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Human Natural Killer Cells Mediate Adaptive Immunity to Viral Antigens

Rana Nikzad[1,2,9], **Laura S. Angelo**[1], **Kevin Aviles-Padilla**[1,3], **Duy T. Le**[1,4], **Vipul K. Singh**[1], **Lynn Bimler**[1,4], **Milica Vukmanovic-Stejic**[5], **Elena Vendrame**[6], **Thanmayi Ranganath**[6], **Laura Simpson**[6], **Nancy L. Haigwood**[7], **Catherine A. Blish**[6,8], **Arne N. Akbar**[5], and **Silke Paust**[1,2,3,4,9,*]

[1]Center for Human Immunobiology, Department of Pediatrics, Texas Children's Hospital, Houston, TX, USA

[2]Translational Biology and Molecular Medicine Graduate Program at Baylor College of Medicine, Houston, TX, USA

[3]The Integrative Molecular and Biomedical Sciences Graduate Program at Baylor College of Medicine, Houston, TX, USA

[4]The Immunology Graduate Program at Baylor College of Medicine, Houston, TX, USA

[5]Division of Infection & Immunity, University College London, UK

[6]Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

[7]Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR USA

[8]Chan Zuckerberg Biohub, San Francisco, CA, USA

[9]Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA, 92037

Abstract

Adaptive immune responses are defined as antigen-sensitization-dependent and antigen-specific responses leading to establishment of long-lived immunological memory. While natural killer (NK) cells have traditionally been considered cells of the innate immune system, mounting evidence in mice and non-human primates warrants reconsideration of the existing paradigm that B and T cells are the sole mediators of adaptive immunity. However, it is currently unknown whether human NK cells can exhibit adaptive immune responses. We therefore tested whether human NK cells mediate adaptive immunity to virally encoded antigens using humanized mice

*To whom correspondence should be addressed: paust@scripps.edu.

Author Contributions

SP designed experiments, RN, LA, KAP, DTL, VKS, MVS, EV, TR, LS, DSA, LB, BK, NH, CAB, ANA, and SP performed experiments and analyzed data, SP, RN and LB performed statistical analyses, and RN, ANA, CAB and SP wrote the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Data and Materials Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

and human volunteers. We found that human NK cells displayed vaccination-dependent, antigen-specific recall responses *in vitro*, when isolated from livers of humanized mice previously vaccinated with human immunodeficiency virus (HIV)-encoded envelope protein. Furthermore, we discovered that large numbers of cytotoxic NK cells with a tissue-resident phenotype were recruited to sites of varicella-zoster virus (VZV) skin test antigen challenge in VZV-experienced human volunteers. These NK-mediated recall responses in humans occurred decades after initial VZV exposure, demonstrating that NK memory in humans is long-lived. Our data demonstrates that human NK cells exhibit adaptive immune responses upon vaccination or infection. The existence of human memory NK cells may allow for the development of vaccination-based approaches capable of establishing potent NK-mediated memory functions contributing to host protection.

One Sentence Summary

Vaccination or childhood infection elicits antigen-specific, long-lived immunological memory responses in human natural killer cells.

Introduction

Natural killer (NK) cells are an essential weapon of the immune system: they kill target cells rapidly, secrete large amounts of cytokines, and mutations preventing their maturation or function cause severe immune deficiencies (1). NK cells survey their environment with a diverse receptor repertoire including activating and inhibitory receptors, cytokine and chemokine receptors, and adhesion molecules (2). Healthy cells escape NK immune attack by expressing robust levels of major histocompatibility complex class I (MHC I) molecules that ligate NK-expressed inhibitory receptors, such as killer immunoglobulin-like receptors (KIRs), and CD94/NKG2A (3). However, infected or malignant cells often downregulate MHC I while expressing ligands for NK-expressed activating receptors, such as NKG2D (4–6) and CD16 (7). Activation through such receptors elicits rapid target-cell killing via the secretion of pore-forming proteins (perforin) and cytotoxic mediators (granzymes) that trigger target-cell apoptosis (8, 9), as well as cytokine and chemokine release.

In humans, NK cells are identified as cells that express both the hematopoietic cell marker CD45 and the glycoprotein CD56 but not CD3, a signaling component of the T cell receptor (10, 11). Both T-box transcription factors T-bet and Eomesodermin (Eomes) are expressed by human NK cells, are crucial for NK development, are differentially expressed during specific developmental stages, and in tissue-specific NK subsets (12, 13). While NK cells were traditionally thought to develop in the bone marrow (BM), mounting evidence suggests that their progenitors can develop *in situ*. About 90 percent of human peripheral blood mononuclear cell (PBMC)-derived NK cells are T-bet^{hi}, Eomes^{lo}, CD56^{lo} and CD16^{hi}, and are considered mature and highly cytotoxic. In contrast, the remaining ten percent of human PBMC-derived NK cells are T-bet^{hi}, Eomes^{hi}, CD56^{hi} and CD16^{lo} (14) and exhibit robust cytokine production, but are considered less mature and less cytotoxic than CD56^{lo} NK cells.

In contrast to PBMC, human tissues harbor an abundance of $CD56^{hi}$, $CD16^{lo}$ NK cells (13, 15). Adult human spleen and liver contain both T-bet and Eomes expressing NK cells (13), and harbor NK cells that express the G-protein coupled C-X-C motif chemokine receptor 6 (CXCR6) (16), which is required for NK memory in mice (17–19). $Eomes^{hi}$ $CXCR6^{hi}$ NK cells in human adult livers and spleens also express the activation or tissue residency marker CD69. The majority of murine and human CXCR6-expressing NK cells are found in the liver, while CXCR6-expressing NK cells are largely absent from human PBMC (12, 15) and from murine spleen (17–19). Human spleen (17), BM and lymph nodes (20) however contain $CXCR6^{+}$ NK cells, but with perhaps a lower frequency than the human liver. In the steady state, the ligand for CXCR6, the transmembrane chemokine C-X-C motif ligand 16 (CXCL16), is abundantly expressed on liver sinusoidal endothelial cells in mice and humans, where it provides homing (16) and survival signals to $CXCR6^{+}$ immune cells (18, 19, 21, 22). Whether human spleen, BM and lymph nodes express significant amounts of human CXCL16 remains to be determined.

While NK cells have traditionally been considered cells of the innate immune system, mounting scientific evidence suggests that NK cells mediate adaptive immune responses (17–19, 23). Using delayed type hypersensitivity (DTH) and viral challenge models (18, 19, 24), we previously reported that murine hepatic $CXCR6^{hi}$ NK cells exhibit all three hallmarks of adaptive immunity in response to haptens and virally-encoded antigens: vaccination-dependence, antigen-specificity and long-lived immunological memory (17–19). Further, our and other published data over the past 12 years has clearly demonstrated that NK memory in mice is B- and T-cell-independent (18, 19, 24–26). Neither the priming of memory NK cells, nor their memory response are dependent on T or B cells. Antigen-primed NK cells are sufficient and required for NK memory to occur as demonstrated by NK cell depletion or adoptive transfer (18, 19, 24, 25). The presence of naïve T and B cells neither augments nor inhibits memory NK cell-mediated recall responses, and the recruitment of antigen-primed NK cells to sites of challenge is antigen specific in mice, even in a competitive setting (18).

Interestingly, NK memory has been demonstrated in nonhuman primates (NHP) (23) and is as such not restricted to rodents. However, these exciting findings beg the question of whether human NK cells are capable of adaptive immune responses, which is currently unknown. We therefore tested whether human NK cells mediate adaptive immune responses using humanized mice and human volunteers. The BLT (bone marrow, liver, thymus) humanized mouse model we use for our studies is particularly suited to test our hypotheses, as it allows for the production of several dozen human donor-matched BLT mice in which a functional human immune system develops, including human HLA-restricted T cells, NK cells, B cells, dendritic cells, neutrophils and monocytes (27–32). Furthermore, BLT mice of a human-donor matched cohort are of identical murine and human genetics, allowing for direct comparisons of immune memory of donor-matched naïve vs. vaccine primed NK cells, and controlled studies of immune responses to vaccination in tissues other than the peripheral blood.

Here, we demonstrate that spleens and livers of BLT mice harbor human NK cells that are tissue resident in phenotype, and similar in phenotype to NK cells found in adult human

liver and spleen. We demonstrate that human NK cells isolated from livers, but not from spleens, of vaccinated BLT mice mediate vaccination-dependent and antigen-specific killing *in vitro*. Evaluating VZV-experienced human adults, we found that large numbers of NK cells are recruited to sites of VZV skin test antigen (VZV-STA) challenge, even decades after initial VZV exposure (33). Interestingly, these memory NK cells phenotypically resemble human tissue resident NK. Our data demonstrates that human NK cells with a tissue resident phenotype mediate adaptive immunity similarly to that of NK cells in mice and NHP.

Results

Spleens and livers of BLT mice harbor tissue-specific human NK cells

Human NK cells develop in BLT mice, are responsive to IL-15, a cytokine important for NK cell development and function (34), kill MHC-I-deficient target cells, and produce IFN-γ upon activation (35). However, little is currently known about the distribution of tissue-specific NK cells in BLT mice and their maturation status. We therefore used multi-parametric flow cytometry to examine the expression of tissue-specific transcription factors and cell-surface markers on splenic and hepatic BLT-derived NK cells, four to six months after reconstitution. Eight to twelve weeks post reconstitution, over 90 percent of hematopoietic cells found in PBMC of our BLT mice were of human origin (Supplementary Figure 1A), and human NK cells were present systemically, including in BLT spleens and livers (Supplementary Figure 1B, C). Four to six months post reconstitution, both T-bet and Eomes expressing NK cells were present in spleens and livers of BLT mice (Figure 1A). Eomes^{hi} CD56^{hi} CXCR6^{hi} NK cells were more abundant in BLT mouse liver, while BLT-derived splenic NK cells were more frequently T-bet^{hi} CD56^{lo} and CXCR6^{lo}, similar to reports examining human adult splenic or hepatic NK cells (13, 15). We next inspected the expression of tissue residency and functional markers on splenic and hepatic BLT-derived NK cells (Figure 1B). Spleen NK cells had a lower frequency of CD56^{hi} NK cells than liver NK cells, as well as a reduced frequency of CXCR6, CD16, NKG2D, and CX3CR1-expressing NK cells, albeit all of these markers were nevertheless expressed on a substantial number of splenic NK cells (Figure 1B). However, we observed no differences in the frequencies of CD69, CD62L, perforin, granzyme B, or IFN-γ-expressing NK cells between BLT spleens and livers (Figure 1B). We conclude that expression of tissue residency markers, lymph node-homing ability, cytotoxic effector functions, and IFN-γ-production are similar between splenic and hepatic NK cells, while the expression of the activating receptors NKG2D and CD16 is reduced on splenic BLT-derived NK cells compared to donor-matched hepatic NK cells.

Splenic and hepatic NK cells are similar in phenotype in BLT mice and human adults

We next compared the phenotypes of BLT-derived splenic and hepatic NK cells to that of human adult donor-derived spleen or liver, and to the current literature (13, 15). Liver samples were of transplant quality, while spleen samples were obtained from newly diagnosed and treatment-naïve pancreatic cancer patients undergoing splenectomy. Only disease-free spleens were used for experimental analyses. With the exception of CD62L, the frequency of CD56^{hi}, CXCR6, CD16, NKG2D, CD94, CD69, NKG2A, CX3CR1, perforin

and granzyme B-positive NK cells was indistinguishable between BLT-derived and adult human donor-derived livers (Figure 2A). Likewise, splenic BLT-derived NK cells were similar to human spleen-derived NK cells, with the exception of the frequencies of CD16, NKG2D, and CD62L-expressing NK cells, which were reduced in BLT mice compared to human adult spleen (Figure 2B). However, these markers were nevertheless robustly expressed on a substantial number of splenic BLT-derived NK cells. As BLT mice do not develop proper lymph node architecture, it may not be surprising that the frequency of CD62L-expressing NK cells is higher in BLT livers and spleens compared to human adult liver and spleen, as these cells are left to circulate in BLT mice but may home to lymph nodes in healthy adult humans. We conclude that human NK cells in BLT mice phenotypically resemble the corresponding tissue-specific NK cells of adult humans, both in the expression of tissue-specific transcription factors and relevant cell surface markers.

Phenotypically mature human NK cells develop in BLT mice

We next used CyTOF mass cytometry to compare the expression of additional markers on BLT NK cells vs. human adult PBMC NK cells, a population we consider predominantly mature in phenotype (Supplementary Figure 2 and Supplementary Table 1). We found that the inhibitory and activating KIR2DL1, KIR2DL3, KIR2DL5, KIR2DS2, KIRDS4, and KIR3DL1 receptors, the activating and costimulatory receptors NKp30, NKp40, NKp46, DNAM-1, 2B4, CD8, NTB-A, and Tactile, the inhibitory receptors CD94/NKG2A and PD-1, as well as the apoptosis-inducing FasL were expressed at a similar frequencies on BLT-derived vs. human adult PBMC-derived NK cells (Supplementary Figure 2). In contrast, the frequencies of CD94/NKG2C, CD57 and TIGIT-expressing human NK cells were reduced in BLT mice compared to adult human PBMC-derived NK cells. As CD57 is a marker for terminally differentiated $NKG2C^{+}$ NK cells that accumulate in CMV-seropositive human adults (36), and TIGIT is a marker of immune exhaustion (37, 38), we did not expect either marker to be robustly expressed in naïve BLT mice. Thus, we conclude that BLT-derived human splenic and hepatic NK cells are similar in phenotype to human adult tissue-matched donor NK cells, supporting the use of this model to analyze whether human NK cells mediate priming-dependent and antigen-specific immunological memory responses after vaccination.

Human hepatic and splenic NK cells respond to vaccination of BLT mice

Whether human NK cells are capable of adaptive immunity has not yet been determined. We chose humanized mice for the testing of two of the three hallmarks of adaptive immunity: vaccination dependence and antigen specificity (17, 19), as the experimental use of donor-matched cohorts of naïve and vaccinated humanized mice allows us to compare antigen-specific recall responses of truly antigen-naïve NK cells to that of donor-matched, antigen-primed NK cells. Such comparisons would be difficult to perform with human samples, as it is not possible to trace the prior antigen exposure of adult humans. To test whether human NK cells in BLT mice respond to vaccination, we vaccinated BLT mice by intravenous and intraperitoneal infusions of syngeneic DC preparations that were loaded for 2 hours in a tissue culture incubator with HIV envelope protein (HIV-env; from HIV-Q23–17 clone), and left donor-matched control BLT mice naïve by infusing them with the same preparation of donor-matched DC but without HIV-Env loading. This protocol elicits strong immunological

memory responses in mice (18). We chose HIV-Env, as both murine and NHP-derived NK cells mediate adaptive immune responses to this clinically important antigen (18, 23), and used a mix of monomeric gp120 and trimeric gp140 for these studies, as it is currently unclear which antigen confirmation triggers superior immunological memory in human NK cells. Fourteen days post vaccination, we examined the phenotypes of splenic and hepatic NK cells isolated from either control or vaccinated human donor-matched BLT mice using CyTOF (Supplemental Figure 3). HIV-Env-primed splenic and hepatic NK cells had decreased expression of select activating receptors and maturation markers (CD16, CD57) and inhibitory receptors (KIR2DL1, KIR2DL5), and increased expression of the adhesion molecules CD2 and CD62L. Interestingly, both splenic and hepatic NK cells further upregulated the inhibitory receptor NKG2A, but only splenic NK cells upregulated perforin. Splenic NK cells, unlike hepatic NK cells, also decreased their expression of the ribosyl ADP-cyclase CD38 (39), the adhesion molecule CD96, and the activating KIRs KIR2DS4 and KIR2DS5 (Supplemental Figure 3). We conclude that splenic and hepatic NK cells in BLT mice respond to DC-based HIV-Env vaccination by global and tissue specific changes in their expression levels of activating and inhibitory receptors, adhesion molecules, and effector function molecules.

Human NK cells mediate antigen-specific and vaccination-dependent recall responses

To test whether splenic or hepatic NK cells mediate vaccination-dependent and antigen-specific recall responses upon vaccination of the host, we performed *in vitro* killing assays with splenic or hepatic NK cells from human-donor matched naïve or HIV-Env-vaccinated BLT mice. All groups of BLT mice were treated weekly with trans-presented human IL-15 prior to immunization to significantly expand the numbers of NK cells in both the spleen and liver of BLT mice, thereby enabling replicate-well experiments (Supplemental Figure 4). BLT mice were either left naïve, immunized via intraperitoneal injection with antigen-free syngeneic DC as a control, or immunized with recombinant HIV-Q23–17 Env (gp140/gp120)-loaded syngeneic DC. Fourteen days after immunization, human NK cells were isolated from the spleens and livers of naïve and HIV-Env vaccinated human-donor-matched BLT mice by flow cytometry-based cell sorting, and the amount of antigen-specific NK-mediated killing determined by comparing killing of syngeneic target cells (DC) by naïve vs. HIV-Env-primed NK cells. Target cells were either loaded with the same HIV-Env preparation used for vaccination (experimental “recall” group), or used as control target cells that were (i) left antigen-free (vaccination requirement control), (ii) loaded with an irrelevant protein antigen, Ovalbumin (Ova; antigen-specificity control #1), or (iii) loaded with an irrelevant pathogen, ultraviolet-inactivated influenza A virus H1N1 PR8 (UV-inactivated H1N1 PR8; antigen-specificity control #2). Human BLT-liver or spleen-derived NK cells were cocultured with CFSE-labeled syngeneic target cells at a 1:1 ratio, for six hours at 37C 5% CO_2, before target cell killing was determined using flow cytometry. We found that HIV-Env-primed hepatic NK cells vigorously killed HIV-Env-loaded syngeneic target cells (DC) *in vitro* (Figure 3A). The killing of HIV-Env-loaded syngeneic target cells by hepatic NK cells was antigen specific, as hepatic NK cells from HIV-Env-vaccinated animals did not kill syngeneic target cells loaded with either UV-inactivated H1N1 RP8 or Ova, nor did they kill antigen-free targets. Killing assays were free of T cells as demonstrated by post-sort analysis (Supplemental Figure 5). Interestingly, splenic NK cells, isolated from HIV-Env-vaccinated

donors, did not kill HIV-Env-loaded syngeneic targets, despite of their similar expression of perforin and granzyme B, when compared to hepatic HIV-ENV specific memory NK cells (Figure 1B), and, as expected, neither did naïve hepatic or naïve splenic NK cells (Figure 3). It is important to note that we did not observe killing of antigen-free ("none") target cells by splenic or hepatic NK cells, as their death rate was similar to that of target cells incubated without NK cells for the duration of the assay, and as a such we are not subtracting significant background killing from our antigen-loaded experimental killing assay group's results. Our findings are similar to previously published results in mice, in which NK memory is also restricted to hepatic NK cells (18). We conclude that in BLT-mice, hepatic human NK cells mediate vaccination-dependent, antigen-specific recall responses, both hallmarks of adaptive immunity.

We next analyzed the expression of CXCR6 on splenic and hepatic human NK cells of BLT mice, as the survival and memory functions of murine hepatic NK cells are dependent on NK cell-expressed CXCR6 (18). We found that the majority of hepatic, but not splenic NK cells express CXCR6 (Figures 1 and 2). Further, BLT-derived hepatic NK cells had similar CXCR6 expression levels and frequencies to human NK cells isolated from healthy human liver (15). Thus, in mice and humanized mice, a large percentage of hepatic NK cells express CXCR6, which localizes these cells to the liver, and hepatic NK cells from mice and humanized mice mediate vaccination-dependent and antigen-specific immunological memory to HIV-Env.

Human NK memory is very long-lived

To test whether human NK cells are capable of the third hallmark of adaptive immunity, longevity, we elicited a DTH response, a hallmark of adaptive immunity, in the skin of VZV-experienced human volunteers via intradermal injection of VZV-STA. To maximize time elapsed between NK priming by VZV infection and VZV-STA challenge, we excluded volunteers who had received Zostavax vaccinations or had a history of shingles. This DTH skin test allows us to examine whether human NK cells are recalled to sites of VZV-STA challenge, decades after the presumed time of NK cell priming to VZV antigen, and to determine memory NK cell phenotypes and effector functions. We chose VZV for our study, as childhood infection with VZV generally confers lifelong immunity in healthy persons, NK cells respond to VZV infection (40, 41), and have been implicated in host protection from VZV-disease (1, 41, 42).

Healthy human volunteers, who were between 22 and 65 years of age and had previously suffered from VZV infection in their youth, were injected intra-dermally with VZV-STA. As a control, sterile saline solution was injected in the opposite arm, and VZV-STA and control blister-fluids analyzed at the same time. We scored the magnitude of the DTH response at all injection sites three days after VZV-STA or saline challenge by measuring the erythema, induration and diameter at the site of injection to produce a clinical score as previously described (33, 43–45). All VZV-STA-challenged donors scored between 3–7 on a scale of 1–10 (10 = maximum score), with higher responses noted in younger individuals as previously reported (44). To avoid contamination of our experimental samples with blood-derived NK cells, we did not perform a punch biopsy on the DTH site, but instead generated

a skin blister above the site of VZV antigen challenge, as blister-fluid is mostly devoid of PBMC (33, 44, 45). Sixteen hours later, we compared NK cell frequencies and phenotypes in the blister-fluids from naïve control and VZV-STA-injected skin to each other and to donor-matched PBMC-derived NK. While we did not detect NK cells in blister-fluids from the sites of intradermal saline control injections, large numbers of NK cells infiltrated blister-fluids above the sites of VZV-STA in all donors, including donors that in had experienced VZV infection many decades earlier (Figure 4A, B).

We next analyzed the cytotoxic functions of VZV blister-fluid-derived human NK cells, by assessing their CD107a expression levels (46–48) *ex vivo*, as CD107a is a functional marker for NK cytotoxicity (23). Our approach is supported by multiple reports demonstrating that cytokine release does not upregulate CD107a expression on the surface of NK cells, but that upregulation correlates significantly with NK-mediated cytotoxicity (47, 48). Further, a statistically significant correlation between NK killing of HIV Gag- or Env-primed target cells and CD107a upregulation has been reported for memory NHP-derived NK cells, supporting our conclusion that CD107a upregulation on NK cells is indicative of antigen-specific cytotoxicity (23). To take our “degranulation snapshot”, we compared the frequency of CD107a-expressing NK cells in freshly harvested VZV-STA challenge blister-fluids to that of donor-matched PBMC-derived NK cells, as naïve skin-associated blister-fluid did not contain sufficient numbers of NK cells for proper statistical analysis (Figure 4a). We discovered that NK cells isolated from VZV-STA challenge blister-fluid expressed significant levels of cell surface CD107a directly *ex vivo*, without NK restimulation or blockade of CD107a internalization, when compared to human donor matched PBMC NK cells (Figure 4C). This data is important, as it demonstrates that NK cells isolated from the site of an antigen recall response are actively killing target cells in blister-fluid of VZV-STA challenge. As NK cells at sites of VZV-STA challenge are cytotoxic, they are unlikely to be bystander immune cells recruited by elevated cytokines or chemokines at the challenge site in skin, as cytokine and chemokine signaling alone does not trigger NK-mediated killing (49). We conclude that NK cells recruited to the site of VZV-STA challenge in VZV immune donors are cytotoxic and distinct both in phenotype and function from donor-matched PBMC-derived NK cells. Their rapid recruitment to sites of VZV-STA challenge decades after initial infection of the host suggests that NK cells play a role in long-term antigen-specific recall responses in humans.

Human memory NK cells express tissue residency markers

We next compared the expression of tissue-resident NK markers, and the expression of the transcription factors T-bet and Eomes on cytotoxic ($CD107a^+$) vs. non-cytotoxic ($CD107a^-$) NK cells in VZV-STA-challenge blister-fluids (Figure 5A,B). We found that $CD107a^+$ VZV-STA blister-fluid NK cells were $CD56^{hi}$, and expressed more CXCR6, NKG2D, and CD69 than $CD107a^-$ NK cells, while CD62L-expresssion was reduced on $CD107a^+$ VZV-STA blister-fluid NK cells (Figure 5A,B), and CD16 expression was similar between both types of blister-fluid NK cells. This may not be surprising, as CD16 (FcγRIII) mediates cytotoxicity in an antibody-dependent way, and NK memory is independent of B cells (17–19, 24).

VZV-STA blister-fluid NK cells, when analyzed in bulk and compared to donor-matched PBMC-derived NK cells, were similarly distinct in their tissue resident phenotype. NK cells at sites of VZV-STA challenge were predominantly $CD56^{hi}$ and more frequently expressed CXCR6, NKG2D, CD69, and CD62L, while the expression of the cytotoxicity receptor CD16 was significantly reduced on VZV-STA challenge blister-fluid NK cells compared to donor-matched PBMC NK cells (Figure 5C). Interestingly, VZV skin antigen challenge blister-fluid NK cells expressed the transcription factor Eomes, with or without co-expression of T-bet, while donor-matched PBMC derived NK cells were $Eomes^{lo}$ and generally expressed T-bet either with or without Eomes co-expression (Figure 5D). While numerous publications have investigated the phenotypes and effector functions of human NK cells in inflamed skin, the phenotype of healthy skin-resident NK cells remains elusive, in large part due to a natural lack of NK cells in uninflamed skin. A single publication describes skin-resident NK cells as NKG2D- and perforin-negative, and unable to lyse target cells unless pre-activated with IL-2 (50). In contrast, VZV-STA blister-fluid NK cells express significant amounts of NKG2D and degranulate *ex vivo* (Figure 5 A-C). We conclude from our data that human NK cells recruited to the site of VZV-STA challenge phenotypically resemble activated human tissue-resident NK cells and participate in lifelong immune memory responses.

Discussion

Currently, vaccinations are the best way to protect public health, having saved over 10 million lives globally in the past 55 years (51). While there is still no preventative HIV vaccine, mounting epidemiological and experimental evidence points to an important role for NK cell-mediated effector functions in host protection from HIV infection (52, 53). Should human NK cells mediate adaptive immune responses more broadly to pathogen-encoded or tumor antigens, then the discovery of NK memory opens the door for vaccination to direct potent memory NK cell-mediated effector functions towards host protection, including protection from HIV, which will require a rapid host protective immune response. We therefore evaluated whether human NK cells mediate all three hallmarks of adaptive immunity to HIV-Env: antigen sensitization-dependence, antigen specificity, and longevity. Using BLT mice and human volunteers, we discovered that human NK cells mediate robust vaccination-dependent recall responses featuring antigen-specificity and longevity.

We performed deep phenotyping of splenic and hepatic BLT-derived NK cells, using CyTOF and multiparameter flow cytometry. We discovered that the expression of maturation markers, activating receptors and inhibitory receptors was mostly consistent between NK cells from spleens and livers of BLT mice and adult human PBMC-derived NK cells. Similarly, the expression of NK cell-associated transcription factors and tissue-specific markers for splenic and hepatic NK cells was indistinguishable between human and BLT-derived NK cells, when compared to adult human spleen and liver derived NK cells (Figure 2). Our data is important in the context of a prior report that describes BLT mouse BM-derived human NK cells as phenotypically immature, eight weeks after reconstitution of NSG mice (34). In contrast, we phenotyped splenic and hepatic NK cells, rather than BM-derived human NK cells, and did so four to six months after transplantation. Thus, human NK cells are either more mature in spleens and livers of BLT mice than in the BM, or they

simply mature over time. In the previously mentioned report, the maturation of human NK cells was only achieved upon infusions of BLT mice with trans-presented human IL-15 (34), while in our study, infusions with trans-presented IL-15 were not required to mature NK cells but to increase their numbers (Supplemental Figure 4). Of note, as BLT mice bear xenografts of human lymphoid tissue and have limited blood volume, we did not perform skin-based DTH experiments, nor did we extensively phenotype BLT-derived PBMC. Despite these caveats, our data supports the conclusion that BLT mice harbor mature, functional human NK cells that not only resemble human NK cells in phenotype but can be primed to become HIV-Env-specific memory NK cells that mediate vaccination-dependent antigen-specific recall responses. When we challenged the skin of VZV-immune volunteers with VZV-STA and examined NK cells recruited to sites of VZV-DTH, we found large numbers of NK cells at sites of VZV-DTH, but minimal NK cell recruitment to saline control challenge (Figure 4A,B). Importantly, NK cells recruited to sites of VZV-DTH were cytotoxic (Figure 4C). We hypothesize that cytotoxic NK cells at sites of VZV skin antigen challenge are killing VZV-loaded target cells of unknown identity, as cytokine release does not upregulate CD107a on NK cells and elevated cytokine or chemokine levels that likely exist at sites of VZV-DTH cannot by themselves trigger NK-mediated cytotoxicity, which requires NK cell activation via the ligation of NK activating receptors (49). The precise nature of these target cells is currently unknown; however, it is tempting to speculate that they may be antigen-presenting cells loaded with injected VZV glycoproteins. Unfortunately, as our study protocol did not include approval to inject volunteers with irrelevant control antigens or to perform VZV-STA challenge in VZV-naïve persons, who are quite rare, our hypothesis cannot be experimentally addressed further at this time. Similar to our previous reports for murine memory NK cells, human memory NK cells resembled tissue-resident NK cells based on their phenotypes and expressed transcription factors (13, 15), and were distinct in phenotype when compared to human donor-matched PBMC-derived NK cells (Figure 5). However, as both human spleen- and liver-derived NK cells include $CXCR6^{+}$ $CD16^{lo}$ $CD69^{+}$ $NKG2D^{+}$ $CD62L^{+}$ T-bet^{lo} and $Eomes^{hi}$ NK cells (13, 15), and CXCR6 expression is not restricted to these tissues in humans (20) it is currently impossible to distinguish whether human memory NK cells originate from the liver or another organ. We were eager to determine whether CXCR6 expression is required for the function and survival of human memory NK. Unfortunately, we found that modulation of the CXCR6-CXCL16 pathway via antibody binding compromised NK viability thereby preventing us from performing this experiment.

Our findings obtained in BLT mice are only partially in agreement to those reported for NHP, where both hepatic and splenic NK cells mediated antigen-specific recall responses (23). However, a direct phenotypic comparison of macaque, human, BLT mouse and murine NK cells remains elusive due to significant species-specific differences in the expression of NK-specific cell surface markers, differences in the availability of antibodies specific to these markers for each species, and as such differences in the way NK cells are identified and isolated for experimental analysis. Reports that analyze the phenotypes of human splenic or hepatic NK cells are rare (13, 15), thus it is currently unclear how related human splenic and hepatic NK cells may be in humans vs. BLT mice, where the frequency of NKG2D- and CD16-expressing NK cells was reduced, while the frequency of perforin and

granzyme B-expressing NK cells was similar (Figure 2B). This leaves the possibilities that memory NK cells in mice and BLT mice are more restricted in their tissue residency, perhaps through a species-specific difference in the expression of ligands important for memory NK cell survival or function, such as CXCL16, while human splenic and hepatic NK cells are similar, thus indistinguishable from each other in VSV-STA blisters. Alternatively, NK cells in humans and NHP could be distinct, with human NK memory restricted to hepatic NK cells only in humans, BLT mice, and mice. The answer to these important questions will require further studies.

Whether NK memory responses in primates are modulated by other immune cell types is currently unknown, and this important question cannot be easily experimentally assessed in humans. In mice, neither antigen-sensitization nor NK cell-mediated recall responses requires the presence of T or B cells, nor are they inhibited by the presence of naïve T or B cells (17–19, 26). However, we cannot formally exclude the possibility that in humans, other immune cells may modulate NK cells and their memory responses through unknown mechanisms, as this is not experimentally testable *in vivo*. It is however important to note that IFN-γ-release by NK cells is not a measure of NK memory, as naïve NK cells, and even umbilical cord blood-derived NK cells are robust IFN-γ producers (54). The mechanisms by which NK cells recognize and distinguish different antigens remains unknown, was not the focus of this study, and is likely distinct from that of NKG2C-expressing NK cells that expand upon CMV infection of humans (36, 55). However, this germline encoded one gene – one agonist activation and expansion may be distinct in its mechanism from that of the human memory NK cells described here, which we hypothesize is the result of fixed genomic rearrangements, rather than epigenetic modifications (17). Future work will need to be directed towards understanding how long-lived, antigen-specific NK memory responses can be targeted towards improved human health via the development of novel clinical diagnostic approaches, vaccines, therapeutic agents, or immunotherapies.

Materials and Methods

Study design

The goals of this study were (i) to investigate the maturation and functional status of human NK cells in the spleens and livers of naïve BLT mice, via direct comparisons of NK cell-expressed cell surface markers and transcription factors between BLT-derived NK cells of the spleen and liver to that of adult human spleen- and liver-derived NK cells, as well as human adult derived PBMC NK cells; (ii) to investigate whether human splenic or hepatic NK cells mediate vaccination-dependent and antigen-specific recall responses to experimental anti-viral vaccines using BLT mice; and (iii) to investigate the longevity of the adaptive NK cell-mediated immune response in human volunteers via delayed type hypersensitivity and examination of responder NK cells at sites of challenge. A minimum of three genetically unrelated human donor-derived BLT cohorts with a minimum of five mice per group were analyzed for each data point, with the exception of Supplemental Figures 2 and 3, for which 5 mice were pooled to obtain sufficient numbers of NK for CyTOF analysis. We used three to seven genetically unrelated human donors with four to nine mice per group to analyze NK cell phenotypes and NK cell-mediated effector functions by flow

cytometry and functional assays. We further used eleven genetically unrelated human volunteers to analyze the longevity of the human NK cell-mediated immunological recall response to VZV skin antigen challenge, using skin-blister-fluid-derived NK cells for our experimental evaluations, as well as human donor-matched PBMC-derived NK cells as a control.

Animals

BLT mice were generated as previously described (29). Briefly, six to twelve-week-old female lymphocyte-deficient NSG mice were transplanted underneath the capsule of the left kidney with small pieces of human fetal liver and thymus (Advanced Bioscience Resources). Immediately after transplantation, NSG recipient mice were infused with $1.5–4\times10^5$ autologous fetal liver-derived $CD34^+$ hematopoietic stem cells (HSC) via the mouse-tail vein. Twelve weeks after transplantation and HSC infusion, the reconstitution efficiency of each animal was determined by flow cytometric analysis of PBMC collected from the sub-mandibular vein. PBMC were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare) as per the manufacturers protocol, washed once in sterile Phosphate Buffered Saline (PBS), supplemented with 2% Fetal Bovine Serum (FBS), resuspended in 0.2 milliliter (ml) PBS 2% FBS, incubated with murine and human Fc-Block (Becton Dickinson, BD) for 10 minutes at 4°C to prevent non-specific antibody staining, stained with antibodies specific to murine CD45 (30-F11; Biolegend, GE Healthcare) and human CD45 (H130; Biolegend), and evaluated for their percentage of human $CD45^+$ by flow cytometry. Mice with ≥ 60% reconstitution with human $CD45^+$ hematopoietic cells were used for experiments. All mice were cared for in the animal facilities of the Center for Comparative Medicine at Baylor College of Medicine (BCM) and Texas Children's Hospital (TCH), and all protocols were approved by the BCM Institutional Animal Care and Use Committee.

Isolation of human $CD34^+$ HSC from fetal liver

A single cell suspension was generated from human fetal liver by mechanical disruption using 40μm nylon mesh. Cells were washed in sterile PBS 2% FBS, and $CD34^+$ cells isolated via a CD34 positive selection kit (Stem Cell Technologies) as per manufacturers protocol.

Generation of HIV envelope glycoproteins

The sequence for the envelope gene (*env*) from HIV-Q23–17, a Clade A Kenyan isolate of HIV-1, was motif-optimized and synthesized as the gene encoding gp140 with the cleavage site intact between gp120 and gp41, truncated just prior to the transmembrane domain of gp41. This *env* gene was cloned into the mammalian cell expression vector pEMC*, modified for enhanced expression under the control of the human cytomegalovirus IE-1 promoter and enhancer (56). Protein expression was performed by transient transfection of human 293 cells, generating a mixture of soluble, secreted Env gp140 and Env gp120 upon partial cleavage of gp140. The protein was purified using lectin affinity chromatography, followed by sizing chromatography to yield protein that was 95% pure (Coomassie gel staining) (57); identity was confirmed by Western blot using HIV Env-specific human mAb b12.

IL-15 treatment of BLT mice used as donors for NK cell killing assays

Before vaccination, BLT mice were treated weekly, for four weeks, with human IL-15Rα.Fc chimera (R&D; 2.5 μg/mouse) and recombinant human IL-15 (Biolegend; 2.5 μg/mouse), to expand their mature NK populations. IL-15 injections were given intraperitoneally using a 30-gauge needle.

Vaccination of BLT mice

Spleens from two to three donor matched naïve BLT mice were processed into single cell suspensions by mechanical disruption. Immune cells were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare) as per the manufacturers protocol (950 *g*, 20 min, no brake) and washed once in sterile PBS supplemented with 2% FBS. Splenocytes ($5–7.5\times10^5$) were plated in 200ul RPMI, supplemented with 10% FBS, and 1% of each of the following: Hepes, non-essential amino acids, Na-pyruvate, L-glutamine, Penicillin / Streptomycin, (R-10 media) in a V-bottom plate. To each well, 20μg of HIV-Env(Q23–17 HIV-1 encoded monomeric gp120 + trimeric gp140), Ova, or UV inactivated influenza A virus PR8 H1N1 was added, and cells were incubated for 2 hours at 37°C 5% CO_2. Cells were then divided into equal amounts and injected intravenously and intraperitoneally into donor matched BLT mice using a 30gauge needle.

Target cell isolation from spleens of naïve BLT mice for *in vitro* killing assays

Spleens were isolated from euthanized naïve BLT donor mice and mechanically disrupted in sterile PBS 2% FBS using frosted glass slides to generate a single cell suspension. Immune cells were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare) as per the manufacturers protocol (950 *g*, 20 min, no brake). Immune cells were washed once in sterile PBS supplemented with 2% FBS, re-suspended in 150 microliters (ul) PBS 2% FBS, incubated with murine and human Fc-Block (BD) for 10 minutes at 4°C, and stained with antibodies specific to murine CD45 (30-F11; Biolegend) and human CD43 (CD43–10G7; Biolegend). $CD43^-$ target cells (B cells and dendritic cells (DC)) were isolated using Fluorescence Activated Cell Sorting (FACS) on a BD ARIA, resulting in ≥ 99.8% purity.

Isolation of human NK cells from BLT mouse livers and spleens

Fourteen days after immunization, single cell suspensions from spleens and livers were generated by mechanical disruption of spleens, using frosted glass slides, and livers, using 40μm nylon mesh. Immune cells where then isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare) as per the manufacturers protocol. Splenic and hepatic immune cells were washed once in sterile PBS 2% FBS, re-suspended in 150ul PBS 2% FBS, incubated with murine and human Fc-Block (BD) for 10 minutes at 4°C, and stained with antibodies specific to mCD45 (30-F11; Biolegend), hCD45 (H130; Biolegend), hCD3 (OKT3; Biolegend), and hCD56 (HCD56; Biolegend) for 30 minutes at 4°C. Human NK cells (≥ 99% pure) were isolated by FACS-based cell sorting of mCD45/$hCD3^-$ hCD45/$hCD56^+$ NK cells, using a BD ARIA (5,000–7,000 events/second).

Research ethics approval for PBMC, adult liver and spleen tissue

All human peripheral blood and adult spleen samples were obtained by written informed consent and the protocol was approved for use by the National Institutes of Health (NIH) and Baylor College of Medicine Institutional Review Boards for the Protection of Human Subjects, and in accordance with the Declaration of Helsinki.

PBMC from healthy donors

All PBMC donors were healthy adult volunteers. To isolate PBMC, peripheral blood was diluted 1:1 with Ca^{++}, Mg^{++} free phosphate buffered saline (PBS) and layered over Ficoll-Pacque (GE Healthcare), centrifuged at 2,000 rpm with no brake for 20 minutes at room temperature (RT) according to the manufacturer's instructions. The lymphocyte layer was removed, washed, counted, and immune cells stained with the indicated antibodies and analyzed using multiparametric flow cytometry. No frozen cells were used for this study.

Adult liver and spleen tissue

Informed patient consent was not required to collect human adult liver perfusates from adult cadaver donors. 50–100 mL of liver perfusate was collected, centrifuged, and the cell pellet was resuspended RPMI-1640 (HyClone) and separated by density centrifugation using Ficoll-Pacque (GE Healthcare) as described above. Cells were washed in PBS, counted, and used immediately for multiparameter flow cytometry. Adult spleen tissue was obtained from patients undergoing pancreatic ductal adenocarcinoma surgery or splenectomy related to chronic pancreatitis. Patients had no known viral infections, were treatment-naïve and did not have involvement of the spleen with cancer. Single cell suspensions were generated from adult spleen tissue by mechanical separation, centrifugation and density centrifugation over Ficoll-Pacque (GE Healthcare). The lymphocyte layer was removed, washed, counted, and immune cells stained with the indicated antibodies and analyzed using multiparametric flow cytometry. No frozen cells were used for this study.

In vitro NK cell killing assay

Target cells were plated in a 96-well V-bottom plates at 5×10^5 cells / well in R-10 media, pulsed with either 5 μg / well Ova, 5 μg / well ultraviolet (UV)-inactivated influenza A H1N1 PR8, or 5 μg / well HIV env, or left antigen-free, and incubated for 24 hours at 37°C 5% CO_2. Cells were then washed twice with sterile PBS and labeled with carboxyfluorescein succinimidyl ester (CFSE; CellTrace, Fisher Scientific) as per the manufacturers protocol for subsequent identification by flow cytometry and washed twice with R-10 media. CFSE-labeled target cells were co-cultured in V-bottom plates with sorted hepatic or splenic syngeneic human NK cells at a 1:1 ratio for six hours at 37°C 5% CO_2. After the six-hour incubation period, plates were immediately placed on ice. NK:Target cell ratios were determined by flow cytometric analyses, using pure populations of CFSE-labeled target cells or CD56-stained NK cells as fluorescence minus one (FMO) controls. NK cell killing was calculated as follows for either naïve or HIV-Env-primed NK cells: Percent Specific Lysis = [1-(Antigen-free target cell ratio / Antigen-loaded target cell ratio)] x 100, whereby antigen-loading was performed with either Ova protein, UV-inactivated influenza A H1N1 PR8, or HIV env.

Staining and Mass Cytometry acquisition

The staining antibody panel is outlined in Supplemental Table 1. All antibodies were conjugated using MaxPar® X8 labeling kits (Fluidigm). Panel was lyophilized in single-use lyospheres (BioLyph, Chaska, MN) to ensure antibody stability. Example of stains and gating strategy for each marker is shown in Supplemental Figure 2. Detailed staining protocols have been previously described (2), with some modifications as the following describes. Briefly, spleen and liver cells were depleted of murine cells using murine CD45 magnetic bead negative selection (Miltenyi). Human NK cells were isolated by magnetic bead negative selection using the NK isolation kit (Miltenyi). Samples were barcoded, using human CD45 live barcoding as described to ensure uniformity of staining (58). Barcoded samples were resuspended in 25 mM cisplatin (Enzo Life Sciences) for 1 minute and then quenched with 100% FBS. Cells were stained for 30 minutes at 4°C , fixed (BD FACS Lyse), permeabilized (BD FACS Perm II), and stained with intracellular antibodies for 45 minutes at 4°C. Cells were resuspended overnight in iridium intercalator (Fluidigm) in 2% paraformaldehyde in PBS and then washed once with PBS and 3 times with Milli-Q water immediately before acquisition on a Helios mass cytometer (Fluidigm).

Mass Cytometry data analysis

Samples were manually debarcoded, using Boolean combination gates in FlowJo v10.1r3. To ensure purity of NK cells, serial negative gating was performed before analysis, as shown in Supplemental Figure 2. Analyses for Figures 1 and 3 were conducted in the open-source statistical package R. viSNE was implemented on gated NK in Cytobank, as described by Amir et al. (59). Mean intensity values were calculated using FlowJo v10.1r3 and heatmaps were created using GraphPad Prism v7.0.

Research ethics approval for VZV glycoprotein skin test challenge

This work was approved by the Ethics Committee of the Royal Free Hospital. Healthy persons who had a history of childhood chickenpox infection (n = 10, median age = 32.9 years, age range = 22–85 years, 6 male, 4 female) were recruited for the study. All volunteers provided written informed consent, and study procedures were performed in accordance with the principles of the Declaration of Helsinki.

Participant exclusion criteria

Individuals with history of neoplasia, immunosuppressive disorders or inflammatory skin disorders were excluded from this investigation. Furthermore, we excluded individuals with co-morbidities that are associated with significant internal organ or immune dysfunction including heart failure, severe chronic obstructive pulmonary disease, diabetes mellitus and rheumatoid arthritis and individuals on immunosuppressive regimes for the treatment of autoimmune or chronic inflammatory diseases. We did not exclude volunteers with a history of uncomplicated hypertension or hypercholesterolemia as this would have prevented the majority of aging volunteers from participating in this study. Individuals who had received Zostavax vaccinations or had a history of shingles were also excluded from this study.

Skin tests

Delayed-type hypersensitivity responses were induced by intradermal injection of 0.5ml VZV antigen. VZV-STA contains viral glycoproteins prepared from the culture fluid of the attenuated VZV Oka parental strain –infected MRC-5 cells (60, 61). VZV antigen (BIKEN, The Research Foundation for Microbial Diseases of Osaka University, Japan) was injected intradermally into sun-unexposed skin of the medial proximal volar forearm as per manufacturer's instructions. Baseline skin erythema was determined as the mean of three measurements using a Derma-Spectrometer (Cortex Technology, Hadsund, Denmark). Skin erythema index (EI), size of induration and palpability were also recorded 3 days after VZV skin challenge, and the change in skin EI calculated by subtracting the baseline from the VZV challenge measurement. The induration size was determined by calculating the mean of two measured perpendicular planes. Induration, palpability, and the change in erythema from baseline were measured on day 3 after VZV skin challenge to generate a clinical score (0–10).

Preparation of suction blister cells and PBMC preparation

To induce skin suction blisters over the site of VZV skin test DTH, the dermis was split from the epidermis by the application of a negative pressure of 25–40 kPa (200–300 mmHg) below atmospheric pressure, via a suction chamber for 2–4 h using a clinical suction pump (VP25; Eschmann), until a unilocular blister measuring 10–15 mm in diameter was formed. Blister fluid was aspirated 16–20 hours after blisters were formed using a sterile 23-G needle and 2-ml syringe (Tyco Healthcare UK Ltd, Gosport, UK; Fig. 1i,j), and microcentrifuged at 650 × *g* for 4 min to pellet the blister-fluid-resident cells. The pellet was resuspended in complete RPMI 1640 medium (Life Technologies) containing 10% human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all obtained from Sigma-Aldrich). Heparinized blood was collected at the time of blister aspiration for PBMC isolation. PBMCs were prepared by density centrifugation on Ficoll-Paque (Amersham Biosciences) and resuspended in complete medium. Erythrocyte and leukocyte numbers were quantified using a hemocytometer, and viability was assessed by trypan blue exclusion.

Flow cytometric analysis

Multiparameter analysis of PBMC and blister NK cell phenotype was performed on an ARIA II (BD Biosciences). PBMCs and blister cells were stained with different combinations of antibodies specific to human CD3(UCHT1;Biolegend), CD8(HIT8a;Biolegend), CD16(3G8;Biolegend), CD45(HI30;Biolegend), CD56(MEM-188;Biolegend), CD62L(DREG-56;Biolegend), CD69(FN50;Biolegend), CD107a(H4A3;Biolegend), NKG2D(1D11;Biolegend), CXCR6(K041E5;Biolegend), T-bet(04–46; BD), and EOMES(WD1928;ThermoFisher). All surface staining was performed for 30 min on ice after prior incubation of cells with Human Fc block and Murine Fc block (BD) for 10 minutes on ice. Following surface staining, cells were fixed and permeabilized using Foxp3 Transcription Factor Fixation/Permeabilization Concentrate and Diluent Kit (eBiosciences, Thermo Fisher), as per the manufacturers protocol, and stained for the expression of the transcription factors T-bet and Eomes. Fluorescence minus one control

stains were performed using PBMC to verify the staining specificity and as a guide for setting markers to delineate positive and negative populations. Gating was set on the live lymphocyte population using forward- and side-scatter profiles to include lymphocytic blasts, followed by single cell gating using forward- and side-scatter heights and widths, before identification of hematopoietic cells using human CD45 staining.

Statistics

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). The Two-way ANOVA with Tukey's multiple comparison test was used when comparing three or more groups. The unpaired t-test with Welch's correction was used when comparing two groups of unpaired data. A paired t-test was used when comparing two groups of paired data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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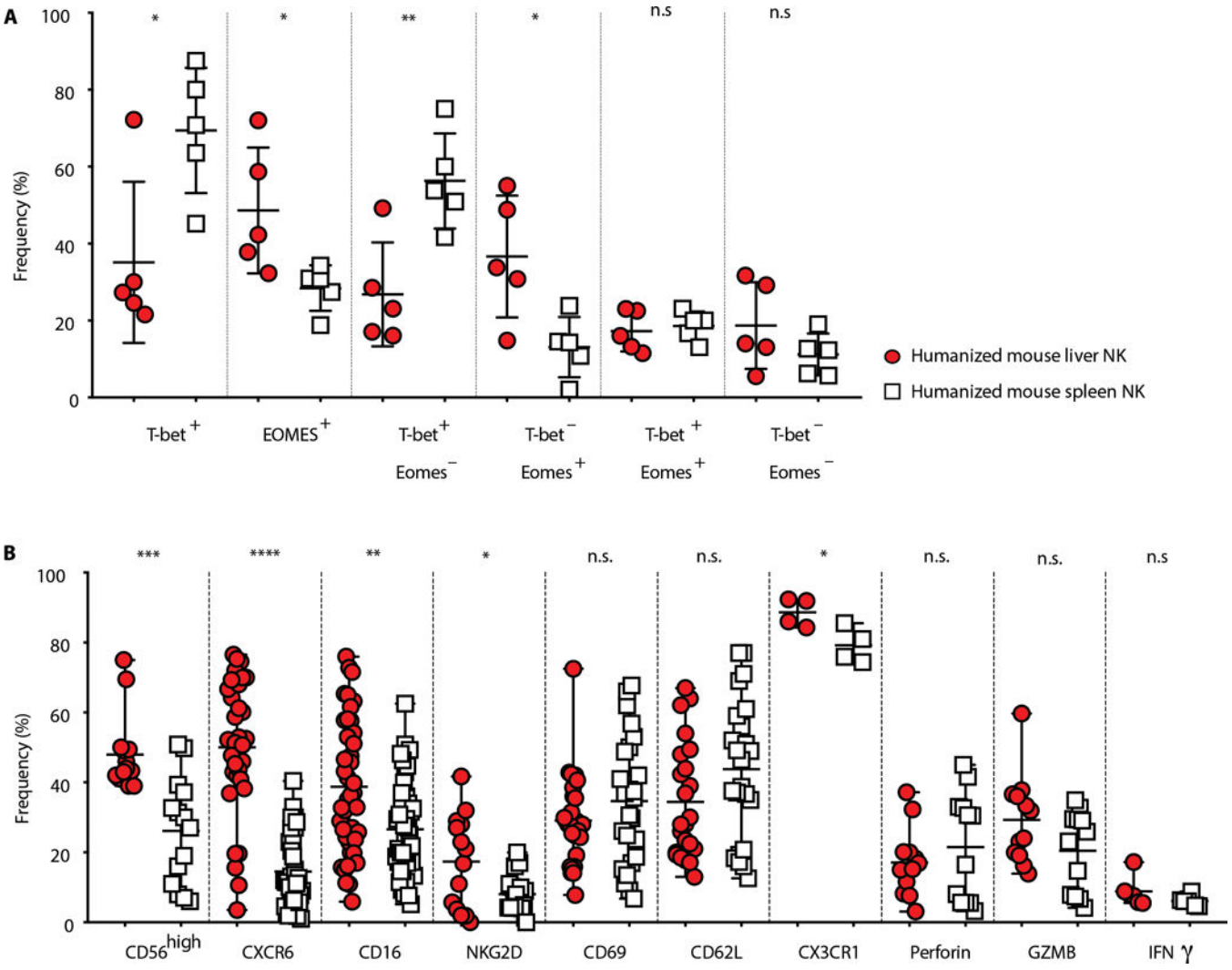

Figure 1. Spleens and livers of BLT mice harbor tissue-specific human NK cells.

Human NK cells were isolated from BLT mice and evaluated by flow cytometry in single cell suspensions. Murine cells were excluded using antibody to mouse CD45. (**A, B**) The frequency of human NK cells expressing the indicated transcription factor (A) or cell surface marker (B) is shown for spleens and livers of naïve BLT mice. Gating was performed on live cells by forward and side scatter areas, single cells, human $CD45^+$/murine $CD45^-$ cells, then $CD56^+/CD3^-$ cells. Five genetically unrelated human donor cohorts (**A**), and six to seven genetically unrelated human donor cohorts of four to nine BLT mice (**B**) were analyzed four to eleven months post transplantation, with the exception of CX3CR1, for which four genetically unrelated human donor cohorts were analyzed. Unpaired t-test with Welch's correction; (**A**) * p = 0.05, ** p = 0.01, *** p< 0.0005; **** p< 0.0001. (**B**) * p< 0.03, ** p = 0.015, *** p< 0.0002; **** p< 0.0001.

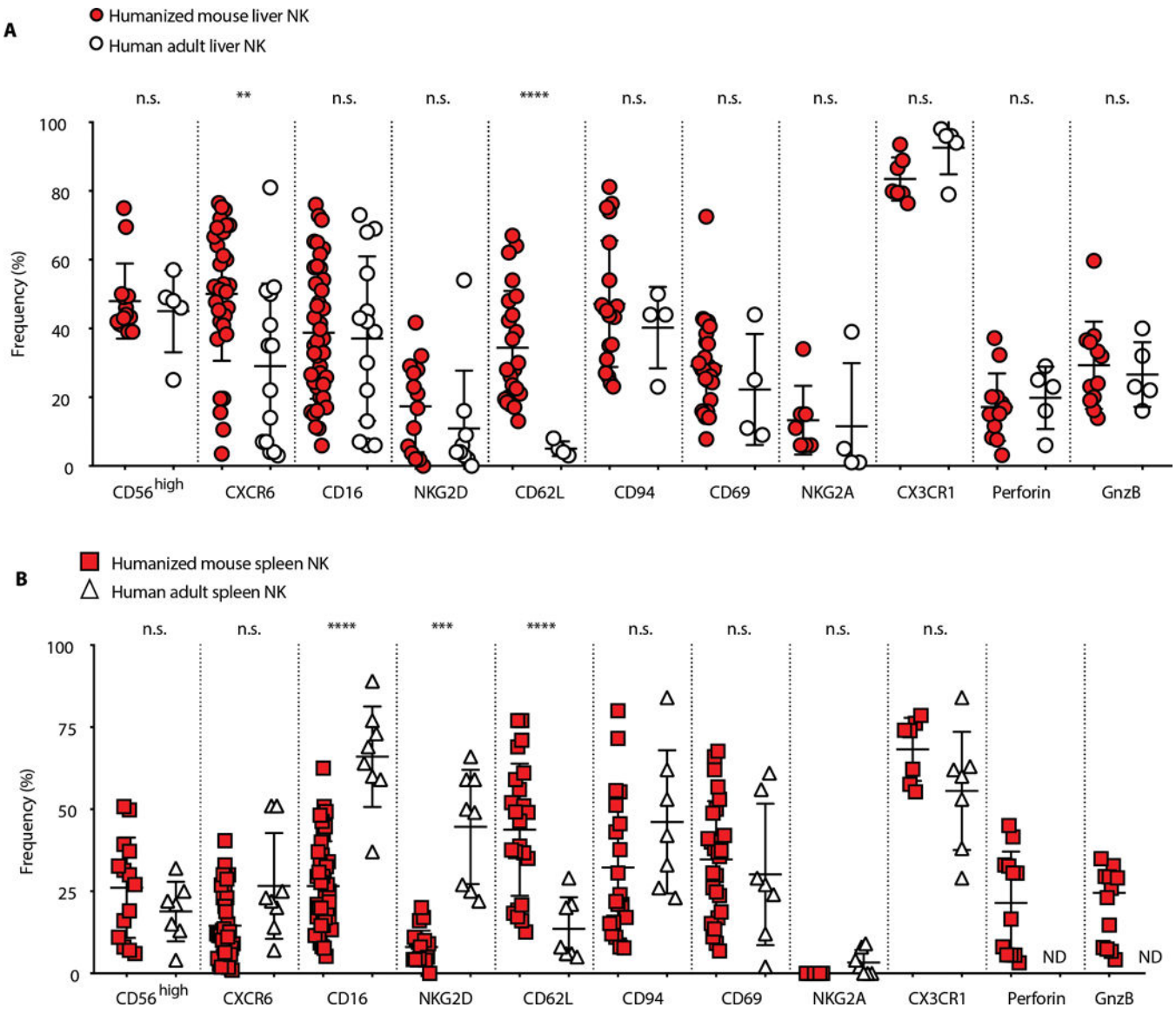

Figure 2. Spleens and livers of BLT mice harbor human NK cells similar in phenotype to human adult tissue-matched NK cells.

Human NK cells were isolated from BLT mice and evaluated by flow cytometry in single cell suspensions. Murine cells were excluded using antibody to mouse CD45. The frequency of human NK cells expressing the indicated cell surface marker is shown for livers (**A**) and spleens (**B**) of naïve BLT mice and is compared to liver and spleen-derived NK cells from adult human donors. Gating was performed on live cells by forward and side scatter areas, single cells, human $CD45^+$/murine $CD45^-$ cells, then $CD56^+/CD3^-$ cells. Seven to ten genetically unrelated human donor cohorts of four to nine BLT mice were analyzed; four to eleven months post transplantation and compared to 4–14 genetically unrelated human donors. Adult human donor-liver NK cells were obtained from transplant-quality livers, while adult human donor-spleen NK cells were obtained from uninvolved spleens from freshly-resected treatment-naïve, HIV- and hepatitis-negative newly diagnosed pancreatic

cancer patients. Each symbol represents one mouse or human donor. Unpaired t-test with Welch's correction; ** p = 0.009; *** p< 0.0005; **** p<0.0001.

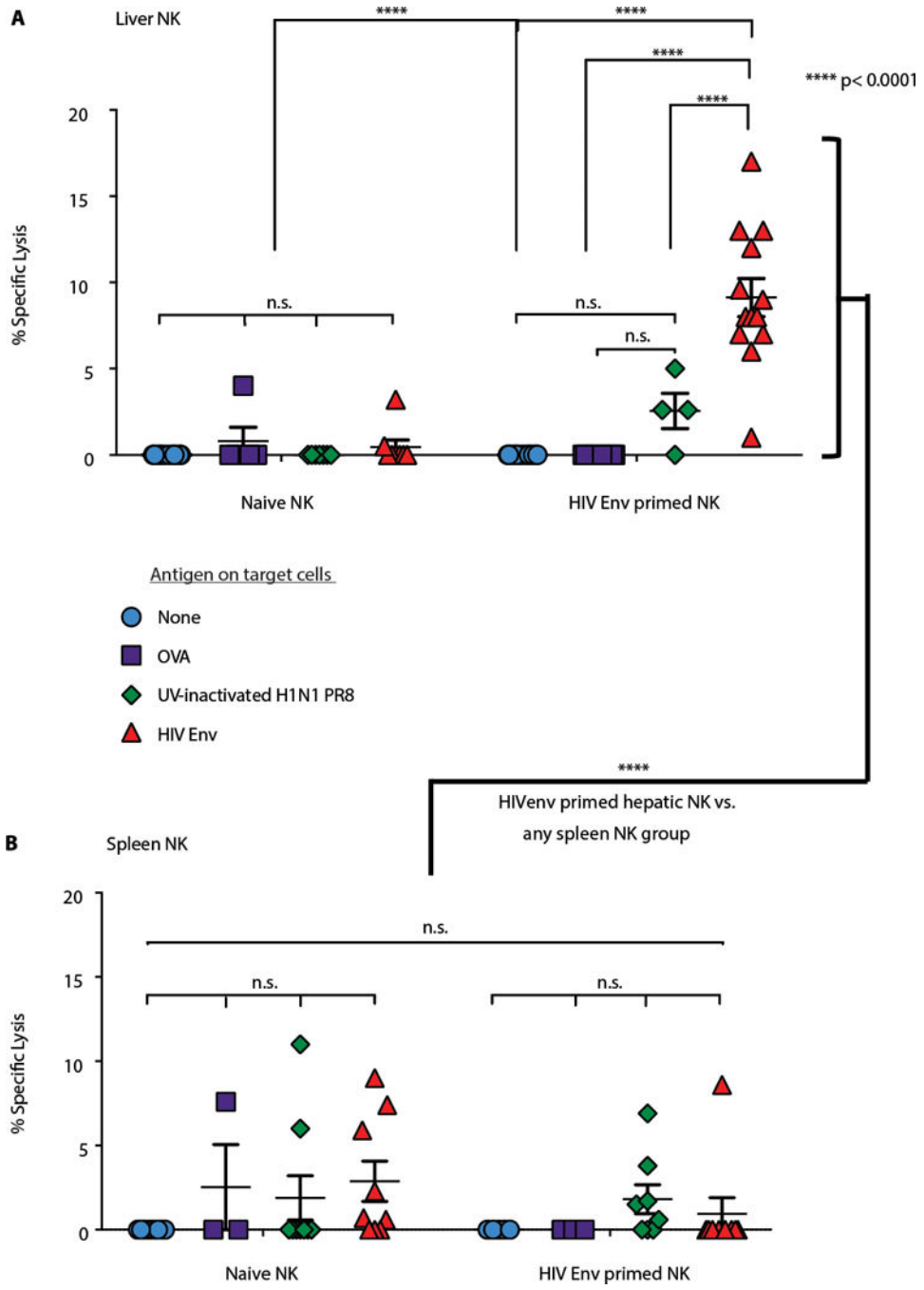

Figure 3. Human hepatic NK cells mediate antigen-specific and vaccination-dependent killing.

Human donor matched BLT mice were left naïve or were immunized by intraperitoneal and intravenous injections with recombinant HIV-Q23–17 Env (gp140/gp120)-loaded syngeneic dendritic cells (HIV-Env). Fourteen days after the immunization, human NK cells were isolated from either naïve or HIV-Env-vaccinated human donor-matched BLT mice by flow cytometry-based cell sorting. The NK cells were cocultured with CFSE-labeled antigen-free, Ova-loaded, UV-inactivated H1N1 PR8 influenza A-loaded, or HIV-Env-loaded syngeneic target cells at a 1:1 ratio, for six hours at 37C 5% CO_2, before target cell killing was

determined using flow cytometry. A total of three (spleen) to four (liver) genetically-unrelated human donor cohorts of five to eight BLT mice were analyzed five months after transplantation, and the data pooled for panels A and B. Two-way ANOVA with Tukey's multiple comparison test. **** $p<0.0001$.

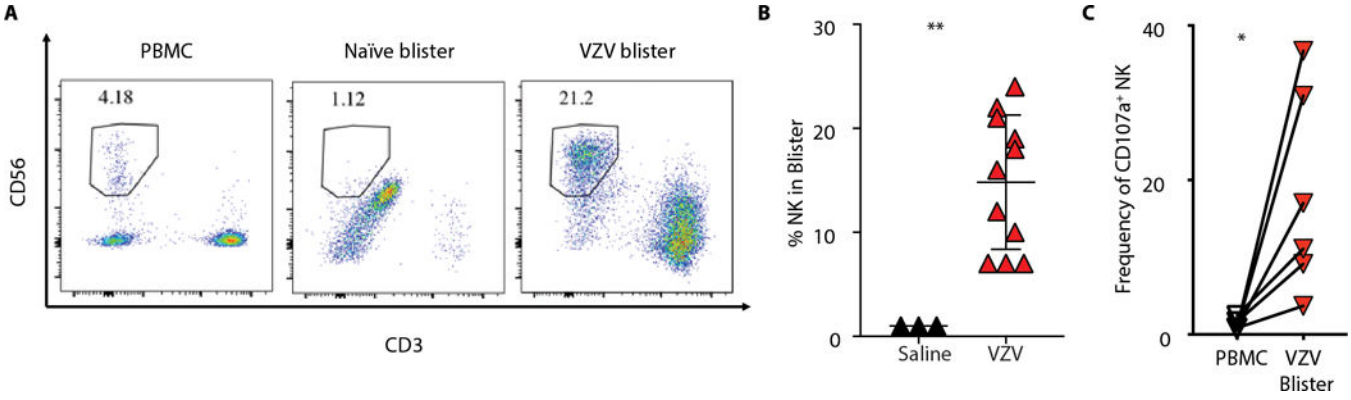

Figure 4. Human NK cell-mediated memory is long-lived.

Human donor matched PBMC and blisters-fluids were isolated on day 3 after VZV-DTH induction and stained for indicated markers. (**A, B**) Frequency of human NK cells (huCD45^{+} CD56^{+}, CD3^{-}) in blister-fluid of naïve (saline-injected; n=3) skin compared to VZV-STA-injected skin (n=11). (**C**) Frequency of actively degranulating, cytotoxic human NK, as judged by cell-surface expression of CD107a (n=6). (**B, C**) Each symbol represents one human donor. Paired t-test * p = 0.027, ** p = 0.0037.

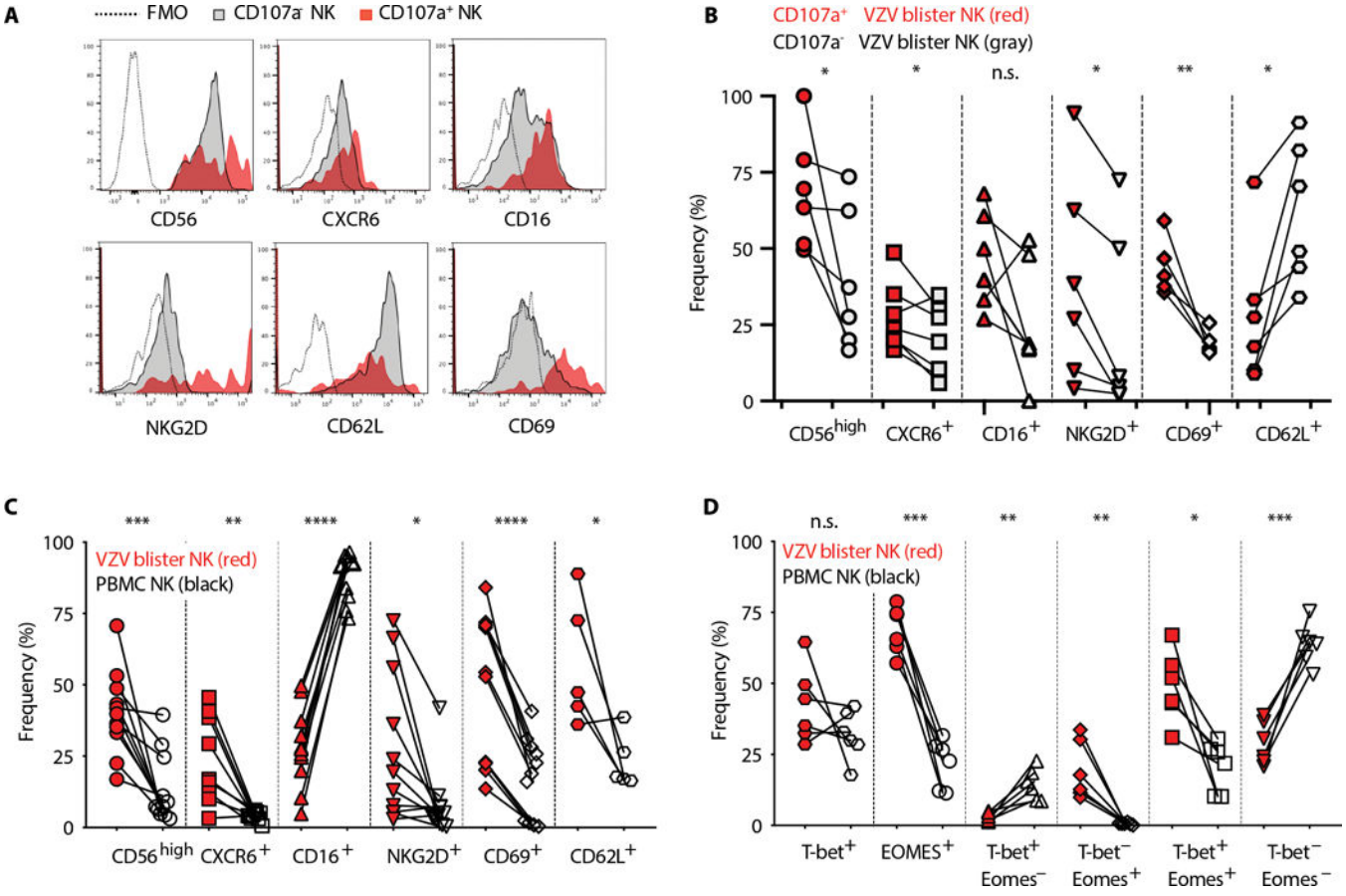

Figure 5. Human NK memory is mediated by NK cells with a hepatic phenotype.

Human donor-matched PBMC and blisters-fluids were isolated on day 3 after VZV glycoprotein challenge and stained for indicated markers. (**A, B**) Analysis of NK-expressed markers on degranulating ($CD107a^+$) vs. non-degranulating ($CD107a^-$) NK cells in blister-fluid of VZV-STA-injected skin. An example of a histogram overlay for each marker is shown in A for a single donor, while 5–7 genetically unrelated human donors were individually analyzed for each marker in B. (**C**) Frequency of human NK cells expressing the indicated markers indicative of a human hepatic NK cell phenotype. 6–11 genetically unrelated human donors were individually analyzed for each marker. (**D**) Frequency of human NK cells expressing the indicated transcription factor master regulators indicative of a human hepatic NK cell phenotype. 6 genetically unrelated human donors were individually analyzed for each marker. Paired t-test; * p = 0.05, ** p = 0.01, *** p< 0.001; **** p<0.0001.