, lessons learned from the generation and maintenance of memory NK cells in mouse models might help to manufacture NK cell populations with the best *in vitro* expansion leading to efficient transduction rates with CARs and to allow potent *in vivo* antitumour activity. These NK cells could be also genetically modified to produce cytokines, such as IL-12 and IL-18, but care must be taken to ensure safety.

Another potential use of concepts from NK cell memory in cancer therapy is suggested by the enhanced antibody-dependent cellular cytotoxicity (ADCC) demonstrated by the newly discovered human FcεRIγ-deficient adaptive NK cells (FIG. 3c). A recent study suggested that the Fc component of rituximab, a CD20-specific therapeutic antibody, is required for the long-term protective effect of this antibody against tumours[79]. Whether Fc receptor-expressing NK cells, and possibly the FcεRIγ-deficient NK cell subset, contributed to the observed long-term protection in this study should be evaluated in future studies.

Conclusions and perspective

Given the importance of immunological memory to protect against disease, it is intriguing that innate immune cells now enter the stage. We are just starting to understand the molecular mechanisms involved in the generation and maintenance of memory NK cells and their interaction with other immune cells. The discovery of memory behaviour in NK cells has catalysed research to determine whether other recently identified innate lymphoid cells have the capacity for immunological memory. These recent developments provide new insights and opportunities to manipulate cells of the innate immune system to augment their activity against infectious disease and cancer.

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Competing interests statement
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