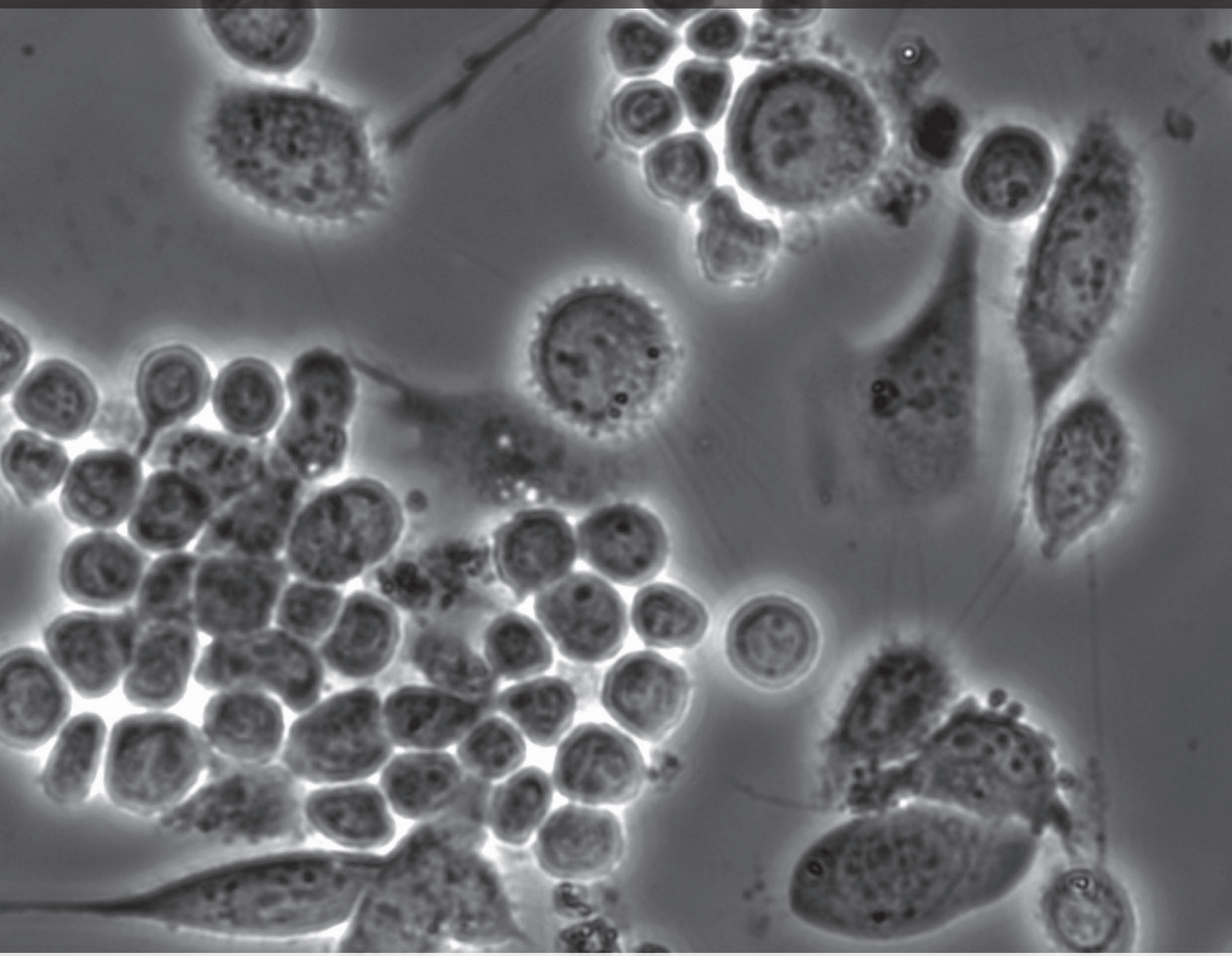
NATURAL KILLER CELLS IN HUMAN DISEASES: FRIENDS OR FOES?

EDITED BY: Vincent Vieillard, Bree Foley and Sandra Lopez-Verges
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-404-4
DOI 10.3389/978-2-88945-404-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

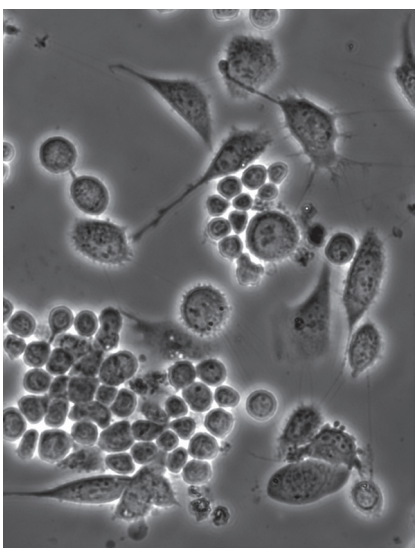
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

NATURAL KILLER CELLS IN HUMAN DISEASES: FRIENDS OR FOES?

Topic Editors:
Vincent Vieillard, Center of Immunology and Infectious Diseases, Pitie-Salpetriere Hospital, France
Bree Foley, Telethon Kids Institution, University of Western Australia, Australia
Sandra Lopez-Verges, Gorgas Memorial Institute of Health Studies and Sistema Nacional de Investigación (SNI), Secretaría Nacional de Ciencia, Tecnología e Innovación (SENACYT), Panama



U2OS human osteosarcoma cells in co-culture with NK92 cells during 24 hours. Image: Gerald Moncayo.

NK cells are lymphocytes of the innate immune system that share some features with adaptive immune cells like T cells. They are well known for their importance to control viral infections and tumor development, but also intracellular bacterial and parasitic infections. A balance between negative and positive signals transmitted via germ line-encoded inhibitory and activating receptors controls the function of NK cells. Activated NK cells respond by killing the infected or tumor cells without prior sensitization, and by producing cytokines and chemokines. It has been shown that NK cells cross-talk with other immune cells, such as dendritic cells and macrophages, can shape T cell and B cell immune responses through direct interactions as well as by virtue of their cytokine/chemokine production. NK cells can also regulate immune responses by killing other immune cells, including activated T cells, or by producing anti-inflammatory cytokines upon excessive inflammation. However, NK cells are not friends in all situations. Indeed, it has been shown in LCMV-infected murine models that, depending on the viral inoculation load, NK cells may either help fight infection or can promote chronic infection. Moreover in cancer models, it has been shown that NK cells can kill anti-tumoral T cells. Recent studies of NK cells in patients with cancer support the notion of detrimental roles of NK cells. Furthermore, studies implicate NK cells in contributing to both graft rejection and tolerance to an allograft. In some autoimmune diseases, like rheumatoid arthritis, NK cells may promote disease pathogenesis.

The scope of this Research Topic is to present and discuss knowledge on the role of NK cells in various diseases settings: viral infections as well as other infections, cancer, transplantation, and autoimmunity. The aim is to discuss how NK cells respond during disease and specifically when, why and how NK cells can be harmful and if they exert different functions (production of specific cytokines, inhibition of other immune cells through other mechanisms beside cytotoxicity) in these situations. Which are the NK cell subsets that play beneficial or deleterious roles in these diseases? Are there different phenotypes associated with protective NK cells (e.g. antiviral, antitumoral) and NK cells involved in disease pathogenesis? How are these diverse NK cells activated and do they function primarily through direct cytotoxicity, ADCC or cytokine and chemokine production? What are the signals or interactions that can change and shape the NK cell response shifting them from protective to harmful?

We thank the authors that submitted reviews and original research manuscripts that help to better understand these questions, with the aim that this will help the scientific community to determine what could be the main future research directions to better understand the role of NK cells in disease protection or development.

Citation: Vieillard, V., Foley, B., Lopez-Verges, S., eds. (2018). Natural Killer Cells in Human Diseases: Friends or Foes? Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-404-4

Table of Contents

frontiers in Immunology

EDITORIAL
published: 07 December 2017
doi: 10.3389/fimmu.2017.01737

Editorial: NK Cells in Human Diseases: Friends or Foes?

Vincent Vieillard[1], Bree Foley[2]* and Sandra L. López-Vergès[3]**

[1] Center of Immunology and Infectious Diseases, Pitié-Salpêtrière Hospital, Paris, France, [2] Telethon Kids Institution, University of Western Australia, Subiaco, WA, Australia, [3] Gorgas Memorial Institute of Health Studies, Panama City, Panama

Keywords: natural killer cells, anti-microbial response, antiviral response, antitumor response, transplantation, uterine natural killer cells

Editorial on the Research Topic

NK Cells in Human Diseases: Friends or Foes?

Natural killer (NK) cells are lymphocytes from the innate immune system that play a protective role against tumors and viral infections. Activated human NK cells respond by killing infected or tumor cells and can shape the adaptive response by producing cytokines and chemokines and by interacting with dendritic cells and other immune cells, and also have memory-like features (1). For these reasons, NK cells are principally considered as protective cells. However, recent discoveries have found that NK cells can have a negative role due to overproduction of pro-inflammatory cytokines, destruction of host cells in autoimmune diseases, in graft-versus-host diseases, or in regulating other immune cells that provide protective immunity, e.g., in some cancers where NK cells counteract tumor-specific infiltrating T cells. The aim of this research topic is to analyze the different situations in which NK cells could have a beneficial or a deleterious role and what are the signals and mechanisms that could explain these differences in behavior. In this era of immunetherapy, understanding of NK-cell biology and its relation to human disease is crucial for rational design of treatments that are efficient but also are safe.

Edited and Reviewed by:
Yenan Bryceson, Karolinska Institute, Sweden

****Correspondence:***
Vincent Vieillard
vincent.vieillard@upmc.fr;
Bree Foley
bree.foley@telethonkids.org.au;
Sandra L. López-Vergès
slopez@gorgas.gob.pa

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *23 October 2017*
Accepted: *23 November 2017*
Published: *07 December 2017*

Citation:
Vieillard V, Foley B and López-Vergès SL (2017) Editorial: NK Cells in Human Diseases: Friends or Foes? Front. Immunol. 8:1737. doi: 10.3389/fimmu.2017.01737

NK CELLS AND ANTI-MICROBIAL RESPONSE

Natural killer-cell responses have been described historically in chronic viral infections, particularly with members of the herpes viral family. Human cytomegalovirus (CMV) infection has been shown to induce the expansion of a specific subset of peripheral blood NK cells that are adaptive-like (2). Other viral infections can also induce these adaptive-like NK cells; however, these cells seem strongly associated with CMV seropositivity (3, 4). Malone et al. investigated if these expansions could be affected by concurrent chronic hepatitis virus infections. More studies are needed to determine if these adaptive-like NK cells in CMV seropositive individuals are more responsive during all infections or only in a subset of them, or during tumor responses, and if this induces a difference in the severity of the disease. Are they as functional in patients with HIV infection? NK-cell function as well as that of other immune cells can be dysregulated during HIV infection and its inflammatory status. As the incidence of some cancers is increased in HIV-infected patients, with NK cells shaping the response to both viral infections and cancer, Leal et al. tried to differentiate anti-HIV and antitumor NK-cell features with the goal to determine if NK cells could concurrently be manipulated in the future to halt HIV disease and HIV malignancies. It is clear that NK cells play a crucial role in controlling viral load and eliminating virus-infected cells; however, they do not always respond with the same intensity to infections. The phenotype of each subset of NK cells, the different alleles of NK-cell receptors, as well as splice variants of these receptors, all play a role in determining NK-cell responses. Shemer-Avni et al. explored the predominance and the difference in activity of splice variants of the NKp46 receptor during respiratory viral

infections, opening the path to their analysis during other infections in which NKp46 has been shown to play a role. Is this mechanism of regulation of receptor activity specific for NKp46 or is it general for all NK receptors?

Natural killer cells are mainly studied for their antiviral activities; however, it has been shown that they could play a role against intracellular bacteria and parasites and even against fungal infections. The role of NK cells in some parasitic infections such as malaria can vary during the course of infection, is heterogeneic, and can also be influenced by host genetics as reviewed by Wolf et al. NK cells kill or inhibit the growth of different fungi. Ogbomo and Mody discussed how NK cells recognize fungi-infected cells and the mechanisms for the induction of granule-dependent cytotoxicity.

NK CELLS AND ANTITUMORAL RESPONSE

Due to the selective pressure of NK cells in tumor development, tumors have developed many escape mechanisms, similarly to viruses, to avoid NK-cell recognition and activation (5). One of these is the regulation of the expression of the ligands of activating receptors. A better understanding of the underlying mechanisms may allow the discovery of a potential therapeutic approach. Moncayo et al. showed that MICA expression seems regulated by cell adhesion and contact and this could play a role in cancer immune evasion. Another escape mechanism could be the alteration of NK-cell behavior by the tumor microenvironment; Stabile et al. reviewed how many components of this microenvironment could influence NK cell developmental programming or the response of distinct NK-cell subsets. Different JAK/STAT pathways could be activated through these components, thus Gotthardt and Sexl discussed how this could shape NK-cell behavior. It is still necessary to determine if and how some STAT family members contribute to the switch from tumor suppression to tumor progression. Also, future studies are needed to demonstrate if by modifying or eliminating some microenvironment components or regulating some JAK/STAT pathways, it is possible to harness NK-cell subsets so they can control once again solid and hematological malignancies.

Through their cytokine and chemokine production as well as their cross talk with other immune cells, NK cells can regulate tissue inflammation and regeneration. Tosello-Trampont et al. showed that it is through this immunoregulatory role that NK cells play an important role in metabolic liver diseases.

NK CELLS AND TRANSPLANTATION

The role of NK cells in graft acceptance or rejection of solid tissues has been largely studied to help in determining the best host–donor match. The treatment of hematological malignancies through allogeneic hematopoietic cell transplantation is still associated with significant morbidity and mortality related to cancer relapse as well as to transplant-related complications including graft-versus-host disease (GvHD). Even if the protective role of NK cells against cancer relapse is well sustained, their role in GvHD is still controversial. Simonetta et al. reviewed and discussed this with the goal of obtaining a model that could show which are the conditions that could induce naturally suppressing NK cells to sustain GvHD. More studies are needed to determine if the adaptive-like NKG2C$^+$ NK cells that play a role in the control of viral infections in HSC and solid transplant recipients contribute to control of GvHD.

UTERINE NK CELLS

Natural killer cells do not only play a crucial role during disease. Uterine NK cells are required to have a healthy pregnancy, as they regulate trophoblast invasion and uterine spiral arterial remodeling in humans. Gaynor and Colucci reviewed the current knowledge on their development and function. The emergence of Zika virus, which can be transmitted from an infected-mother to the fetus resulting in strong neurological consequences for the fetus, shows the importance of studying uterine NK cells as they could play a role in Zika virus control at the level of the placental barrier.

In conclusion, we want to express our gratitude to all the authors who have contributed to this research topic and to the reviewers for their valuable work showing that many components can shape NK cell behavior and thus have consequences on human disease.

AUTHOR CONTRIBUTIONS

SL-V wrote the editorial; VV and BF edited the editorial.

FUNDING

This work was done with no specific founding. SL-V is member of the Sistema Nacional de Investigación (SNI) of SENACYT, Panama.

REFERENCES

1. Cerwenka A, Lanier LL. Natural killer cell memory in infection, inflammation and cancer. *Nat Rev Immunol* (2016) 16:112–23. doi:10.1038/nri.2015.9
2. Lopez-Verges S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57+ NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci U S A* (2011) 108:14725–32. doi:10.1073/pnas.1110900108
3. Björkström NK, Svensson A, Malmberg K-J, Eriksson K, Ljunggren H-G. Characterization of natural killer cell phenotype and function during recurrent human HSV-2 infection. *PLoS One* (2011) 6:e27664. doi:10.1371/journal.pone.0027664
4. Hendricks DW, Balfour HH, Dunmire SK, Schmeling DO, Hogquist KA, Lanier LL. Cutting edge: NKG2C(hi)CD57+ NK cells respond specifically to acute infection with cytomegalovirus and not Epstein-Barr virus. *J Immunol* (2014) 192:4492–6. doi:10.4049/jimmunol.1303211

5. Guillerey C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. *Nat Immunol* (2016) 17:1025–36. doi:10.1038/ni.3518

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 08 May 2017
doi: 10.3389/fimmu.2017.00525

Cytomegalovirus-Driven Adaptive-Like Natural Killer Cell Expansions Are Unaffected by Concurrent Chronic Hepatitis Virus Infections

*David F. G. Malone[1], Sebastian Lunemann[2], Julia Hengst[1,3], Hans-Gustaf Ljunggren[1], Michael P. Manns[3,4], Johan K. Sandberg[1], Markus Cornberg[3,4], Heiner Wedemeyer[3,4] and Niklas K. Björkström[1]**

[1] Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden, [2] Department for Viral Immunology, Heinrich Pette Institute, Hamburg, Germany, [3] Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany, [4] German Center for Infection Research, Partner Site Hannover-Braunschweig, Hannover-Braunschweig, Germany

OPEN ACCESS

Edited by:
Sandra Laurence Lopez-Verges, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama

Reviewed by:
Alessandro Moretta, University of Genoa, Italy
Aimee Beaulieu, Rutgers New Jersey Medical School, USA

***Correspondence:**
Niklas K. Björkström
niklas.bjorkstrom@ki.se

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *05 January 2017*
Accepted: *19 April 2017*
Published: *08 May 2017*

Citation:
Malone DFG, Lunemann S, Hengst J, Ljunggren H-G, Manns MP, Sandberg JK, Cornberg M, Wedemeyer H and Björkström NK (2017) Cytomegalovirus-Driven Adaptive-Like Natural Killer Cell Expansions Are Unaffected by Concurrent Chronic Hepatitis Virus Infections. Front. Immunol. 8:525. doi: 10.3389/fimmu.2017.00525

Adaptive-like expansions of natural killer (NK) cell subsets are known to occur in response to human cytomegalovirus (CMV) infection. These expansions are typically made up of NKG2C$^+$ NK cells with particular killer-cell immunoglobulin-like receptor (KIR) expression patterns. Such NK cell expansion patterns are also seen in patients with viral hepatitis infection. Yet, it is not known if the viral hepatitis infection promotes the appearance of such expansions or if effects are solely attributed to underlying CMV infection. In sizeable cohorts of CMV seropositive hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis delta virus (HDV) infected patients, we analyzed NK cells for expression of NKG2A, NKG2C, CD57, and inhibitory KIRs to assess the appearance of NK cell expansions characteristic of what has been seen in CMV seropositive healthy individuals. Adaptive-like NK cell expansions observed in viral hepatitis patients were strongly associated with CMV seropositivity. The number of subjects with these expansions did not differ between CMV seropositive viral hepatitis patients and corresponding healthy controls. Hence, we conclude that adaptive-like NK cell expansions observed in HBV, HCV, and/or HDV infected individuals are not caused by the chronic hepatitis infections *per se*, but rather are a consequence of underlying CMV infection.

Keywords: natural killer cells, cytomegalovirus, killer-cell immunoglobulin-like receptor, hepatitis B virus, hepatitis C virus, hepatitis delta virus

INTRODUCTION

Infection with cytomegalovirus (CMV) is associated with expansion of a subset of NKG2C-expressing natural killer (NK) cells (1). Such expansions have also been observed in CMV seropositive individuals infected with other infections such as hantavirus (2), HIV-1 (3, 4), and EBV (5). Expanded NK cell populations expressing the activating NKG2C receptor, or CD2 in the case of homozygous

Abbreviations: CMV, cytomegalovirus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; KIR, killer-cell immunoglobulin-like receptor; NK, natural killer; PBMC, peripheral blood mononuclear cell.

deletion of the NKG2C gene (6), are often dominated by the expression of a single inhibitory killer-cell immunoglobulin-like receptor (KIR) receptor (7). NK cell populations with these phenotypic traits have memory-like features including the lack of adaptor proteins EAT-2 (8) and FcεR1γ (9, 10) and have distinct epigenetic characteristics compared to non-expanded NK cells (8, 10, 11).

Cytomegalovirus-driven NK cell-expansions have been reported in viral hepatitis infections (12). Furthermore, increased NKG2C-expressing NK cell populations have been observed in patients with chronic hepatitis B virus (HBV) infection (13) or hepatitis C virus (HCV) infection (14). NK cell expansions in CMV seropositive HCV-infected patients share phenotypic traits with expansions found in healthy controls including loss of the FcεR1γ adaptor (15) and superior capacity to respond to CD16 stimulation (12, 15). However, the extents to which different hepatitis virus infections contribute to or modify these NK cell expansions are unknown.

To study this, we examined NK cell expansions in HBV-, HCV-, and hepatitis delta virus (HDV)-infected patients in the context of underlying CMV infection by studying the expression of differentiation markers and inhibitory KIRs on NK cells. The results suggest that the presence of adaptive-like NK cell expansions seen in HBV, HCV, and HDV infections is primarily caused by the underlying CMV infection, and not significantly influenced by the hepatitis virus infections *per se*.

MATERIALS AND METHODS

Patient Material

Peripheral blood mononuclear cells (PBMCs) from patients used in this study were collected at the outpatient clinic of the Department of Gastroenterology, Hepatology, and Endocrinology at Hannover Medical School in Germany. A total of 24 HBV, 18 HCV, and 28 HDV patients were analyzed, of which 20, 12, and 24 were CMV seropositive, respectively. The detailed clinical characteristics of these patients have been reported previously (16). PBMCs from 29 healthy donors, of whom 23 were CMV seropositive, were collected at the Karolinska University Hospital, Stockholm, Sweden. PBMCs were isolated through standard density-gradient separation and cryopreserved for deferred analysis.

Monoclonal Antibodies and Viability Stains

The following monoclonal antibodies were used in the study: CD14-Horizon-V500, CD19-Horizon-V500 (BD Biosciences), CD3-PE-Cy5, CD56-ECD, CD57-PacificBlue, NKG2A-APC (Beckman Coulter), CD4-PE-Cy5, KIR3DL1-Alexa700 (Biolegend), KIR2DL1-biotin, KIR2DL3-FITC, NKG2C-PE (RnD systems), Aqua Dead Cell Stain Kit 405 nm, Strepavidin-Qdot-605, and Qdot-700 (Thermofisher).

Flow Cytometry

Flow cytometry staining was performed as previously described (17). In brief, cryopreserved PBMCs were thawed and washed twice prior to 30 min incubation in the dark at room temperature with the desired antibody combination. Subsequently, the PBMCs were washed once and incubated for 20 min in the dark at room temperature with streptavidin-conjugates. Cells were subsequently washed twice and fixed in 2% PFA for 10 min in the dark at 4°C. Data were acquired on a BD LSRFortessa and analyzed with FlowJo software version 9.8 (LLC, OR, USA).

CMV Serology

Cytomegalovirus IgG serology on hepatitis samples was determined at Hannover Medical School, Hannover; serology on the healthy control samples was determined at Karolinska Institutet, Sweden; both centers used Abbott ARCHITECT Anti-Cytomegalovirus IgG tests.

Statistical Methods, Analysis Strategies, and Outlier Identification

Since expanded NK cell subsets have narrow KIR-repertoires often dominated by a single inhibitory receptor, different statistical strategies aimed at identifying outliers have previously been utilized to identify NK cell expansions (7, 18, 19). For cohorts significantly larger than ours, the Chauvenet criterion has been applied to select for NK cell expansions combined with cellular maturation (7, 18). However, along with our smaller cohorts, the lower expression of some KIRs during hepatitis infections may affect selection. In line with how we previously identified NK cell expansions (19), a variation of the Tukey's range test accounting for the cohort size was utilized to identify potential NK cell expansions. In more detail, $CD56^{dim}$ NK cells were subdivided into subsets based upon their expression of NKG2A and NKG2C. Subsequently, the expression repertoire of KIR2DL1, KIR2DL3, and KIR3DL1 was assessed. Particular KIR-defined subpopulations were considered as expanded populations if their population frequency was greater than one interquartile range above quartile three in each cohort. Threshold population sizes of either 5 or 20% were both analyzed. Further ensuring that the particular KIR-expressing NK cell subpopulation was a substantial constituent of the NK cell subset (e.g., $NKG2A^{-}NKG2C^{+}$), it was required to be greater than quartile one of the NK cell subset. Finally the geometric MFI of CD57 on the KIR-expressing NK cell subpopulation had to be greater than the geometric MFI of the subject's total $CD56^{dim}$ NK cells, ensuring that the identified outliers contained terminally differentiated NK cells (7, 20). Identifications of NK cell expansions were performed using Microsoft Excel version 14.4.3 (Microsoft Corporation, Washington, USA). Data were analyzed using Prism version 5.0d (GraphPad Software Inc., CA, USA). For the discrete statistics, non-parametric tests were used as outlined in the respective figure legends.

RESULTS

Validation of CMV-Associated Alterations in NK Cell Differentiation Status

First, NK cell differentiation-associated receptors NKG2A, CD57, and NKG2C, as well as inhibitory receptors KIR2DL1, KIR2DL3,

and KIR3DL1 were stained for on CD56^{dim} NK cells (**Figure 1A**). Expression of these markers was assessed in relation to CMV serostatus in the entire study cohort. As expected from previous reports (1, 21), CMV infection was associated with a higher frequency of CD56^{dim} NK cells expressing NKG2C and CD57, and with lower NKG2A expression compared to CMV seronegative individuals. However, CMV serostatus did not significantly alter expression of individual KIRs (**Figure 1B**).

Viral Hepatitis Does Not Alter NK Cell Differentiation as Assessed in CMV Seropositive Patients and Controls

Of the 88 individuals assessed in the entire study cohort, 79 were confirmed CMV seropositive permitting us to study the influence of hepatitis infection on a CMV seropositive background in relation to NK cell differentiation (**Figure 1C**). No major differences in expression of NKG2A, NKG2C, or CD57 were observed when comparing CD56^{dim} NK cells in CMV seropositive chronic hepatitis patients with corresponding CMV seropositive healthy controls (**Figure 1C**).

A recently published study including the same patient cohorts indicated that there was no overall difference in the expression of inhibitory KIRs on NK cells during hepatitis infection compared to healthy controls (16). However, in that report expression patterns of individual KIRs were not assessed. Here, a more detailed examination was performed. NK cells from CMV seropositive HCV-infected patients showed a significant decrease in expression of KIR2DL1 and KIR3DL1 relative to healthy controls, while expression of KIR2DL3 was unaltered (**Figure 1C**). HBV infection and HDV infection had no significant impact on KIR expression (**Figure 1C**).

Presence of CMV-Driven NK Cell Expansions Remain Unaltered in Chronic Viral Hepatitis

As described, expansions of NKG2C$^+$ NK cells with a specific KIR-repertoire driven by CMV infection have been observed in chronic hepatitis virus infections (12). However, whether hepatitis virus infection affects the prevalence of these expansions is unknown. Adaptive-like NK cell expansions have previously been identified based on deviations in KIR expression profiles, using statistical strategies such as the Chauvenet criterion (7, 18) and the Tukey's range test (19), combined with cellular differentiation. As our analysis included patients with different hepatitis infections, likely affecting receptor expression to varying degrees, each group was analyzed separately. NK cell subsets were assessed for the presence of expansions (see Materials and Methods) and quantified (**Figures 2A–C**). For both NKG2A$^-$NKG2C$^-$ and NKG2A$^-$NKG2C$^+$ NK cell subsets, there was no overall difference in the number of CMV seropositive individuals with NK cell expansions, irrespective of chronic hepatitis virus infections (**Figure 2D**). Since only 9 out of 88 studied patients were CMV seronegative, it was not possible to determine the influence of chronic viral hepatitis on potential expansions within this cohort. Together, we conclude that there is no difference between the prevalence of adaptive-like NK cell expansions observed in CMV seropositive subjects with HBV, HCV, and HDV infection as compared to CMV seropositive healthy controls.

DISCUSSION

Adaptive-like expansions of certain NK cell subsets are known to occur in response to human CMV infection. As described, such expansions are typically made up of NKG2C$^+$ NK cells with specific KIR expression patterns. Such expansions have been observed in several viral infections including viral hepatitis infections (12, 15). Yet, it is not known if viral hepatitis infection as such contributes to or promotes the appearance of such expansions, or if effects are primarily due to underlying CMV infection. Utilizing a CMV seropositive patient cohort with different forms of chronic viral hepatitis (HBV, HCV, and HDV), we confirmed the existence of large populations of adaptive-like CD57 expressing NK cells. However, the presence of these expansions was largely unrelated to hepatitis status. Since the vast majority of studied subjects were CMV seropositive, it was not possible to determine the influence of chronic viral hepatitis infections on potential adaptive-like NK cell expansions in CMV seronegative individuals. Based on results obtained in this study, we conclude that the observed expansions in hepatitis virus infected individuals (12) are attributed largely, if not exclusively, to underlying CMV infection.

Except for expression of NKG2C combined with a narrow KIR-repertoire, adaptive-like NK cell expansions have also been reported to express high levels of CD57, LILRB1, and CD2 while largely lacking NKG2A, NKp30, CD161, EAT-2, and FcεR1γ expression (2, 6–8). Although the adaptive NK cells display unique functional characteristics (2, 6–8), the phenotypic diversity within adaptive-like expanded subsets appears lower as compared to, e.g., the conventional CD56^{dim} NK cell subset (22, 23). It is currently unclear to which the degree the phenotype of adaptive-like NK cell expansions is affected in settings of disease, e.g., during chronic viral infections. In this study, we utilized expression of NKG2C, KIRs, and CD57 combined with absence of NKG2A to identify adaptive-like NK cell expansions. We could not detect any differences in expression of NKG2A, CD57, and NKG2C when comparing patients with controls. However, it is still plausible that other phenotypic traits associated with adaptive-like expansions are uniquely affected during chronic viral hepatitis.

It has been previously shown that KIR2DL2/3 is the most frequently expressed KIR on expanded adaptive-like NK cells during chronic hepatitis virus infections (12). While, we were unable to *KIR* genotype the present patient cohort, to allow us to precisely examine KIR2DL2 expression, we did observe maintenance of KIR2DL3 expression and down-regulation of KIR2DL1 and KIR3DL1 in chronic hepatitis C compared to healthy controls (**Figure 1C**). This is in line with previous results from other groups (13, 24). Preservation of expression of KIR2DL3 may be due either to immune adaptation or selective pressure in response to hepatitis infection. Intriguingly, it was recently shown that HCV sequence variation in core protein-derived HLA-presented

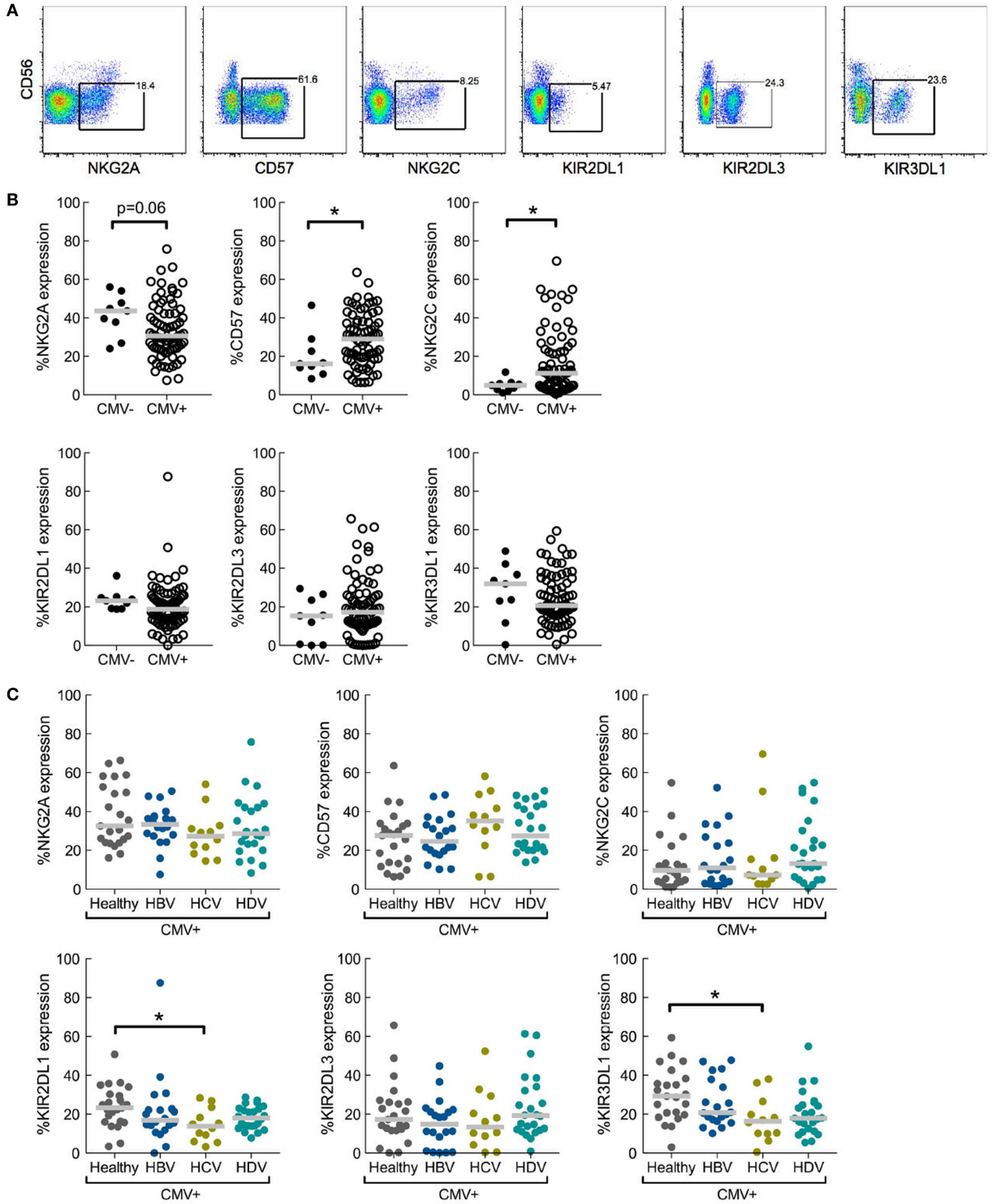


FIGURE 1 | Role of underlying cytomegalovirus (CMV) infection in chronic viral hepatitis infections with respect to effects of natural killer (NK) cell differentiation and killer-cell immunoglobulin-like receptor (KIR) expression. **(A)** Representative staining of differentiation-associated receptors on $CD56^{dim}$ NK cells. Data shown from total lymphocytes have CD3, CD4, CD14, CD19, and dead cell marker positive cells excluded. **(B)** Expression of NKG2A, CD57, NKG2C, KIR2DL1, KIR2DL3, and KIR3DL1 on $CD56^{dim}$ NK cells in CMV seronegative (CMV^-) or seropositive (CMV^+) individuals, irrespective of hepatitis status; CMV^- $n = 9$ and CMV^+ $n = 79$. Statistical analysis performed with Mann–Whitney test. **(C)** Expression of NKG2A, CD57, NKG2C, KIR2DL1, KIR2DL3, and KIR3DL1 on $CD56^{dim}$ NK cells in CMV seropositive individuals split into healthy ($n = 23$) and hepatitis virus infected [$n = 20$, 12, and 24 for hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis delta virus (HDV), respectively]. Statistical analysis performed with Kruskal–Wallis test and Dunn's multiple comparison. Gray bar indicates median. *$p < 0.05$.

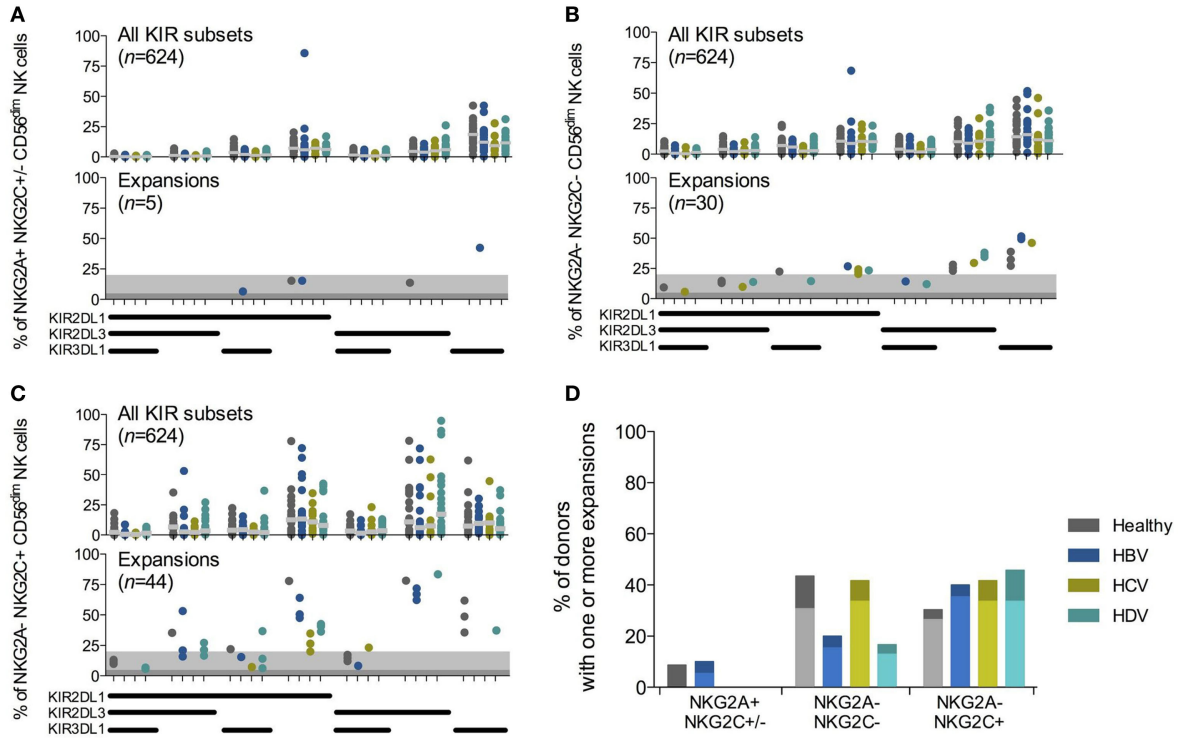


FIGURE 2 | **Chronic viral hepatitis does not affect the frequency of cytomegalovirus (CMV)-driven natural killer (NK) cell expansions**. **(A–C)** Killer-cell immunoglobulin-like receptor (KIR)-repertoire and adaptive-like NK cell expansions within NKG2A^{+}NKG2C$^{+/-}$ **(A)**, NKG2A^{-}NKG2C^{-} **(B)**, and NKG2A^{-}NKG2C^{+} **(C)** CD56^{dim} NK cell populations in CMV seropositive individuals. Upper-row figures display all KIR subpopulations [n = 23, 20, 12, and 24 for healthy individuals and hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis delta virus (HDV) infected individuals, respectively]. Gray bar indicates median. Bottom-row figures display the identified expansions. The dark gray shading indicates a 5% and the light gray shading a 20% threshold for confirmation of expansions. **(D)** Percentage of CMV seropositive individuals that have at least one measured expansion within the CD56^{dim} NK cell population. Twenty percent threshold results depicted in light colored bars. Chi-squared test for all NK cell subsets: healthy, $p < 0.02$; HBV, $p < 0.04$; HCV, $p < 0.08$; HDV, $p < 0.01$; and between NKG2A^{-} subsets: healthy, $p = 1$; HBV, $p = 0.27$; HCV, $p = 1$; HDV, $p < 0.17$. Five percent threshold results depicted as light and dark bars, Chi-squared test for all NK subsets: healthy, $p < 0.03$; HBV, $p < 0.074$; HCV, $p < 0.03$; HDV, $p = 0.0004$; and between NKG2A^{-} subsets: healthy, $p < 0.55$; HBV, $p < 0.3$; HCV, $p = 1$; HDV, $p < 0.06$.

epitopes alters the binding of KIR2DL3 to its HLA-ligand with functional consequences for NK cells suggesting a potential pathway for viral escape via KIR2DL3 (25).

There are contradictory results regarding NKG2C expression on NK cells during hepatitis infections. A systematic review suggested increased levels of NKG2C on CD56^{dim} NK cells during HBV infections relative to healthy donors (26). Further, levels of NKG2C in HBV infections were shown to be greater than those in HCV infections (13), while others have seen increased NKG2C during HCV infection (24). Data from our cohorts do not confirm these reports. As CMV serostatus associates with NKG2C expression, it is possible that these reported discrepancies would disappear had CMV serological status been considered. Indeed, such bias inferred by CMV was previously reported for individuals infected with HIV-1 (4).

In this study, we employed a non-parametric method to identify expanded NK cell populations. In brief, CD56^{dim} NK cells were separated into subsets based on expression of NKG2A and NKG2C, further subdivided based upon their expression profile of KIR2DL1, KIR2DL3, and KIR3DL1, and assessed for KIR-expressing NK cell expansions. NK cell expansions were in all assessed patient groups and healthy controls primarily confined within the NKG2A^{-}NKG2C^{+} or the NKG2A^{-}NKG2C^{-} groups of CD56^{dim} NK cells (**Figures 2A–C**). Few, if any, expansions had an NKG2A^{+} phenotype (**Figure 2A**). Although NK cell expansions have predominantly been described as NKG2C^{+} (18), NKG2C^{-} CD2^{+} NK cell expansions have also been reported (6). Therefore, we speculate that the NKG2A^{-}NKG2C^{-} NK cell expansions we observed may have had another activating receptor associated with their function, e.g., CD2 or activating KIRs. Taken together, the results validated our statistical strategy for identification of expansions, permitting us to compare how common expanded NK cell subsets were in CMV seropositive individuals with chronic viral hepatitis infections compared to healthy controls.

The NK cell expansions that we identified may be the reported adaptive-like NK cells, which lack the adaptor protein FcεR1γ and have been associated with reduced liver damage in HCV infection (15). Thus, the role for such cells, with increased levels of IFN-γ production in response to CD16 stimulation (10), should be thoroughly assessed in acquisition and progression of hepatitis virus infections in future studies.

Taken together, these results suggest that the NK cell differentiation status and the presence of NK cells with an adaptive phenotype, when controlled for CMV, remain unaffected by chronic viral hepatitis infection.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of Hannover Medical School, Hannover, Germany with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Hannover Medical School, Hannover, Germany.

AUTHOR CONTRIBUTIONS

DM performed most of the experiments, contributed to study design, acquisition of data, analysis, and drafting of the manuscript; SL performed most of the experiments, contributed to study design, and acquisition of data; JH contributed to study design and analysis; MM, JS, and MC contributed to study design and data interpretation; H-GL and HW contributed to study design, data interpretation, drafting the manuscript, and supervision of the work; NB contributed to study design, data analysis, drafting of the manuscript, and supervised the work.

ACKNOWLEDGMENTS

The authors would like to thank A. Heim for performing CMV serology assays. This work was funded by the International Research Training Group 1273 supported by the German Research Foundation (DFG), the Center Research Grants 738 (project B2), 841 (project A7), and 900 (project A5) supported by the DFG, the Deutsches Zentrum für Infektionsforschung, the German Liver Foundation, the Swedish Research Council, the Swedish Cancer Society, the Stockholm County Council, the Swedish Foundation for Strategic Research, the Swedish Society for Medical Research, the Cancer Research Foundations of Radiumhemmet, Knut and Alice Wallenberg Foundation, the Novo Nordisk Foundation, Åke Wiberg's Foundation, the Stockholm County Council, the Tobias Foundation, and the Karolinska Institutet.

REFERENCES

1. Lopez-Vergès S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57$^+$NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci U S A* (2011) 108:14725–32. doi:10.1073/pnas.1110900108
2. Björkström NK, Lindgren T, Stoltz M, Fauriat C, Braun M, Evander M, et al. Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J Exp Med* (2011) 208:13–21. doi:10.1084/jem.20100762
3. Zhou J, Amran FS, Kramski M, Angelovich TA, Elliott J, Hearps AC, et al. An NK cell population lacking FcRγ is expanded in chronically infected HIV patients. *J Immunol* (2015) 194:4688–97. doi:10.4049/jimmunol.1402448
4. Gumá M, Cabrera C, Erkizia I, Bofill M, Clotet B, Ruiz L, et al. Human cytomegalovirus infection is associated with increased proportions of NK cells that express the CD94/NKG2C receptor in aviremic HIV-1-positive patients. *J Infect Dis* (2006) 194:38–41. doi:10.1086/504719
5. Saghafian-Hedengren S, Sohlberg E, Theorell J, Carvalho-Queiroz C, Nagy N, Persson J-O, et al. Epstein-Barr virus coinfection in children boosts cytomegalovirus-induced differentiation of natural killer cells. *J Virol* (2013) 87:13446–55. doi:10.1128/JVI.02382-13
6. Liu LL, Landskron J, Ask EH, Enqvist M, Sohlberg E, Traherne JA, et al. Critical role of CD2 co-stimulation in adaptive natural killer cell responses revealed in NKG2C-deficient humans. *Cell Rep* (2016) 15:1088–99. doi:10.1016/j.celrep.2016.04.005
7. Béziat V, Liu LL, Malmberg J-A, Ivarsson MA, Sohlberg E, Björklund AT, et al. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood* (2013) 121:2678–88. doi:10.1182/blood-2012-10-459545
8. Schlums H, Cichocki F, Tesi B, Theorell J, Béziat V, Holmes TD, et al. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity* (2015) 42:443–56. doi:10.1016/j.immuni.2015.02.008
9. Hwang I, Zhang T, Scott JM, Kim AR, Lee T, Kakarla T, et al. Identification of human NK cells that are deficient for signaling adaptor FcRγ and specialized for antibody-dependent immune functions. *Int Immunol* (2012) 24:793–802. doi:10.1093/intimm/dxs080
10. Lee J, Zhang T, Hwang I, Kim A, Nitschke L, Kim M, et al. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. *Immunity* (2015) 42:431–42. doi:10.1016/j.immuni.2015.02.013
11. Luetke-Eversloh M, Hammer Q, Durek P, Nordström K, Gasparoni G, Pink M, et al. Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in NKG2Chi natural killer cells. *PLoS Pathog* (2014) 10:e1004441. doi:10.1371/journal.ppat.1004441
12. Béziat V, Dalgard O, Asselah T, Halfon P, Bedossa P, Boudifa A, et al. CMV drives clonal expansion of NKG2C(+) NK cells expressing self-specific KIRs in chronic hepatitis patients. *Eur J Immunol* (2012) 42:447–57. doi:10.1002/eji.201141826
13. Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology* (2009) 137:1151–60, 1160.e1–7. doi:10.1053/j.gastro.2009.05.047
14. Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, et al. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. *Gastroenterology* (2010) 138:325–35.e1–2. doi:10.1053/j.gastro.2009.08.066
15. Oh JS, Ali AK, Kim S, Corsi DJ, Cooper CL, Lee S-H. NK cells lacking FcεRIγ are associated with reduced liver damage in chronic hepatitis C virus infection. *Eur J Immunol* (2016) 46:1020–9. doi:10.1002/eji.201546009
16. Lunemann S, Malone DF, Hengst J, Port K, Grabowski J, Deterding K, et al. Compromised function of natural killer cells in acute and chronic viral hepatitis. *J Infect Dis* (2014) 209:1362–73. doi:10.1093/infdis/jit561
17. Björkström NK, Fauriat C, Bryceson YT, Sandberg JK, Ljunggren H-G, Malmberg K-J. Analysis of the KIR repertoire in human NK cells by flow cytometry. *Methods Mol Biol* (2010) 612:353–64. doi:10.1007/978-1-60761-362-6_24
18. Béziat V, Traherne J, Malmberg J-A, Ivarsson MA, Björkström NK, Retière C, et al. Tracing dynamic expansion of human NK-cell subsets by high-resolution analysis of KIR repertoires and cellular differentiation. *Eur J Immunol* (2014) 44:2192–6. doi:10.1002/eji.201444464
19. Ivarsson MA, Stiglund N, Marquardt N, Westgren M, Gidlöf S, Bjorkstrom NK. Composition and dynamics of the uterine NK cell KIR repertoire in menstrual blood. *Mucosal Immunol* (2016) 10:322–31. doi:10.1038/mi.2016.50
20. Björkström NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* (2010) 116:3853–64. doi:10.1182/blood-2010-04-281675
21. Hendricks DW, Balfour HH, Dunmire SK, Schmeling DO, Hogquist KA, Lanier LL. Cutting edge: NKG2C(hi)CD57+ NK cells respond specifically to

acute infection with cytomegalovirus and not Epstein-Barr virus. *J Immunol* (2014) 192:4492–6. doi:10.4049/jimmunol.1303211
22. Björkström NK, Ljunggren H-G, Michaëlsson J. Emerging insights into natural killer cells in human peripheral tissues. *Nat Rev Immunol* (2016) 16:310–20. doi:10.1038/nri.2016.34
23. Horowitz A, Strauss-Albee DM, Leipold M, Kubo J, Nemat-Gorgani N, Dogan OC, et al. Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med* (2013) 5:208ra145. doi:10.1126/scitranslmed.3006702
24. Szereday L, Meggyes M, Halasz M, Szekeres-Barthó J, Pár A, Pár G. Immunological changes in different patient populations with chronic hepatitis C virus infection. *World J Gastroenterol* (2016) 22:4848–59. doi:10.3748/wjg.v22.i20.4848
25. Lunemann S, Martrus G, Hölzemer A, Chapel A, Ziegler M, Körner C, et al. Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C*03:04 and modulate NK cell function. *J Hepatol* (2016) 65:252–8. doi:10.1016/j.jhep.2016.03.016
26. Zhang Q-F, Shao J-Y, Yin W-W, Xia Y, Chen L, Wang X, et al. Altered immune profiles of natural killer cells in chronic hepatitis B patients: a systematic review and meta-analysis. *PLoS One* (2016) 11:e0160171. doi:10.1371/journal.pone.0160171

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 15 February 2017
doi: 10.3389/fimmu.2017.00161

Expression of NKp46 Splice Variants in Nasal Lavage Following Respiratory Viral Infection: Domain 1-Negative Isoforms Predominate and Manifest Higher Activity

*Yonat Shemer-Avni[1†], Kiran Kundu[1,2†], Avishai Shemesh[1,2], Michael Brusilovsky[1], Rami Yossef[1], Mesfin Meshesha[1], Semaria Solomon-Alemayehu[1], Shai Levin[1], Orly Gershoni-Yahalom[1], Kerry S. Campbell[3] and Angel Porgador[1,2]**

[1] The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel, [2] National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer Sheva, Israel, [3] Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA, USA

The natural killer (NK) cell activating receptor NKp46/NCR1 plays a critical role in elimination of virus-infected and tumor cells. The *NCR1* gene can be transcribed into five different splice variants, but the functional importance and physiological distribution of NKp46 isoforms are not yet fully understood. Here, we shed light on differential expression of NKp46 splice variants in viral respiratory tract infections and their functional difference at the cellular level. NKp46 was the most predominantly expressed natural cytotoxicity receptor in the nasal lavage of patients infected with four respiratory viruses: respiratory syncytia virus, adenovirus, human metapneumovirus, or influenza A. Expression of NKp30 was far lower and NKp44 was absent in all patients. Domain 1-negative NKp46 splice variants (i.e., NKp46 isoform d) were the predominantly expressed isoform in nasal lavage following viral infections. Using our unique anti-NKp46 mAb, D2-9A5, which recognizes the D2 extracellular domain, and a commercial anti-NKp46 mAb, 9E2, which recognizes D1 domain, allowed us to identify a small subset of NKp46 D1-negative splice variant-expressing cells within cultured human primary NK cells. This NKp46 D1-negative subset also showed higher degranulation efficiency in term of CD107a surface expression. NK-92 cell lines expressing NKp46 D1-negative and NKp46 D1-positive splice variants also showed functional differences when interacting with targets. A NKp46 D1-negative isoform-expressing NK-92 cell line showed enhanced degranulation activity. To our knowledge, we provide the first evidence showing the physiological distribution and functional importance of human NKp46 splice variants under pathological conditions.

Keywords: splice variants, natural killer, NCR, NKp46, respiratory viral infection

OPEN ACCESS

Edited by:
Sandra Laurence Lopez-Verges, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama

Reviewed by:
Stephen K. Anderson, National Cancer Institute-Frederick, USA
Rafael Solana, University of Córdoba, Spain

***Correspondence:**
Angel Porgador
angel@bgu.ac.il

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *13 December 2016*
Accepted: *31 January 2017*
Published: *15 February 2017*

Citation:
Shemer-Avni Y, Kundu K, Shemesh A, Brusilovsky M, Yossef R, Meshesha M, Solomon-Alemayehu S, Levin S, Gershoni-Yahalom O, Campbell KS and Porgador A (2017) Expression of NKp46 Splice Variants in Nasal Lavage Following Respiratory Viral Infection: Domain 1-Negative Isoforms Predominate and Manifest Higher Activity. Front. Immunol. 8:161. doi: 10.3389/fimmu.2017.00161

INTRODUCTION

Acute viral respiratory tract infections represent a major public health and economic problem, affecting all age groups and causing severe disease, especially in infancy and old age. Various respiratory viruses, such as respiratory syncytial virus (RSV), influenza viruses, parainfluenza viruses, and adenoviruses may cause respiratory illness. In particular, RSV is the leading cause of hospitalization for respiratory tract illnesses in young children (1). Cellular immune responses play a major role in viral clearance. However, they can play a pivotal role in contributing to the pathogenesis of chronic diseases, e.g., asthma and apnea, in some cases (2, 3). The reported clinical manifestations for viral respiratory infections are largely overlapping, suggesting common regulatory pathways. Despite extensive diagnostic investigations of the operative cellular immune responses to respiratory infections, the role of natural killer (NK) cells and their involvement in immune responses to acute upper and lower respiratory tract infections remain largely unknown (4). NK cells have been found to be the major (25%) immune cells in non-squamous nasal lavage (5), but the specific type of NK-cells or their activity were not studied.

Natural killer cell activity is regulated by a balance between signals delivered by inhibitory and activating receptors. The natural cytotoxicity receptors (NCRs) NKp46 (NCR1), NKp44 (NCR2), and NKp30 (NCR3), as well as NKG2D, are the main activating receptors involved in mediating NK cell function in health and disease (6, 7). NCR and NKG2D mRNAs are subjected to alternative mRNA splicing events, resulting in the generation of splice variants that could mediate different and even opposing functions for the same receptor. For NKp30, three splice variants were reported: NKp30a, NKp30b, and NKp30c. NKp30a and NKp30b are considered as immune stimulatory isoforms and lead to increased IFN-γ and TNF-α secretion, while NKp30c leads to IL-10 secretion and immunosuppression. NKp30 splice variants have been studied by us and others in cancer, viral infection, and pregnancy (8–13). Also, three splice variants for NKp44 were reported: NKp44-1, NKp44-2, and NKp44-3. We were the first to report that the ITIM-positive NKp44-1 splice variant could serve as an inhibitory receptor when co-incubated with target cells expressing PCNA (14), and our findings with regard to the NKp44-PCNA inhibitory axis were corroborated by others (15). In acute myeloid leukemia (AML), sole expression of NKp44-1 was associated with poor survival of newly diagnosed patients (14). A NKp44-1-dominant inhibitory profile predominated in healthy pregnancy gestation, but preponderance of a NKp44 activation phenotype, was associated with pregnancy disorder (10). For NKG2D, it was reported that a truncated human NKG2D splice isoform negatively regulates NKG2D-mediated function (16).

Interestingly, no studies of the function and distribution of NKp46 splice variants have been published to date. Here, we studied the expression of NCRs in nasal lavage following respiratory viral infection and observed that NKp46 was expressed abundantly. The NKp46 receptor has five main splice variants divided into a group that contains both extracellular domains (3 isoforms) and a group that does not express the D1 domain (2 isoforms) (11, 17). Interestingly, the D1-negative NKp46 splice variants were the predominant isoforms expressed in nasal lavage following viral infection. We further showed that the D1-negative NKp46 splice variants are more active in both a NK cell line and primary human NK cells.

MATERIALS AND METHODS

Antibodies and Fusion-Ig Proteins

The following fluorochrome conjugated anti-human monoclonal antibodies were used for flow cytometry: anti-CD3 (PE, IQproducts), anti-CD56 (allophycocyanin, BioLegend), anti-CD16 (Pacific blue, BioLegend), anti-NKp46 (Alexa Fluor 647, clone 9E2, BioLegend), anti-NKp46 (iFluro 488, clone D2-9A5, Gen-Script), and anti-CD107a (BrilliantViolet 421, BioLegend). Purified anti-human monoclonal antibodies used for cell stimulation were anti-NKp30 (clone 210847, R&D Systems) and anti-NKp46 (clone D2-9A5). Monoclonal antibody D2-9A5 was generated using hybridoma technology described previously (18). HRP-conjugated goat anti-mouse IgG, Fcγ fragment-specific (Jackson ImmunoResearch Laboratories) antibody was used as secondary antibody. Recombinant NKp46 receptor Ig proteins, respectively, NKp46-D1 Ig, NKp46-D2 Ig, NKp46-Full Ig, and Human Fc were generated as described previously (19, 20).

Generation of NKp46 Domain 1$^+$ and NKp46 Domain 1$^-$ isoform-expressing NK Cell Lines and Cell Culture

NKp46 full length, i.e., domain 1$^+$ (isoform a; matching NM_004829.5) and NKp46-D2, i.e., domain 1$^-$ (isoform d; matching NM_001242356.1) cDNA were N-terminal FLAG-tagged and cloned into pBMN-IRES-GFP retroviral vector. NK-92 cells (ATCC CRL-2407), derived from human NK cell leukemia were then transduced with the constructs to produce NK-92-NKp46-Full (NK92-46Full) and NK-92-NKp46-D2 (NK92-46D2) cell lines, respectively. Retroviral transduction was performed as previously described (21–24). NK-92 cell lines were then grown in Alpha-MEM medium (Gibco, Life Technologies) supplemented with 10% horse serum, 10% FBS, 0.2 mM myo-inositol (Sigma), 0.1 mM β-mercaptoethanol (Sigma), 0.02 mM folic acid (Fisher Scientific), 100 IU/mL of recombinant human IL-2 (PeproTech), and 1% penicillin/streptomycin (Life Technologies). HEK293T, SV40 large T Ag-transfected HEK293 cells (CRL-11268) were used as target cell lines. HEK293T cells were cultured in DMEM (Gibco, Life Technologies) medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cell lines were grown in a 5% CO_2 humidified 37°C incubator.

Isolation and Culture of Primary Human NK Cells

Natural killer cell isolation was done from peripheral blood of healthy volunteer donors, who were recruited by informed consent as approved by the Ben-Gurion University of The Negev Institutional Review Board, using the RosetteSep Human NK cell Enrichment Cocktail kit (Stem Cell Technologies). After purification, cells were cultured in CellGro stem cell serum-free growth

medium (CellGenix) supplemented with 10% heat-inactivated human plasma from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, 1× MEM non-essential amino acids, 1% penicillin/streptomycin, 10 mM HEPES (Life Technologies), and 300 IU/ml human IL-2 (PeproTech) (23, 25).

Samples

Respiratory specimens, mainly nasal washes, were routinely obtained from hospitalized children with acute respiratory tract infection or from healthy children undergoing elective procedures (control group). Samples of nasopharyngeal washing (NPW) were tested for respiratory virus infections by real-time PCR (26). Briefly, nucleic acid extraction was performed using NucliSense EasyMag (Biomerieux, Marcy l'Etoile, France), according to the manufacturer's instructions. One milliliter of aspirate was extracted into 50 µl of elution solution. Amplification was carried out in a final volume of 10 µl, using the RNA Ultrasense One-step qRT-PCR system (Invitrogen, Carlsbad, CA, USA) with 4 µl of nucleic acid and four sets of primers and probes to detect four viruses, and an internal control (IC) set (27). Samples positive for respiratory syncytia virus (RSV), adenovirus (ADV), human metapneumovirus (HMPV), or influenza A (FLUA H1N1 strain), along with healthy controls, were included in the study.

Ethics Statement

The number of the Ethics Committee approval is 0113-14-SOR. The approval is to get respiratory specimens from children under 5 years old (hospitalized or healthy controls undergoing elective procedures). It includes the requirement to sign a written informed consent by the parents.

RNA Extraction, Reverse Transcription, Real-time Reverse Transcriptase Polymerase Chain Reaction (qRTPCR), and Primer Set Efficiencies

qRTPCR for NCRs

The sets of primers and probes used to detect the NCRs were all designed to span an intron or a splice junction, and thus test differentially spliced RNA transcripts, avoiding testing of DNA in the samples. The primers and probes were described in Table S1 in Supplementary Material. Each sample (extracted as described above, from NPW) was tested in parallel for NKp30, NKp44, and NKp46. Amplification was carried out in a final volume of 20 µl, using the RNA UltraSense One-Step qRT-PCR system (Invitrogen, Carlsbad, CA, USA) with 4 µl of nucleic acid and the relevant set of primers and probes. Samples that were positive for NKp46 were further analyzed for the NKp46 isoforms, specifically the presence of D1 domain, see Table S2 in Supplementary Material for primers–probes combinations.

Flow Cytometry

Human primary NK cells (10^5 cells/condition) were incubated with CD3-PE, CD56-APC, and CD16-Pacific blue mAbs (1 µg/ml) on ice for 1 h, washed and NK purity was checked. Primary NK cells from day 14, 21, and 28 culture were double stained on ice with anti-NKp46-Alexa Fluor 647 (clone 9E2) and anti-NKp46-iFluro 488 (clone D2-9A5) mAbs (1 µg/ml) for 1 h, washed and assessed for isoform development. Flow cytometry was done using on a Gallios instrument (Beckman Coulter) and FACS data were analyzed using FlowJo software (Tree Star, Inc.).

Cell Stimulation and Flow Cytometry-Based Degranulation Assay

For the NK cell degranulation assay, 96 well U-bottom plates were coated with 1.5 and 5 µg/ml of anti-NKp46 mAb (clone D2-9A5) and anti-NKp30 (clone 210847) mAb overnight at 4°C. After washing, 10^5 human primary NK cells were added for each condition along with BrilliantViolet 421-conjugated anti-CD107a mAb (1:400 final dilutions). After 4 h of incubation (37°C, 5% CO_2), cells were washed and stained again with the same anti-CD107a mAb (1:400 final dilution) and anti-NKp46 conjugated with Alexa Fluor 647 (clone 9E2). Percentages of $CD107a^+$ cells were assessed from both 9E2-positive and -negative population. The NK-92 cell line was transduced to express NKp46 full-IRES-GFP (NK92-46Full) and the NK-92 cell line expressing NKp46 D2-IRES-GFP (NK92-46D2) were separately co-incubated for 4 h (37°C, 5% CO_2) with HEK293T cells using a E:T ratio of 1:1, 1:2, or 1:5 along with BrilliantViolet 421-conjugated anti-CD107a mAb (1:400 final dilutions). Then, the cells were washed and stained again with BrilliantViolet 421-conjugated anti-CD107a mAb (1:400 final dilutions). NK-92 cells expressing the same level of GFP (correlating with equivalent levels of NKp46 isoform expression) were gated and percentages of $CD107a^+$ cells were analyzed. Flow cytometry was done using Gallios instrument (Beckman Coulter) and FACS data were analyzed using FlowJo software (Tree Star, Inc.).

ELISA Assay

ELISA plates were coated overnight at 4°C with 1 µg/ml of recombinant NKp46 receptor Ig proteins (NKp46-D1 Ig, NKp46-D2 Ig, and NKp46-Full Ig) and Human Fc as control. After washing, the plates were blocked with skimmed milk followed by incubation with 1 µg/ml of anti-NKp46 mAb (clone 9E2 or clone D2-9A5). Detection was done using HRP-conjugated goat anti-mouse IgG (1:1000 final dilution). OD was measured at 650 nm with a Dynex Technologies MRX microplate reader.

Statistical Analysis

Graphics and statistical analysis were performed using GraphPad/Prism5, OriginPro8, and Microsoft Office/Excel software. Statistical significance was calculated using Mann–Whitney *U* or Wilcoxon rank-sum test and One-way ANOVA. $p < 0.05$ was considered statistically significant.

RESULTS

Expression of NCRs, NKp46 Isoforms, and Cytokines in Nasal Lavage

The assessment of NCR expression in the upper airways during viral infection was carried out using qRTPCR analysis. Three sets of primers and probe (Tables S1 and S2 in Supplementary Material) were designed for each of the three NCRs (NKp46,

NKp44 and NKp30). Samples taken from nasal lavage (using nasal wash) of hospitalized patients with respiratory tract infection were tested. The most prevalent viruses that are found in these patients were chosen: 34 infected with RSV (respiratory syncytial virus) and 12 infected with HMPV, both ssRNA(+) viruses from the *Pneumoviridae* family; 24 infected with ADV, dsDNA viruses; and 14 infected with FLUA (influenza A) a segmented ssRNA(−) virus from the *Orthomyxoviridae*. Fifteen samples of healthy controls that arrived to the hospital for elective surgery were included in the study as well. Expression of the three NCRs was tested along with an IC, which is used to monitor for RNA extraction and for the PCR process (ERV-3 transcript, see Table S1 in Supplementary Material). To summarize, 83% (70 out of 84) of the infected samples were positive for NKp46 receptor, 18% (15 out of 84) were positive for NKp30, and none were positive for NKp44 (**Figure 1A**, detailing for the different viruses). All NKp30-positive samples were also positive for NKp46.

In the healthy control samples, 27% (4 out of 15) were positive for NKp46 and none was positive for NKp30 or NKp44 (**Figure 1A**). However, NKp46 expression in the nasal lavage of the infected samples was considerably higher as compared to the NKp46 expression in the nasal lavage of the four NKp46$^+$ healthy controls (**Figure 1B**). The fact that NKp46 was widely detected in samples from infected individuals (88, 79, 71, and 92% for RSV, ADV, FLUA, and HMPV, respectively, **Figure 1A**), but not in most healthy individuals, suggests that NKp46 is a preferred receptor in the immune cells infiltrating the nasal lavage during viral infections regardless of the type of viral infection.

Natural killer cells, the prototypical member of group 1 innate lymphoid cells (ILCs) express NKp46 (28, 29) and can invade nasal lavage (30, 31).

We, next, investigated the expression of NKp46 isoforms in the nasal lavage following respiratory viral infection. Differential expression of NKp30 and NKp44 isoforms was reported for different diseases (8–10, 12–14, 16); yet, the distribution of NKp46 isoforms was not studied. The NKp46 receptor has five main splice variants divided into two groups; one group (3 splice variants) contains the domain 1 (D1) region (contains Exon 3) and the other group (2) does not contain the D1 region (lacks Exon 3) (17). We previously published that domain 2 (D2) but not domain 1 (D1), of NKp46 is involved in the recognition of cellular ligands and that recognition of viral hemagglutinins (HA) involve the hinge region connecting domain 2 to the NK cell membrane (32). Therefore, we assessed the expression of D1-positive NKp46 mRNAs in nasal lavage following respiratory viral infection. The samples were screened by qRTPCR, and the results are shown in **Figure 2**. In parallel, each assay included three positive controls: NK cell lines (positive control cell line that contains D1-negative and D1-positive NKp46 isoforms), isolated PBMCs, and whole blood samples of healthy adult individuals (the latter two are depicted in **Figure 2**). **Figure 2** shows that most of the samples from the nasal lavage expressed NKp46 that lacked the D1 domain (75, 71, 57, and 77% for RSV, ADV, FLUA, and HMPV, respectively). In contrast, none of the positive controls was negative for expression of the NKp46 D1 isoforms (**Figure 2** for PBMC/blood controls).

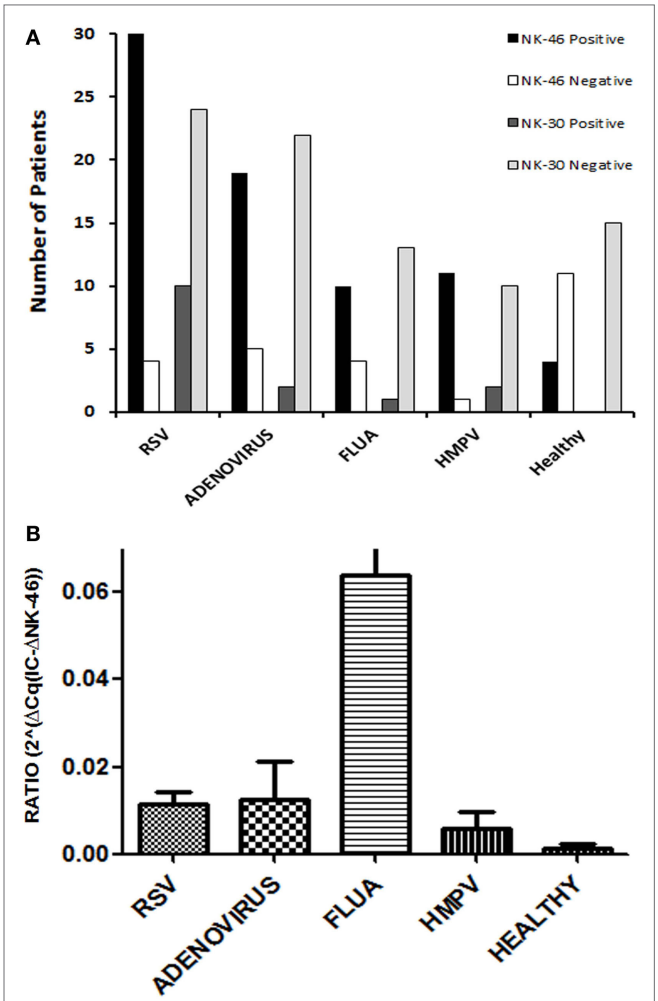


FIGURE 1 | Viral respiratory infections are accompanied by a specific pattern of natural cytotoxicity receptor (NCR) expression. Nucleic acids were extracted from nasal wash samples, obtained from patients with respiratory viral infections, respectively, respiratory syncytial virus, adenovirus, human metapneumovirus, and influenza A, along with healthy controls and then were tested for mRNA expression of the three NCRs (NKp30, NKp44, and NKp46) by qRTPCR. **(A)** Depicted are the number of patients positive and negative for expression of NKp46, as well as NKp30. Purified PBMCs from healthy controls or whole blood served as a positive control in each test. NKp44 receptor expression was not found in any of the samples. **(B)** NKp46 mRNA expression was compared between healthy and each of the virus-infected groups. Graph bars show mean ± SEM. Statistical significance was calculated by Mann–Whitney *U* or Wilcoxon rank-sum test. $p < 0.05$ was considered as statically significant.

NK-92 Cell Line Expressing the NKp46-Isoform d (D1-Negative) is Functionally More Active Than NK-92 Cell Line Expressing the Canonical NKp46-Isoform a (D1-Positive)

The NKp46 receptor consists of two Ig-C2-like ectodomains, D1 and D2 (33). As aforementioned, the NKp46 receptor has five main splice variants divided into a group that contains both

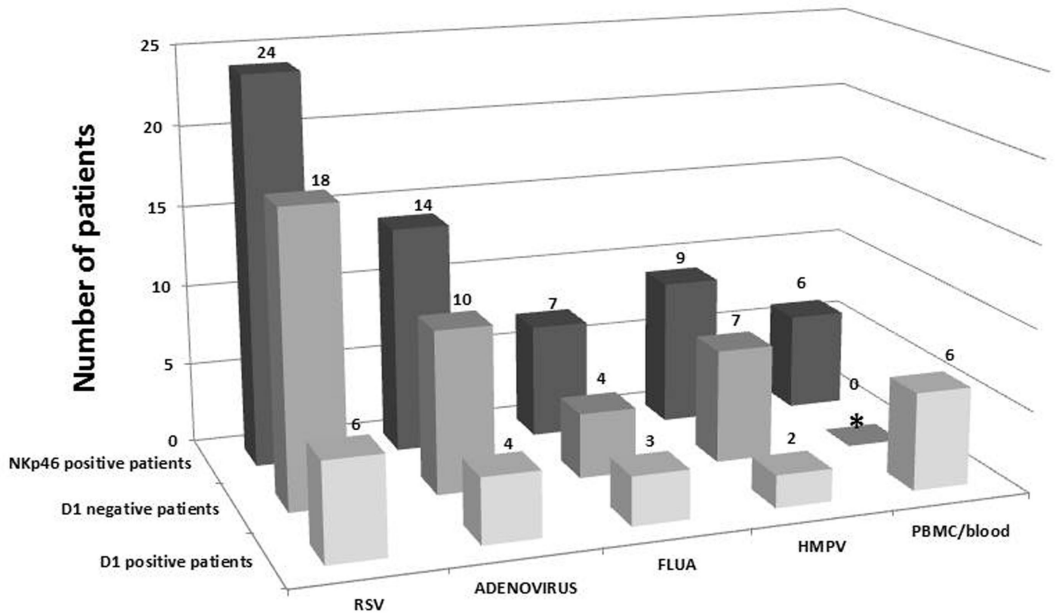


FIGURE 2 | Expression of D1-positive NKp46 isoforms in nasal wash is rare. The presence or absence of NKp46 D1-positive isoforms in samples were tested using two sets of specific primers and probes that span exon4 and exon5 (for NKp46 total) and the D1-positive region (Exon3, see Tables S1 and S2 in Supplementary Material for details). Shown are the total number of NKp46 receptor-positive samples that were found in each type of virus tested, and distribution of D1-positive isoforms and D1-negative isoforms (calculated from the subtraction of D1-positive samples from the number of samples positive for total NKp46). Purified PBMCs from healthy controls or whole blood served as a positive control in each test. *Low levels of D1-negative splice variants were shown for PBMCs in a different qRTPCR assay with a Fw-primer targeting the conjugated Ex2–Ex4 sequence (following the exclusion of the Domain-1 coding Exon 3). However, in this Figure, D1-negative is calculated as described above.

ectodomains (3 isoforms) and a group that does not express the D1 domain (2 isoforms) (11, 17). We, thus, investigated the function of a representative from each group: the canonical NKp46 receptor, i.e., NKp46 full (isoform a; matching NM_004829.5) and the single domain NKp46-D2 (isoform d; matching NM_001242356.1) (**Figure 3A**).

We generated NK-92 cell lines expressing splice variants a (D1-positive) and d (D1-negative) using a lentiviral gene delivery approach (NK92-46Full and NK92-46D2, respectively) and studied degranulation activity of these two cell lines upon interaction with cancer cells. We measured the CD107a surface expression as a degranulation marker. NK92-46Full and NK92-46D2 were co-incubated with HEK293T cell line in an E:T ratio of 1:1, 1:2, 1:5 for 4 h and CD107a surface staining was performed. Since GFP was co-expressed with the transduced NKp46 splice variant, we could measure CD107a expression for NK92-46Full and NK92-46D2 expressing the same levels of transduced NKp46. We observed that for each E:T ratio, degranulation efficacy was significantly higher for NK92-46D2 as compared to NK92-46Full cells (**Figure 3B**, summary of 3 experiments).

Cultured Human Primary NK Cells Develop a NKp46 D1-Negative-Dominant Subpopulation Manifesting Higher Activity

We previously developed the anti-NKp46 mAb, D2-9A5, which recognizes NKp46D2 (34). An ELISA assay to test for reactivity of the commercial anti-NKp46 mAb, clone 9E2, with recombinant NKp46D1-Ig, NKp46D2-Ig, and NKp46full-Ig revealed the recognition of NKp46D1 and NKp46full, but not NKp46D2 (Figure S1 in Supplementary Material). In contrast, D2-9A5 mAb recognized NKp46D2 and NKp46full, but not NKp46D1 (Figure S1 in Supplementary Material). Neither mAb reacted with negative control human Fc (Figure S1 in Supplementary Material). Similarly, the 9E2 mAb stained very positively NK92-46Full cells but to a significantly lesser extent the NK92-46D2 cells, while D2-9A5 mAb stained both cell lines similarly (Figure S2 in Supplementary Material). The low staining of NK92-46D2 by 9E2 antibody probably reflects the endogenous NKp46 expressed by NK-92 cells. Since both mAbs stain different domains, cross-reactivity is not a likely scenario. Yet, we did test for cross-reactivity by staining purified primary human NK cells either with each of the mAbs alone or by double staining with both mAbs. Figure S3 in Supplementary Material shows that the intensity of single staining with each of the mAbs was similar to its staining intensity in the double staining condition; thus demonstrating that 9E2 does not block D2-9A5 and *vice versa*. Therefore, we could use these antibodies together in flow cytometry analysis to test for the presence of a primary human NK subset in healthy controls that expresses only/mostly the D1-negative splice variant, as predicted from the RTPCR results from the nasal lavage of patients infected with respiratory viruses (**Figure 2**).

Since we could not retrieve live NK cells from nasal lavage of infected patients, we tested PBMCs. In freshly purified NK

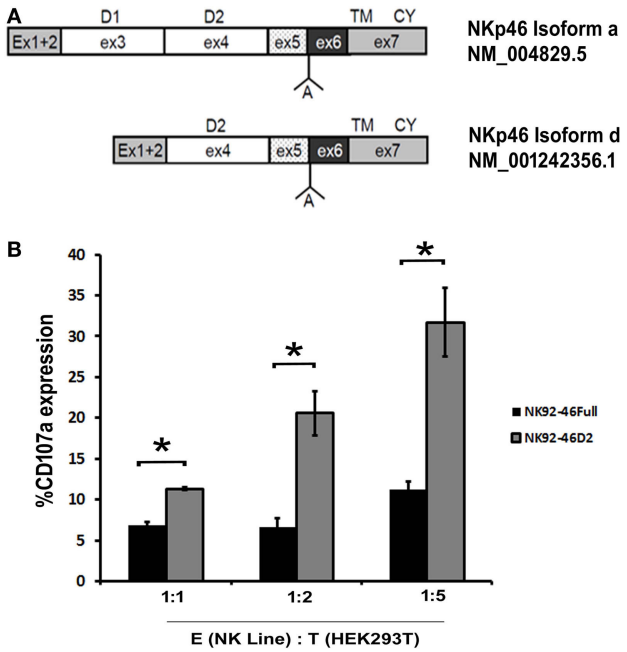


FIGURE 3 | Natural killer (NK)-92 cell line expressing the NKp46 D1-negative isoform is functionally more active than NK-92 cell line expressing the canonical NKp46. **(A)** Schematic diagram of the canonical NKp46 receptor, i.e., NKp46 full (isoform a; matching NM_004829.5) and D1-negative splice variants of NKp46 receptor, i.e., NKp46 D2 (isoform d; matching NM_001242356.1). Domain 1(D1) within exon3, domain 2(D2) within exon4, transmembrane domain, and cytoplasmic domain (CY) within exon7 are marked. "A" shown between exons 5 and 6 note the Alanine AA existing in these isoforms. **(B)** To show degranulation efficiency, NK-92 cell line expressing NKp46 full, IRES-GFP (NK92-46Full), and NK-92 cell line expressing NKp46 D2, IRES-GFP (NK92-46D2) were co-incubated for 4 h with HEK293T cells then washed and stained for CD107a. Percentages of CD107a positive cells were analyzed by flow cytometry. The results are from three experiments. Graph bars show mean ± SEM. $p < 0.05$, ANOVA.

cells, we could not observe a subset that expresses only the NKp46D1-negative splice variants. Yet, when we cultured human primary NK cells in the presence of 300 IU of recombinant human IL-2, they gradually developed a small but very distinct subpopulation that express only NKp46D1-negative splice variants, i.e., cells that were stained with D2-9A5 mAb, but not with 9E2 mAb. The D2-9A5^{+}9E2^{-} NK subset was evident at levels of 5% (14-day culture), 8% (21-day culture), and 10% (28-day culture) (**Figures 4A,B**). To investigate the functional relevance of this subset in primary NK cells, we assessed the degranulation potential of these cells as compared to the D2-9A5^{+}9E2^{+} cultured NK cells. We stimulated the cells with anti-NKp30 or with anti-NKp46 (D2-9A5) mAbs and tested CD107a expression. **Figure 4C** shows that cultured NK cells lacking the D1 domain (D2-9A5^{+}9E2^{-}) were significantly more active as compared to cultured NK cells expressing the D1 domain (D2-9A5^{+}9E2^{+}). Taken together, our data reveal the evolution of NKp46 splice variants within primary NK cells over time, and their differential ability to release cytolytic granules.

DISCUSSION

We showed that NKp46 is not expressed regularly in the nasal lavage of healthy controls, but this receptor was highly expressed in the human nasal lavage from patients infected with various respiratory viruses (**Figure 1**). NKp46 is constitutively expressed by NK cells that are part of group 1 ILCs (28, 29). Other ILCs, such as intraepithelial (lung and intestine) ILC1 and NCR^{+} ILC3 subsets, also expressed NKp46 (28). Most non-NK ILC subsets that constitutively express NKp46, also manifest constitutive expression of NKp44; in contrast, peripheral blood NK cells express NKp44 only after stimulation. Therefore, our failure to observe any NKp44 expression in the human nasal lavage in parallel with NKp46 expression, indicates that the ILCs migrating to the nasal lavage upon respiratory nasal infection, are probably NK cells (35, 36).

Natural killer cells are capable of migrating to virus-infected tissue (30, 31) and specifically for IV infection, CCR2 was shown in mice to mediate the migration of NK cells during influenza virus infection (37). The immunopathology of upper respiratory tract viral infection is characterized by a complex interplay between respiratory virus, resident lung epithelial cells and infiltrating innate and adaptive immune cells. NK cells are one of the important immune cell types recruited into the infected respiratory tract to clear virus and resolve the infection. In addition to directly killing virus-infected cells, NK cells produce significant amounts of IFN-γ to augment cytotoxic T lymphocyte activity (31). NKp46 is a primary activating receptor mediating NK cell antiviral function (6, 7); a number of virus-derived ligands recognized directly by NKp46 have been reported. We have shown that NCRs, particularly NKp46, are involved in the functional recognition of viral HA and hemagglutinin-neuraminidases (38–41). Other groups have similarly reported the involvement of NKp46 and other NCRs in the recognition of viruses (42, 43).

The NKp46 receptor can be encoded by five major splice variants that can be divided into a group that contains both ectodomains (3 isoforms) and a group that does not express the D1 domain (2 isoforms) (11, 17). We showed that D1-positive splice variants are rare in the nasal lavage; therefore, the D1-negative splice variants are the dominant splice variants in the nasal lavage following respiratory viral infection (**Figure 2**). These results are in concordance with our previous reports that the NKp46 recognition site of viral ligands involve the membrane-proximal domain (domain 2) and the hinge peptide connecting NKp46-D2 to the cell membrane, but does not involve the membrane distal domain D1 (32). Differential tissue distribution of NCR splice variants has been previously reported for NKp30 and NKp44, but this is the first report of differential distribution of NKp46 splice variants. It was previously reported that NKp30 and NKp44 splice variant profiles differ between decidua basalis NK (dNK) cells and peripheral blood NK (pNK) cells. The NKp44-1 splice variant was shown to be significantly expressed in dNK cells compared to pNK cells, and the decidual cytokine milieu can change the splice variant profile of pNK cells to mimic that of dNK cells (11). Our recent observations also showed that a NKp44-1$^{\text{dominant}}$ inhibitory profile predominates in dNK cells in healthy pregnancy gestation, whereas a NKp44-2/3$^{\text{dominant}}$ activation profile proved to be more

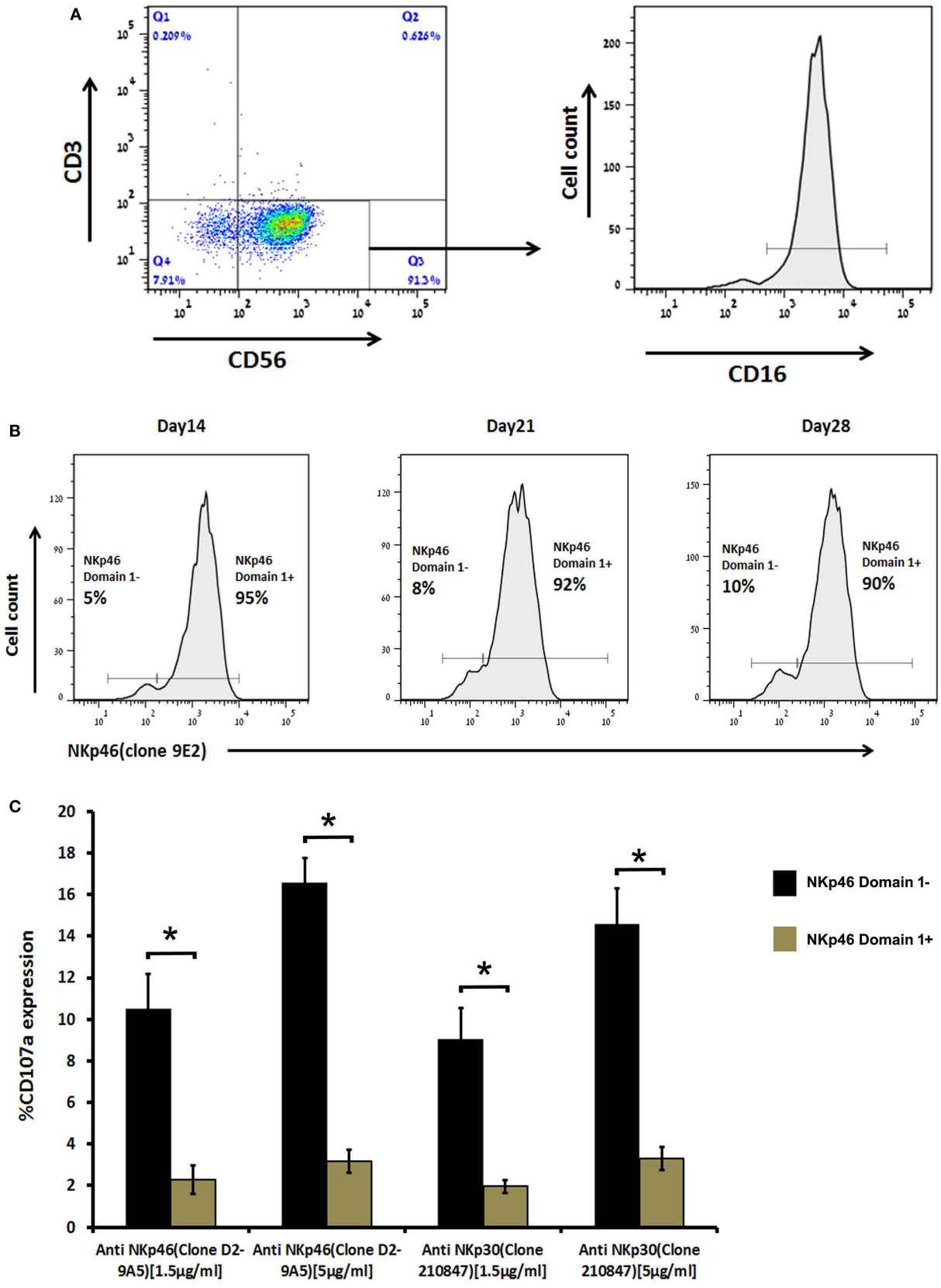


FIGURE 4 | Continued

FIGURE 4 | **Continued**

Human primary natural killer (NK) cells when cultured *in vitro* became enriched with a distinct subpopulation expressing mostly the NKp46 D1-negative isoforms (NKp46 D2) that are functionally more active. **(A)** Primary NK cells were stained with anti-CD3, anti-CD56, and anti-CD16 mAb to determine the percentage of $CD3^-$ $CD56^+$ NK cells and their CD16 expression. **(B)** Primary NK cells were double stained with anti-NKp46-Alexa Fluor 647 (clone 9E2) and anti-NKp46-iFluor 488 (clone D29A5) mAb at day 14, 21, and 28 of culture to show the development of NKp46 D1-negative isoforms. **(C)** When primary NK cells of day 28 culture were cross-linked for 4 h in a 96-well plate, coated with anti-NKp46 mAb (clone D29A5) and anti-NKp30 (clone 210847), then double stained with anti-CD107a and anti-NKp46 (clone 9E2), NK cells expressing the NKp46 D1-negative isoform became more active. Staining results were analyzed by flow cytometry. The results are from one representative experiment of triplicates. Graph bars show mean ± SEM ($p < 0.05$, ANOVA.

prevalent in cases of spontaneous abortions. No significant differences were observed in NKp30 splice variant expression profiles in dNK cells when comparing healthy gestation and spontaneous abortions (44). Recently we also reported that among the three different splice variants of NKp44, the inhibitory NKp44-1 isoform was significantly associated with poor survival of AML patients (14). In the case of gastrointestinal sarcoma tumors (GIST), distinct NKp30 splice variant profiles were observed in peripheral blood NK cells, as compared to healthy individuals (8). In that study, the immunosuppressive NKp30c isoform was expressed more prominently in individuals with GIST (8).

The differential distribution of NKp30 and NKp44 splice variants was associated with alterations in function of the different splice variants. In particular, inhibitory splice variants were identified and characterized for both NKp30 and NKp44 (8, 14, 44). In our studies with NK cell lines transfected with different NKp46 splice variants, we showed a functional difference between a D1-positive and a D1-negative splice variant, with a tendency of the D1-negative splice variant to exert a better NK cell degranulation response (**Figure 3**). Yet, this difference could not define the D1-positive splice variant as a suppressor variant. We were able to use anti-NKp46 mAbs that specifically stain domain 1 or domain 2, to identify primary human NK cells that more readily express D1-negative splice variants (Figures S1–S3 in Supplementary Material). NK cells expressing only the NKp46 D1-negative splice variants were negligible in peripheral blood; yet, following prolonged *in vitro* culture with IL-2, a small but distinct NK subset expressing mostly the D1-negative splice variants was observed. In accordance with the results from the NK cell lines, these D1-negative primary human NK cells exhibited a significantly better degranulation response, as compared to the other IL-2 cultured NK cells expressing both D1-negative and D1-positive NKp46 transcripts (**Figure 4**). We previously reported the evolution of a NK cell subset expressing a specific splice variant of NKp44 (14, 44), and the microenvironment of placenta, as well as tumor, was enriched with TGF-β and other cytokines that can actively modulate NKp44 splice variant expression. The development of NK cells expressing inhibitory NKp44 and NKp30 splice variants, orchestrated by cytokine milieu within placenta and tumor, provides a unique mechanism of immune tolerance.

The question whether or not the two splice variant groups (D1-negative and D1-positive NKp46 isoforms) are mutually exclusive, or can exist within the same NK cell, remained to be further explored. Yet, our D1–D2 antibody staining results of IL-2-maintained primary NK cells indicate the development of NK cell subset expressing mostly the D1-negative splice variants. Single-cell genomic analysis assays should be performed to assess the RNA splice variant distribution in the level of the single cell. Following the setting of single NK cell expressing mostly the D1-negative splice variants, the second subject to be resolved is whether the NK cells migrating to an active site are comprised mostly from cells expressing the same pattern of splice variants. Our results with the nasal lavage following respiratory viral infection point to a setting of NK cells manifesting similar phenotype of NKp46 D1-negative splice variants. Our recent studies with NKp44 splice variants indicate that dominant expression of splice variant (NKp44 isoform 1) can be observed in NK clones (14); moreover, we showed that NK cell subset in specific organs, e.g. the first trimester decidua in normal pregnancy, has the same splice variant profile which is mostly NKp44 isoform 1 (44).

To recapitulate, for the first time, this study demonstrates alterations in the distribution of NCR1 splice variants within nasal mucosa following respiratory viral infections, as well as the ability of cytokine stimulation to modulate the NKp46 splice variant expression profile, which may be representative of conditions in the infected respiratory tract. In addition, we also provide evidence for functional differences between the NCR1 splice variants, since the NKp46 domain 1-negative isoform proved to be functionally more active.

AUTHOR CONTRIBUTIONS

YS-A, KK, AS, and AP planned the experiments. MM, SS-A, AS, and SL performed the PCR experiments. KK, MB, and OG-Y performed the NK and FACS experiments. YS-A, KK, RY, AS, and AP analyzed the experiments. YS, KK, and AP wrote the manuscript. KC and AP edited the manuscript.

FUNDING

This work was supported by the Israel Science Foundation grant 1188/16 (AP), the US/Israel Binational Science Foundation grant 2015344 (AP and KC), the Israeli Ministry of Science and Technology/German Cancer Research Center program Grant CA172 (AP), and the Israeli Ministry of Science and Technology grant 54180 (AP).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00161/full#supplementary-material.

REFERENCES

1. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev* (2010) 23(1):74–98. doi:10.1128/CMR.00032-09
2. Murdoch JR, Lloyd CM. Chronic inflammation and asthma. *Mutat Res* (2010) 690(1–2):24–39. doi:10.1016/j.mrfmmm.2009.09.005
3. Deckers J, Branco Madeira F, Hammad H. Innate immune cells in asthma. *Trends Immunol* (2013) 34(11):540–7. doi:10.1016/j.it.2013.08.004
4. Pavia AT. Viral infections of the lower respiratory tract: old viruses, new viruses, and the role of diagnosis. *Clin Infect Dis* (2011) 52(Suppl 4):S284–9. doi:10.1093/cid/cir043
5. Horvath KM, Herbst M, Zhou H, Zhang H, Noah TL, Jaspers I. Nasal lavage natural killer cell function is suppressed in smokers after live attenuated influenza virus. *Respir Res* (2011) 12(1):102. doi:10.1186/1465-9921-12-102
6. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* (2011) 89(2):216–24. doi:10.1038/icb.2010.78
7. Koch J, Steinle A, Watzl C, Mandelboim O. Activating natural cytotoxicity receptors of natural killer cells in cancer and infection. *Trends Immunol* (2013) 34(4):182–91. doi:10.1016/j.it.2013.01.003
8. Delahaye NF, Rusakiewicz S, Martins I, Menard C, Roux S, Lyonnet L, et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat Med* (2011) 17(6):700–7. doi:10.1038/nm.2366
9. Prada N, Antoni G, Commo F, Rusakiewicz S, Semeraro M, Boufassa F, et al. Analysis of NKp30/NCR3 isoforms in untreated HIV-1-infected patients from the ANRS SEROCO cohort. *Oncoimmunology* (2013) 2(3):e23472. doi:10.4161/onci.23472
10. Shemesh A, Tirosh D, Sheiner E, Tirosh NB, Brusilovsky M, Segev R, et al. First trimester pregnancy loss and the expression of alternatively spliced nkp30 isoforms in maternal blood and placental tissue. *Front Immunol* (2015) 6:189. doi:10.3389/fimmu.2015.00189
11. Siewiera J, Gouilly J, Hocine HR, Cartron G, Levy C, Al-Daccak R, et al. Natural cytotoxicity receptor splice variants orchestrate the distinct functions of human natural killer cell subtypes. *Nature Commun* (2014) 6:10183. doi:10.1038/ncomms10183
12. Messaoudene M, Fregni G, Enot D, Jacquelot N, Neves E, Germaud N, et al. NKp30 isoforms and NKp46 transcripts in metastatic melanoma patients: unique NKp30 pattern in rare melanoma patients with favorable evolution. *Oncoimmunology* (2016) 5(12):e1154251. doi:10.1080/2162402X.2016.1154251
13. Mantovani S, Mele D, Oliviero B, Barbarini G, Varchetta S, Mondelli MU. NKp30 isoforms in patients with chronic hepatitis C virus infection. *Immunology* (2015) 146(2):234–42. doi:10.1111/imm.12495
14. Shemesh A, Brusilovsky M, Hadad U, Teltsh O, Edri A, Rubin E, et al. Survival in acute myeloid leukemia is associated with NKp44 splice variants. *Oncotarget* (2016) 7(22):32933–45. doi:10.18632/oncotarget.8782
15. Horton NC, Mathew SO, Mathew PA. Novel interaction between proliferating cell nuclear antigen and HLA I on the surface of tumor cells inhibits NK cell function through NKp44. *PLoS One* (2013) 8(3):e59552. doi:10.1371/journal.pone.0059552
16. Karimi MA, Aguilar OA, Zou B, Bachmann MH, Carlyle JR, Baldwin CL, et al. A truncated human NKG2D splice isoform negatively regulates NKG2D-mediated function. *J Immunol* (2014) 193(6):2764–71. doi:10.4049/jimmunol.1400920
17. Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, et al. Molecular cloning of NKp46: a novel member. *J Exp Med* (1998) 188(5):953–60. doi:10.1084/jem.188.5.953
18. Yossef R, Gur C, Shemesh A, Guttman O, Hadad U, Nedvetzki S, et al. Targeting natural killer cell reactivity by employing antibody to NKp46: implications for type 1 diabetes. *PLoS One* (2015) 10(2):e0118936. doi:10.1371/journal.pone.0118936
19. Bloushtain N, Qimron U, Bar-Ilan A, Hershkovitz O, Gazit R, Fima E, et al. Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. *J Immunol* (2004) 173(4):2392–401. doi:10.4049/jimmunol.173.4.2392
20. Hecht ML, Rosental B, Horlacher T, Hershkovitz O, De Paz JL, Noti C, et al. Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences. *J Proteome Res* (2009) 8(2):712–20. doi:10.1021/pr800747c
21. Brusilovsky M, Cordoba M, Rosental B, Hershkovitz O, Andrake MD, Pecherskaya A, et al. Genome-wide siRNA screen reveals a new cellular partner of NK cell receptor KIR2DL4: heparan sulfate directly modulates KIR2DL4-mediated responses. *J Immunol* (2013) 191(10):5256–67. doi:10.4049/jimmunol.1302079
22. Purdy AK, Campbell KS. Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR). *Cancer Biol Ther* (2009) 8(23):2211–20. doi:10.4161/cbt.8.23.10455
23. Brusilovsky M, Radinsky O, Cohen L, Yossef R, Shemesh A, Braiman A, et al. Regulation of natural cytotoxicity receptors by heparan sulfate proteoglycans in – cis: a lesson from NKp44. *Eur J Immunol* (2015) 45(4):1180–91. doi:10.1002/eji.201445177
24. Hershkovitz O, Jivov S, Bloushtain N, Zilka A, Landau G, Bar-Ilan A, et al. Characterization of the recognition of tumor cells by the natural cytotoxicity receptor, NKp44. *Biochemistry* (2007) 46(25):7426–36. doi:10.1021/bi7000455
25. Hadad U, Thauland TJ, Martinez OM, Butte MJ, Porgador A, Krams SM. NKp46 clusters at the immune synapse and regulates NK cell polarization. *Front Immunol* (2015) 6:495. doi:10.3389/fimmu.2015.00495
26. Lieberman D, Lieberman D, Shimoni A, Keren-Naus A, Steinberg R, Shemer-Avni Y. Identification of respiratory viruses in adults: nasopharyngeal versus oropharyngeal sampling. *J Clin Microbiol* (2009) 47(11):3439–43. doi:10.1128/JCM.00886-09
27. Lieberman D, Lieberman D, Shimoni A, Keren-Naus A, Steinberg R, Shemer-Avni Y. Pooled nasopharyngeal and oropharyngeal samples for the identification of respiratory viruses in adults. *Eur J Clin Microbiol Infect Dis* (2010) 29(6):733–5. doi:10.1007/s10096-010-0903-5
28. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol* (2016) 17(7):758–64. doi:10.1038/ni.3482
29. Nau D, Altmayer N, Mattner J. Mechanisms of innate lymphoid cell and natural killer T cell activation during mucosal inflammation. *J Immunol Res* (2014) 2014:8. doi:10.1155/2014/546596
30. Culley FJ. Natural killer cells in infection and inflammation of the lung. *Immunology* (2009) 128(2):151–63. doi:10.1111/j.1365-2567.2009.03167.x
31. Newton AH, Cardani A, Braciale TJ. The host immune response in respiratory virus infection: balancing virus clearance and immunopathology. *Semin Immunopathol* (2016) 38(4):471–82. doi:10.1007/s00281-016-0558-0
32. Arnon TI, Achdout H, Lieberman N, Gazit R, Gonen-Gross T, Katz G, et al. The mechanisms controlling the recognition of tumor- and virus-infected cells by NKp46. *Blood* (2004) 103(2):664–72. doi:10.1182/blood-2003-05-1716
33. Foster CE, Colonna M, Sun PD. Crystal structure of the human natural killer (NK) cell activating receptor NKp46 reveals structural relationship to other leukocyte receptor complex immunoreceptors. *J Biol Chem* (2003) 278(46):46081–6. doi:10.1074/jbc.M308491200
34. Gur C, Porgador A, Elboim M, Gazit R, Mizrahi S, Stern-Ginossar N, et al. The activating receptor NKp46 is essential for the development of type 1 diabetes. *Nat Immunol* (2010) 11(2):121–8. doi:10.1038/ni.1834
35. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity* (2013) 38(4):769–81. doi:10.1016/j.immuni.2013.02.010
36. Bernink JochemÂ H, Krabbendam L, Germar K, deÂ Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and -23 control plasticity of CD127+ group 1 and group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity* (2015) 43(1):146–60. doi:10.1016/j.immuni.2015.06.019
37. van Helden MJ, Zaiss DM, Sijts AJ. CCR2 defines a distinct population of NK cells and mediates their migration during influenza virus infection in mice. *PLoS One* (2012) 7(12):e52027. doi:10.1371/journal.pone.0052027
38. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* (2001) 31(9):2680–9. doi:10.1002/1521-4141(200109)31:9<2680::AID-IMMU2680>3.0.CO;2-A

39. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* (2001) 409(6823):1055–60. doi:10.1038/35059110
40. Porgador A. Natural cytotoxicity receptors: pattern recognition and involvement of carbohydrates. *ScientificWorldJournal* (2005) 5:151–4. doi:10.1100/tsw.2005.22
41. Mandelboim O, Porgador A. NKp46. *Int J Biochem Cell Biol* (2001) 33(12):1147–50. doi:10.1016/S1357-2725(01)00078-4
42. Brandstadter JD, Yang Y. Natural killer cell responses to viral infection. *J Innate Immun* (2011) 3(3):274–9. doi:10.1159/000324176
43. Ma Y, Li X, Kuang E. Viral evasion of natural killer cell activation. *Viruses* (2016) 8(4):95. doi:10.3390/v8040095
44. Shemesh A, Kugel A, Steiner N, Yezersky M, Tirosh D, Edri A, et al. NKp44 and NKp30 splice variant profiles in decidua and tumor tissues: a comparative viewpoint. *Oncotarget* (2016) 7(43):70912–23. doi:10.18632/oncotarget.12292

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 09 March 2017
doi: 10.3389/fimmu.2017.00212

NK Cells: Uncertain Allies against Malaria

*Asia-Sophia Wolf, Samuel Sherratt and Eleanor M. Riley**

Department of Immunology and Infection, London School of Hygiene and Tropical Medicine, London, UK

Until recently, studies of natural killer (NK) cells in infection have focused almost entirely on their role in viral infections. However, there is an increasing awareness of the potential for NK cells to contribute to the control of a wider range of pathogens, including intracellular parasites such as *Plasmodium* spp. Given the high prevalence of parasitic diseases in the developing world and the devastating effects these pathogens have on large numbers of vulnerable people, investigating interactions between NK cells and parasitized host cells presents the opportunity to reveal novel immunological mechanisms with the potential to aid efforts to eradicate these diseases. The capacity of NK cells to produce inflammatory cytokines early after malaria infection, as well as a possible role in direct cytotoxic killing of malaria-infected cells, suggests a beneficial impact of NK cells in this disease. However, NK cells may also contribute to overproduction of pro-inflammatory cytokines and the consequent immunopathology. As comparatively little is known about the role of NK cells later in the course of infection, and growing evidence suggests that heterogeneity in NK cell responses to malaria may be influenced by KIR/HLA interactions, a better understanding of the mechanisms by which NK cells might directly interact with parasitized cells may reveal a new role for these cells in the course of malaria infection.

Keywords: NK cells, malaria, inflammatory cytokines, cytotoxic killing, KIR

OPEN ACCESS

Edited by:
Vincent Vieillard, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Salim Iqbal Khakoo, University of Southampton, UK
Stephen Noel Waggoner, Cincinnati Children's Hospital Medical Center, USA

***Correspondence:**
Eleanor M. Riley
eleanor.riley@lshtm.ac.uk

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *07 January 2017*
Accepted: *15 February 2017*
Published: *09 March 2017*

Citation:
Wolf A-S, Sherratt S and Riley EM (2017) NK Cells: Uncertain Allies against Malaria. Front. Immunol. 8:212. doi: 10.3389/fimmu.2017.00212

INTRODUCTION

Natural killer (NK) cells are a subset of lymphocytes that contribute to the control of cancers and infections through the production of pro-inflammatory cytokines and the destruction of damaged, dysfunctional or infected host cells *via* cytotoxic activity [reviewed in Ref. (1)]. They typically constitute about 10% of peripheral blood mononuclear cells (PBMCs), although there is considerable variation between individuals. The activity of NK cells is regulated by binding of antibody–antigen complexes to the Fc receptor CD16 (2), expression of a large range of activating and inhibitory receptors used to directly "read" the surface of potentially infected or dysfunctional cells [reviewed in Ref. (3, 4)], and expression of receptors for cytokines such as interleukin (IL)-12, IL-15, IL-18 and IL-2 [reviewed in Ref. (5)]. Healthy cells express ligands for inhibitory NK cell receptors, ensuring that they are "ignored" by patrolling NK cells, but these ligands are downregulated on damaged or diseased cells, while activating signals (so-called "stress ligands") may be upregulated, making the cells clear targets for NK cell-mediated destruction. Moreover, pro-inflammatory cytokines can override ligand-mediated inhibitory signals, thereby allowing NK cells to participate in systemic immune responses by producing inflammatory cytokines (6–8).

Although traditionally classed as innate lymphocytes, recent work has suggested that NK cells may participate in adaptive immune responses and may also exhibit immunological "memory" or "memory-like" responses leading to significantly higher cytokine production and enhanced cytotoxic responses upon restimulation. This topic was recently comprehensively reviewed by Cerwenka and Lanier (9), but, in brief, enhanced NK cell responses have been described after infection with viruses, after exposure to haptens, and after *in vitro* stimulation with cytokines. Very recently, enhanced responses of human peripheral blood NK cells have also been observed *ex vivo* after influenza vaccination (10). While there is some evidence in murine systems, and more recently in rhesus macaques (11), that these "memory" NK cell responses may be antigen specific, this has only been shown definitively for liver-resident NK cells (12, 13) and the only well-characterized receptor–ligand interaction is the mouse Ly49 receptor family binding murine cytomegalovirus (MCMV) ligands (14–17). In the case of human CMV (HCMV), the functionally equivalent interaction is mediated by heterodimeric CD94/NKG2A and CD94/NKG2C receptors which recognize peptides from HCMV bound to human leukocyte antigen (HLA)-E (18) and which induce characteristic expansions of the $NKG2C^+$ NK cell subset and epigenetic modifications of the NK cell genome (19–22) [reviewed in Ref. (23)]. However, in many cases such as in studies on malaria, rabies, and influenza, these enhanced secondary responses are at least partly attributable to indirect activation of NK cells by memory T cell-derived IL-2 rather than to true "memory" on the part of NK cells themselves (10, 24–26). This proxy recall response was first identified during influenza vaccination by He et al. (27) and then by Horowitz et al. (24) in response to rabies vaccination. Subsequent studies have demonstrated a similar IL-2-dependent effect in response to malaria-infected erythrocytes (25). Regardless of the underlying mechanism, this raises the intriguing possibility that NK cells may contribute substantially to immune responses after malaria vaccination, and preliminary studies have already demonstrated enhanced NK cell activation in response to increased T cell IL-2 production in individuals vaccinated with the RTS,S/AS01 malaria vaccine (26).

Given this evidence, there is considerable interest in gaining a better understanding of the mechanisms by which NK cells are activated during malaria infections and whether this is beneficial or detrimental. Such research will serve to clarify the basic functions of NK cells during infection with intracellular protozoa and, potentially, to target an effective immune mechanism during vaccine development. In this review, we summarize the current state of knowledge of the role of NK cells during malaria infection and malaria vaccination, both in humans and in experimental murine infections.

MECHANISMS OF NK CELL ACTIVATION

Natural killer cells were classically considered "natural" killers because, unlike T cells, they do not require prior exposure to antigen before being able to engage and kill target cells, although it is now understood that they require a complex process of education and licensing in order to become fully functional (7, 28). During infection, the main functions of NK cells are cytokine production and cytotoxic killing of infected host cells. These activities can be triggered by three distinct but complementary activation pathways: cytokine activation, antibody-dependent cell-mediated cytotoxicity (ADCC), and loss of inhibitory signaling due to downregulation or mismatching of major histocompatibility complex (MHC) class I (the missing-self hypothesis). NK cells can be activated *via* a plethora of host (target) cell surface receptors, including activating members of the killer cell immunoglobulin-like receptor (KIR) family that bind to MHC molecules [reviewed in Ref. (29)], killer lectin-like receptors (KLRs) such as NKG2D homodimers and CD94/NKG2A and CD94/NKG2C heterodimers that interact with HLA-E, and natural cytotoxicity receptors (NCRs) such as NKp30 and NKp46 which are believed to recognize pathogen-encoded ligands [reviewed in Ref. (30)]. The outcome of these interactions can be direct lysis of the target cell by the NK cell, which implies an important role for NK cells in killing infected or diseased cells. NK cells also constitutively express receptor subunits for IL-15, IL-18, and IL-12, as well as the low-affinity receptor for IL-2. The high-affinity IL-2 receptor α chain (CD25) is upregulated upon activation, allowing NK cells to become activated by cytokines as a result of local or systemic inflammation (8, 31).

Natural killer cells are also significant producers of inflammatory cytokines during early infection, prior to the priming, clonal expansion and activation of antigen-specific T cells (32–35); in particular, NK cells are an important source of interferon gamma (IFN-γ). *In vitro*, NK cells are capable of producing IFN-γ after activation by exogenous cytokines, in particular IL-12 and IL-18 (8, 31, 36, 37). However, during infection, various cytokines are produced at different times from diverse cellular sources, allowing NK cell responses to be finely controlled so that pathogen growth and dissemination is constrained while simultaneously limiting the pathology caused by uncontrolled inflammation (38–40). For example, during infection with two classic Th1-inducing pathogens, *Salmonella enterica* and *Plasmodium falciparum*, NK cells require activating signals from three distinct sources: a priming or pre-activation signal of either IL-2 or IL-15 from T cells or dendritic cells (DCs) respectively, IL-12 and IL-18 from macrophages, and direct contact with macrophages (41, 42). Activation *via* these pathways allows synthesis of IFN-γ by NK cells that induces downstream transcription of IFN-controlled genes, thereby leading to macrophage activation and killing of target cells or pathogens.

MALARIA SYMPTOMS AND SPREAD

Perhaps the most infamous family of protozoan parasites affecting humans is *Plasmodium,* the causative agent of malaria in a range of mammalian and non-mammalian hosts. The parasites are carried by numerous species of mosquito vectors of the *Anopheles* genus and are transmitted by female mosquitoes during hematophagy. During a single bite, between a dozen and several hundred motile and infectious malaria sporozoites are inoculated into the skin of the mammalian host from the salivary glands of the mosquito; they then disseminate to the liver *via* the blood stream to initiate the intravertebrate developmental

pathway (43–45). The developmental pathway of the *Plasmodium* parasite within its vertebrate host comprises multiple life stages exhibiting both constitutive and stage-specific genomic and proteomic expression (46). Of particular clinical relevance is the intra-erythrocytic stage during which merozoites enter erythrocytes and develop into trophozoites and large, multinucleated schizonts. These schizonts then lyse their host cells in order to release the next generation of merozoites into the circulation and repeat the hematic replication cycle (**Figure 1**).

In humans, this intra-erythrocytic stage of the parasite life cycle causes the symptoms of malaria infection. The lysis of erythrocytes can lead to severe anemia, and structural changes induced in the red blood cell membrane by the parasite can lead to vascular sequestration (i.e., adherence of parasitized erythrocytes to vascular endothelium, causing blockage of small blood vessels). Anemia and sequestration can in turn lead to systemic lactic acidosis due to reduced oxygen delivery to the tissues, and in the brain can lead to cerebral malaria, resulting in seizures, coma, and potentially death [reviewed in Ref. (47)]. Excessive inflammatory immune responses, characterized as high levels of IL-1, IL-6, IFN-γ, and TNFα, exacerbate these direct effects of parasitemia, leading to immunopathology (39, 48–51). However, an insufficient inflammatory response is conversely associated with increased parasitemia and poorer outcomes (39, 40), indicating that an optimal immunological response to malaria is a delicate balancing act. The nature of this paradox will be discussed in detail later in this review.

It is estimated that 3.2 billion people are currently at risk of developing malaria, and in 2015 alone there were an estimated 212 million cases of malaria leading to approximately 429,000 deaths worldwide; of these deaths, 70% were in children under 5 years old (52). The burden of malaria falls most heavily on Sub-Saharan Africa and South Asia; 80% of cases and 90% of deaths occurred in Africa, with the Democratic Republic of the Congo and Nigeria alone accounting for an estimated 35% of global malaria mortality. Current methods for controlling the spread of malaria include the use of long-lasting insecticide-treated bed nets, residual indoor spraying with insecticides, chemoprevention in vulnerable individuals, and combination drug treatment of infected individuals (52). While these tools have curbed the transmission of malaria in the last decades, it is widely accepted by both researchers and public health officials that sustainable malaria control or elimination would be facilitated by a highly effective malaria vaccine, the development of which requires a greater scientific understanding of the interaction between the human immune system and *Plasmodium* parasites.

There are currently six species of *Plasmodium* that are known to cause malaria in humans (53), in addition to three capable of causing similar symptoms in mice [reviewed in Ref. (54)] (**Table 1**). Of the six species capable of infecting humans, *P. falciparum* and *P. vivax* are responsible for most malaria deaths worldwide. *P. falciparum* is responsible for most malaria deaths in Africa (and therefore most global deaths due to Africa's disproportionate malarial burden) and is the most studied human strain of malaria, which is greatly assisted by its tolerance for *in vitro* laboratory culture. By contrast, *P. vivax* has so far been unamenable to long-term laboratory culture. This is a major impediment to further understanding of this species, contributing to its continued status as a major health burden across Asia and South America.

MALARIA IN MICE AND THE IMPORTANCE OF NK CELLS

A rapid and robust pro-inflammatory immune response is essential for control of malaria parasitemia. Much of the research underpinning this observation has been performed in mice infected with species of *Plasmodium* that naturally infect wild rodents, and has shown a crucial role for IFN-γ in parasite control and clearance (55–57) [reviewed in Ref. (58)] and a decreased likelihood of mice developing the severe symptoms of cerebral malaria (59). It is worth noting, however, that the effects of IFN-γ during *Plasmodium* infection vary depending on the amount produced, the time course of cytokine production, and the particular characteristics of the *Plasmodium* strain involved (**Table 2**). IFN-γ is a crucial mediator of antimalarial effector mechanisms and is thought to act primarily by activating macrophages to phagocytose merozoites and parasitized erythrocytes in both an opsonization-dependent (60) and opsonization-independent manner (61), and by inducing macrophages to produce parasiticidal free radicals such as nitric oxide (NO) and superoxides, which combine to form short-lived but highly damaging peroxynitrite capable of efficiently killing infected erythrocytes (62).

Given the capacity of NK cells to secrete large amounts of IFN-γ very quickly (63, 64), it is reasonable to assume that NK cells may contribute to control of malaria infections, and indeed several studies have demonstrated a crucial role for NK cells in the production of cytokines during murine malaria infections (55, 65). Murine splenic, hepatic and peripheral blood NK cells have been shown to significantly upregulate their production of pro-inflammatory cytokines such as IFN-γ and TNFα in response to both erythrocytic and hepatic stages of *Plasmodium yoelii* (55, 66), as well as blood-stage *Plasmodium chabaudi* (55, 65). It has also been demonstrated that experimental depletion of NK cells in mice infected with *P. yoelii* or *P. chabaudi* results in a decrease in IFN-γ production with a corresponding increase in parasitemia (57, 65), suggesting that NK cells contribute significantly to the early production of pro-inflammatory cytokines that is associated with an improved clinical outcome. Additionally, NK cells play an important role in reciprocal activation of DCs for cytokine production and CD4 T cell priming during murine malaria infections (67, 68), placing them at the interface of innate and adaptive immunity.

In pre-erythrocytic stages of infection, IFN-γ produced by proliferating hepatic NK cells inhibits the growth of hepatic schizonts (66, 69). Type I interferons (IFN-α and -β) produced by plasmacytoid DCs (pDCs) are thought to be important drivers of hepatic NK cell activation (69–71) as mice deficient in IFNAR (the IFN-α/β receptor) were unable to reduce the burden of liver-stage parasites in *P. yoelii* non-lethal infections (69, 72). However, some studies have suggested that NK T cells and/or non-conventional γδ T cells may play a greater role than NK cells in driving the early cytokine-driven inflammatory response (69, 73). This uncertainty may be partly due to variability in the time

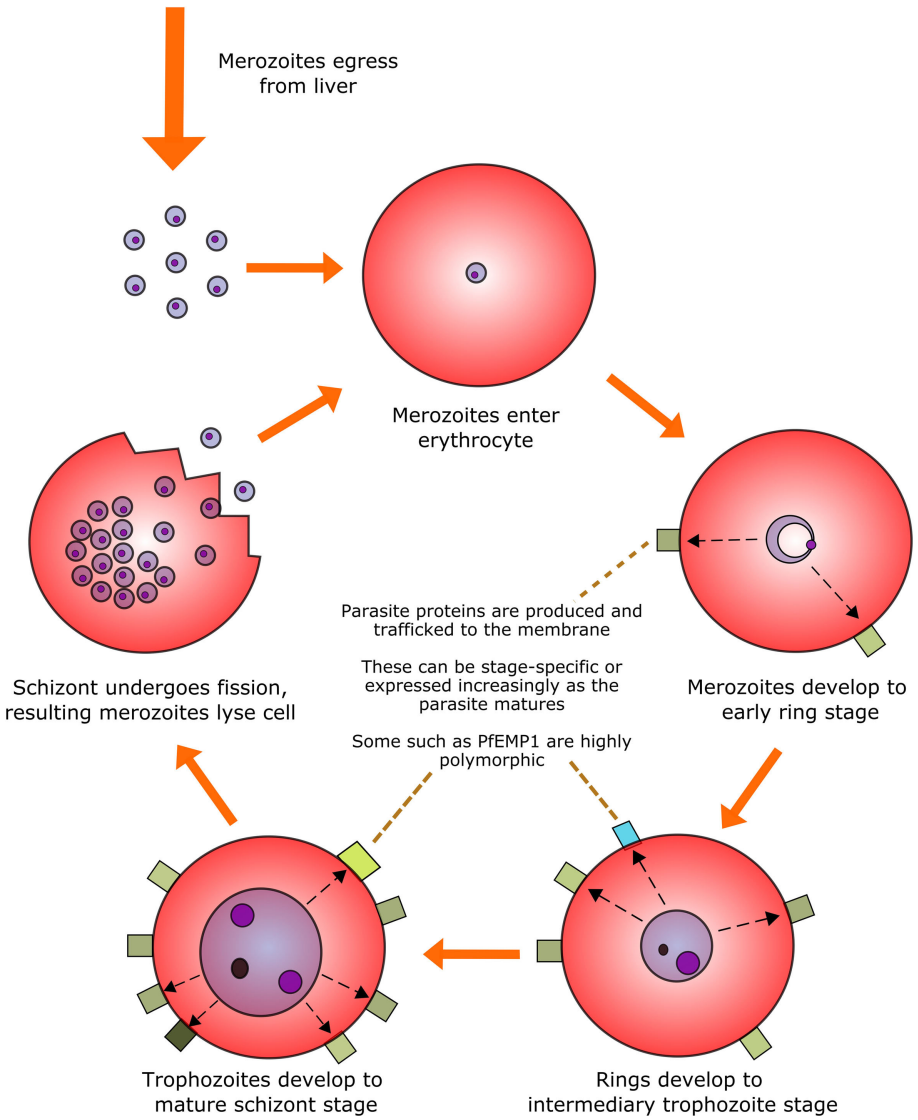


FIGURE 1 | **The intra-erythrocytic asexual replication cycle of malaria parasites**. After initial infection of the host and replication in the liver, merozoites are released into the blood stream where they penetrate healthy erythrocytes. The merozoite will then metabolize host hemoglobin to fuel its own development into the mature schizont stage (over the course of approximately 48 h in the case of *P. falciparum*) while restructuring the erythrocytic membrane to aid in nutrient transfer, rosetting and sequestration, and producing proteins, some of which are exported to the erythrocyte surface. The mature schizont will then undergo replicative fission, forming 8–32 merozoites which lyse the cell membrane and re-enter the bloodstream.

points of infection analyzed, as well as differences between strains of *Plasmodium* infection and murine models. In a dynamic system such as the immune system, with high levels of redundancy, it is likely that more than one cell type or mechanism of protection contributes to the outcome of infection.

The role of IL-15 in priming NK cells has not been extensively studied in mouse models of malaria, but work by Ing et al. indicates that DC-derived IL-15 enhances NK cell production of IFN-γ in combination with IL-12 (67, 74); this is consistent with *in vitro* studies of human NK cells showing that IL-15 is an important priming signal for NK cell activation (38), and that combinations of cytokines are required to drive IFN-γ production by NK cells (8, 31, 32, 75). IL-12 is a key driver of IFN-γ production (31, 33, 76), and loss of IL-12 results in decreased IFN-γ responses, higher parasitemia, and less effective malaria-specific antibody responses (77, 78). In tandem with this, IL-2 signaling is thought to promote full activation of NK cells (based on human *in vitro* studies by Horowitz et al. (63)). For many years, it was believed that CD25, the high-affinity IL-2 receptor subunit, was not expressed on murine NK cells (79) and reports on murine malaria infections tended to support this view (66), but more recent work has found a clear role for CD25 expression, primarily driven by IL-18 but further enhanced by IL-12, during murine malaria infections (64) and MCMV infection (80). Furthermore, there

TABLE 1 | List of *Plasmodium* species causing malaria infection in mice or humans.

Species	Common mammalian host(s)
Plasmodium falciparum	*Homo sapiens*
Plasmodium vivax	*H. sapiens*
Plasmodium malariae	*H. sapiens*
Plasmodium ovale curtisi	*H. sapiens*
Plasmodium ovale wallikeri	*H. sapiens*
Plasmodium knowlesi	*H. sapiens/Macaca fascicularis*
Plasmodium chabaudi	Various *Rodentia* species including murids
Plasmodium berghei	Various *Rodentia* species including murids
Plasmodium yoelii	Various *Rodentia* species including murids

TABLE 2 | List of experimental *Plasmodium* infection models in mice.

Species	Strain	Characteristics
Plasmodium yoelii	Py17X	Non-lethal, self-resolving. Also known as PyNL or PyXNL
	Py17XL	Lethal. Also known as PyL
	PyYM	Lethal
Plasmodium berghei	ANKA	Lethal. Causes experimental cerebral malaria in "susceptible" C57BL/6 mice; "resistant" BALB/c mice subsequently die of severe anemia
	NK65	Lethal. Does not cause cerebral malaria
Plasmodium chabaudi	AS	Non-lethal in C57BL/6 mice, lethal in "susceptible" A/J mice

appears to be a strong correlation between CD25 expression and IFN-γ production, which supports a role for T cell-derived IL-2 in maintaining or driving NK cell responses in both humans and mice (63, 64). The capacity to respond to T cell-mediated signals may also indicate a role for NK cells beyond early infection.

In murine models of virulent malaria infections, excessive IFN-γ or TNF production can lead to severe immunopathology (81–84) suggesting that, although NK cells are beneficial during early immune responses to malaria, they may contribute to the detrimental effects of excessive systemic inflammation (83). There is also evidence that NK cells may recruit T cells to the brain during murine *Plasmodium berghei* infections and therefore contribute to the development of experimental cerebral malaria (83, 85). In keeping with this, the anti-inflammatory cytokines IL-10 and TGF-β have been shown to regulate the pro-inflammatory immune response during malaria infection, counteracting the pathological effects of inflammatory cytokines and promoting healthy resolution of the immune response after initial stimulation (86–88). Neutralization of TGF-β is lethal in a normally non-lethal *P. chabaudi chabaudi* infection (88), and lack of IL-10 in IL-10$^{-/-}$ mice leads to increased IFN-γ, TNFα and IL-12 production and exacerbated pathology and mortality (86, 89). IL-10 and TGF-β appear to show some overlap in function and can individually down-regulate pro-inflammatory responses (87), although both are suggested to modulate macrophage activation (89, 90). The regulatory receptors CTLA-4 and PD-1 are also thought to be important regulators of inflammation and are frequently co-expressed on activated T cells during infection; blockade of either receptor has been shown to induce lethal cerebral malaria in normally resistant BALB/c mice (91). However, overexpression of TGF-β or IL-10 very early in infection inhibits the pro-inflammatory response and impedes parasite clearance (89). Similarly, blockade of CTLA-4 drives excessive inflammation and exacerbates pathology in mice infected with a lethal strain of *P. yoelii*, but mediates lower peak parasitemia and swifter parasite clearance in a non-lethal model (81). There is some evidence in other disease models, including from other protozoan infections, for NK cells as producers of IL-10 (92–95), drawing parallels with CD4 T cells that can produce both IFN-γ and IL-10. To date, IL-10-producing NK cells have not been reported in the context of malaria exposure or infection, but it is certainly possible that "regulatory" NK cells might be found to contribute to healthy resolution of the inflammation associated with malaria infections.

NK CELLS AND CYTOKINE RESPONSES TO MALARIA IN HUMANS

Evidence that NK cells contribute to the antimalarial immune response in experimental murine models has naturally provoked interest in establishing whether the same is true for human *Plasmodium* infections. As anticipated from experimental animal studies, numerous studies conducted on human populations have revealed positive associations between IFN-γ production and protection against malaria infection [reviewed in Ref. (96)]. IFN-γ production by PBMCs has been found to be associated with mild rather than severe malaria in children, and children with mild malaria who had detectable IFN-γ responses also demonstrated delayed incidence of reinfection within 1 year of initial infection (97). More recently, long-term protection against experimental malaria infection after vaccination with whole *P. falciparum* sporozoites while under protective antimalarial drug cover (98) has been associated with both IFN-γ and T cell IL-2 production by PBMCs restimulated *in vitro* with sporozoite antigen (99).

However, as previously demonstrated in mice, excessive pro-inflammatory cytokine production is also associated with onset of clinical disease and immunopathology in humans. An early study in African children by Riley et al. in 1991 demonstrated an association between IFN-γ production after *in vitro* stimulation with malaria antigens and an increased likelihood of developing fever and malaise during *in vivo* infection (49). A later study by Walther et al. found an association between the presence of IFN-γ in plasma in the first few days after experimental malaria infection and fever (39). Other studies have suggested that IFN-γ, TNFα, and IL-12 production by PBMCs are associated with lower parasite densities and higher hemoglobin concentrations, but also with increased incidence of febrile episodes in Ghanaian children (50, 51). High ratios of TNFα to IL-10 have also been linked to severe malaria in children from this region (48).

These studies indicate that excessive pro-inflammatory cytokine responses to human malaria infections correlate with more severe clinical symptoms but better parasite clearance. In concert with this evidence, several studies suggest that overproduction of regulatory cytokines has a negative effect

on parasite clearance. A 2005 study by Walther et al. revealed that excessive production of anti-inflammatory cytokines such as TGF-β and IL-10 early in infection is linked to reduced ability to control parasite growth (39), and a 2006 study by Prakash et al. indicated that regulatory cytokines were upregulated in patients with cerebral malaria (40). It is interesting to note, however, that this study also found an association between high levels of TNFα, but not IFN-γ, and the development of cerebral malaria (40). This perhaps suggests that classing cytokines as simply pro- or anti-inflammatory, and therefore "good" or "bad", may be too simplistic when investigating the antimalarial immune response. While clinical immunity to malaria in both mice and humans (defined here as clearance of parasites in the absence of overt clinical symptoms) does seem to require a precise balance between early pro-inflammatory responses needed to kill parasites and regulatory anti-inflammatory responses needed to prevent immune pathology, increasing evidence suggests that the equilibrium between the two is highly complex. For example, Walther et al. noted an association between a higher frequency of FOXP3$^-$ CD4$^+$ IFN-γ$^+$ IL-10$^+$ effector T cells in the peripheral blood of children with uncomplicated compared to severe clinical malaria (100), while Jagannathan et al. identified an increased risk of future episodes of malaria in individuals with this same population of FOXP3$^-$ CD4$^+$ IFN-γ$^+$ IL-10$^+$ T cells (101). Clearly, the immune response to malaria requires a far more complex investigation than simply stating which cytokines are produced and which are not, and the specific cellular sources of cytokines, the quantity produced, and the timing of their production relative to the time course of infection appear to be key determinants of the outcome of infection.

For a long time, it was assumed that classical αβ CD4 and CD8 T cells were the primary source of malaria-induced IFN-γ as a result of studies where lymphocyte proliferation and IFN-γ production were measured utilizing techniques that could not differentiate between T cells and NK cells (102–104). In more recent times, the advent of intracellular cytokine staining and consequent single cell analysis of cytokine production by flow cytometry has revealed considerable redundancy in the cellular sources of IFN-γ, with γδ T cells and NK cells also producing IFN-γ in response to malaria-infected erythrocytes (32, 105, 106). However, while many different lymphocyte populations are capable of producing IFN-γ, they vary in their relative contributions to the overall IFN-γ response at different stages of infection. The exact proportion of the cytokine response ascribed to NK cells or T cells appears to vary based on the time point examined [reviewed in Ref. (96)] and thus inconsistencies in the literature regarding the major sources of IFN-γ among PBMCs exposed to infected erythrocytes likely reflect differing experimental conditions and differences in the time points chosen for analysis. Very few studies have attempted to establish the full range of cellular sources of IFN-γ over the course of infection (82, 107) [reviewed in Ref. (108)]. In particular, few studies have assessed IFN-γ production in the first 18 h of exposure, and we have shown that assessing IFN-γ production from 24 h coculture onward risks missing important earlier contributions from NK cells (63, 106).

A function for NK cells as early responders in human malaria infection was first suggested in the early 1980s (109), and since that time an increasing number of studies have investigated the role of NK cells in the human antimalarial response. Most of these data are from *in vitro* studies culturing PBMCs or purified NK cells with *P. falciparum*-infected erythrocytes, although a few studies have investigated IFN-γ production and cytotoxic responses *ex vivo* among infected individuals (25, 110–112). *In vitro* studies of PBMCs from malaria-naive individuals have shown that *P. falciparum*-infected erythrocytes can induce NK cells to produce IFN-γ within 6 h of coculture (32), though this response appears to be somewhat heterogeneous between individuals (113). This heterogeneity may be partly explained by NK cell receptor repertoires (114), as evidence suggests that this response may require direct physical contact between NK cells and infected erythrocytes (51, 106). The role of NK cell receptors in determining the magnitude of the IFN-γ response is also supported by evidence from mouse models (83, 115, 116). Optimal NK cell activation also appears to require IL-12 and IL-18 produced by myeloid accessory cells (106), IL-2 from memory CD4$^+$ T cells (63), and physical contacts between NK cells and myeloid accessory cells (42, 117, 118), the molecular basis for which has yet to be fully identified. The requirement for IL-12 and IL-2 for optimal NK cell IFN-γ production may explain the association between production of these cytokines and improved clinical outcome in human *P. falciparum* infections (40).

There are many potential *trans* costimulatory signals that could be provided by myeloid accessory cells activated after exposure to infected erythrocytes [reviewed in Ref. (119)], but so far a role has only been demonstrated for interactions between the adhesion molecules ICAM-1 and LFA-1 (118). The role of DCs in human malaria is less clear, but they appear to contribute to NK cell activation by early cytokine production, possibly after activation by parasitized erythrocytes *via* the CD36 scavenger receptor (42, 120). pDCs are a major contributor of type I IFNs in humans during infection with other pathogens due to expression of TLR7 and TLR9 (121) and are hypothesized to play a similar role in malaria infection (120); for example, mouse models support a role for pDCs in NK cell activation during acute infection (67, 71, 72, 122). Similarly, our unpublished work has shown that *in vitro* NK cell responses to *P. falciparum*-infected red blood cells are enhanced by low levels of IL-15, consistent with data from mouse models (74); IL-15 is likely to be *trans*-presented by DCs [reviewed in Ref. (123)].

Recent evidence from mice for the development of "adaptive" or "memory-like" NK cell responses after infection, antigen sensitization, or exposure to inflammatory cytokines (15, 124) is now beginning to be supported by similar (though currently limited) data from human studies (10, 19). NK cells have been shown to contribute to increased IFN-γ responses to malaria antigens after vaccination (26, 98, 99, 125), although initial studies suggest this may be a proxy effect due to priming of antigen-specific CD4 T cells to secrete IL-2 rather than a reflection of intrinsic changes within the NK cell population itself (63). Notably, enhanced IFN-γ production by NK cells from individuals experimentally infected with malaria up to 20 weeks after initial infection appears to be dependent on the presence of both IL-2 and T cells (25).

NK CELL CYTOTOXICITY AGAINST MALARIA

In addition to the well-established role for NK cells in cytokine production in response to infected erythrocytes, there is limited but growing evidence to suggest that NK cells may also be capable of directly killing *Plasmodium*-infected cells through cytotoxic activity. Cytotoxic granzymes have been detected in the plasma of people with blood-stage malaria infections (111), suggesting that parasite-infected erythrocytes may be targets of NK cell or $CD8^+$ T cell cytotoxic activity *in vivo*. Peripheral blood NK cells from experimentally infected malaria-naive volunteers and naturally infected Cameroonian children have also been shown to release cytotoxic mediators when cultured *in vitro* with hepatocytes infected with liver-stage *Plasmodium* (111), with a similar result reported in Kenyan adults and children in response to infected erythrocytes (126). NK cell cytotoxicity has similarly been observed against hepatic-stage parasites in mice (66).

Natural killer cells have also repeatedly been observed forming stable conjugates with infected erythrocytes *in vitro* (32, 113, 117) (**Figure 2**). In 2005, Baratin and colleagues found that immortalized NK92 cells selectively bound to infected, but not uninfected, erythrocytes (117) (**Figure 2A**), while Artavanis-Tsakonas et al. (106) and Korbel et al. (113) observed conjugate formation between *P. falciparum*-infected erythrocytes and freshly isolated human NK cells from multiple individuals (**Figure 2B**). These conjugates can be observed by light microscopy and can be counted by flow cytometry as singlet events that stain for both NK cell markers and erythrocyte membrane markers (106); these results have been replicated by our group using both flow cytometry markers and a transgenic *P. falciparum* strain expressing green fluorescent protein (127).

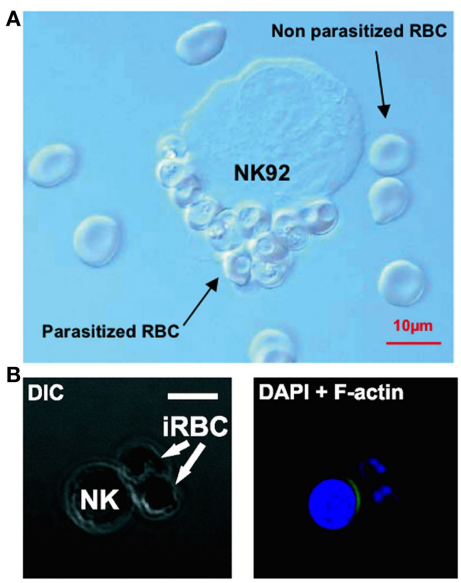


FIGURE 2 | Evidence of natural killer (NK) cells conjugating to malaria-infected erythrocytes. NK cells selectively form conjugates or "rosettes" with infected, but not uninfected, erythrocytes. Baratin et al. observed conjugate formation between the NK92 cell line and *P. falciparum*-infected erythrocytes by light microscopy **(A)**. [Figure from Baratin et al. (118). Copyright under Creative Commons license CC-BY.] Korbel et al. observed conjugate formation *via* confocal microscopy **(B)** where NK cells from some donors showed actin relocalization to the site of contact with *P. falciparum*-infected erythrocytes, possibly indicating formation of an immune synapse. [Figure used with permission from Korbel et al. (113). Copyright 2005. The American Association of Immunologists, Inc.]

Conjugates have been shown to form rapidly (within 30 min), although the proportion of NK cells forming conjugates varies considerably between donors (106, 113), suggesting that the receptors involved in recognition of infected erythrocytes are either polymorphic or are variably expressed on human NK cells. Additionally, in cells from some individuals there is evidence of NK cell cytoskeletal actin rearrangement at the point of contact with infected erythrocytes (113) (**Figure 2B**). Actin rearrangement at the immune synapse between an NK cell or $CD8^+$ T cell and target cells is a prelude to migration of cytotoxic granules toward a target cell (128); as such, these data suggest the formation of a functional cytotoxic synapse between NK cells and infected erythrocytes. More recently, in a humanized mouse model capable of sustaining a *P. falciparum* infection, Chen et al. showed that parasitemia was significantly reduced in the presence of NK cells, and NK cells were directly observed interacting with and killing *P. falciparum*-infected erythrocytes using video microscopy, providing the clearest evidence yet of NK cell cytotoxicity against malaria-infected cells (129).

While there is growing evidence for cytotoxic killing of parasitized cells by NK cells and for functional physical interactions between them, the NK cell receptors and ligands on infected erythrocytes that may mediate these interactions are unknown. Healthy erythrocytes are not known to express either classical or non-classical HLA class I molecules that might represent ligands for inhibitory NK cell receptors such as KIRs or NKG2A, nor are they known to express any known ligands for activating NK cell receptors such as the NCRs. Healthy erythrocytes are therefore generally assumed to go unnoticed by NK cells. However, malaria infection induces numerous perturbations of the erythrocyte membrane [reviewed in Ref. (130)], which may result in the presentation of as yet undiscovered ligands for NK cell receptors.

One parasite-derived molecule that has been widely implicated in interactions between infected erythrocytes and other host cells (and is thus a prime candidate for mediating NK cell interactions with infected erythrocytes) is *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1). *Pf*EMP1 can bind to the adhesion molecules ICAM-1, PECAM, and VCAM, the scavenger receptor CD36, and chondroitin sulfate A (CSA), a glycosaminoglycan modification of many cell surface proteins including those on NK cells (131–133) [reviewed in Ref. (134)]. Polymorphic variants of *Pf*EMP1 display different avidities for these various ligands [reviewed in Ref. (134)]. Baratin et al. (118) have shown that binding of *Pf*EMP1 to CSA mediates binding of infected erythrocytes to NK92 cells, but that this interaction is not required for subsequent activation of the NK cells. In 2007, Mavoungou et al. suggested that *Pf*EMP1 binds to NKp30, a member of the NCR family (135), but a subsequent study by

Chen et al. indicated that none of the NCRs (NKp30, NKp44, or NKp46), nor the unrelated activating receptor NKG2D, are required for conjugate formation with infected erythrocytes (129). CD36 expressed on DCs may mediate DC activation by infected erythrocytes (120), but CD36–*Pf*EMP1 interactions have not been shown to occur on NK cells.

Alternatively, a study by Böttger et al. proposed that the presence of membrane-bound heat shock protein 70 (a "self" stress ligand) on the surface of infected erythrocytes may be sufficient to trigger release of granzyme B from NK cells, leading to subsequent erythrocyte death (136); however, this has not been subsequently confirmed. Other studies have demonstrated that, when compared to live and intact infected erythrocytes, dead or lysed infected erythrocytes do not fully activate NK cells (32, 51), which may narrow down possible candidate ligands, although it is possible that this simply reflects a reduced capacity of dead parasite material to fully activate myeloid accessory cells or IL-2-producing $CD4^+$ T cells rather than evidence of a reduced capacity to bind NK cells directly.

HETEROGENEITY IN NK CELL RESPONSES TO MALARIA

In vitro, NK cell responses to infected erythrocytes differ greatly between individuals, although individuals' responses are consistent over time (106, 113, 114). This diversity likely arises from a number of sources including the strength of the cytokine and costimulatory signals provided to NK cells by myeloid accessory cells (42), differential NK cell maturation status dependent on age and infection with human cytomegalovirus (137) [reviewed in Ref. (138)], frequencies of malaria-reactive or cross-reactive IL-2-secreting $CD4^+$ T cells (25, 63, 98, 99), and genetically determined differences in the expression of NK cell activating and inhibitory receptors, which set the threshold for NK cell activation (7, 114) [reviewed in Ref. (139)]. Among these, there is evidence that genetic diversity genetic diversity in KIRs may contribute to the heterogeneity of the response to infected erythrocytes.

The KIR locus contains genes for both activating (short-tailed) and inhibitory (long-tailed) KIR, and heterogeneity in gene content combined with allelic polymorphism at individual loci and stochastic expression of individual receptors at the cellular level leads to extensive haplotypic diversity and highly diverse NK cell populations within an individual (140). There are two distinct families of KIR haplotypes, comprising combinations of KIRs commonly inherited together. The A haplotype encodes mainly inhibitory receptors with KIR2DS4 the only activating receptor, while the B haplotype encodes more balanced combinations of inhibitory and activating receptors (141) [reviewed in Ref. (142, 143)]. However, genes in the centromeric and telomeric regions of the two haplotypes can recombine during meiosis, leading to hybrid centromeric A/telomeric B haplotypes and *vice versa* (144, 145) [reviewed in Ref. (29)].

Heterozygous carriage of the AB KIR haplotypes appears to be associated with increased IFN-γ production *in vitro* in response to iRBC compared to either AA or BB homozygous individuals (114). Similarly, AB heterozygosity was suggested to be protective during clinical malaria infection, as individuals carrying c-AB2/t-AA (i.e., individuals with heterozygous A and B centromeric KIR genes in combination with telomeric A haplotype genes) were more likely to have asymptomatic malaria infections rather than uncomplicated or severe symptomatic malaria (146). As carriage of both A and B haplotypes is likely to increase the total number of different KIR that can be an expressed by an individual, heterozygosity may increase the proportion of NK cells that express a KIR capable of binding self-HLA and are therefore "licensed" [reviewed in Ref. (28, 147)] and, as such, may be more responsive to activation by pathogens. Conversely, in a Gambian population, Yindom et al. suggested that an AA KIR haplotype may be protective during malaria infection and that carriage of activating KIRs is associated with higher mortality (148); this may suggest that NK cell responses contribute to the over-exuberant inflammatory responses that are associated with severe disease, either because they express a particular activating KIR that recognizes a, as yet unknown, ligand on infected erythrocytes or because the balance of activating to inhibitory KIR expressed by B haplotype-bearing NK cells lowers the threshold for activation [reviewed in Ref. (149)]. To date, the largest genetic association study of KIR and malaria susceptibility, conducted in Thailand, reported that KIR2DL3 in association with its ligand HLA-C1 is associated with an increased risk of cerebral malaria compared to uncomplicated malaria and that this combination of KIR2DL3–HLA-C1 is significantly less common in malaria-endemic areas than might be expected; the authors proposed that this was evidence of natural selection (150). One hypothesis that might unify all of these findings is that protection is mediated by KIR2DL2, a B haplotype KIR, which binds HLA-C1 with higher affinity than KIR2DL3, an A haplotype KIR (151); thus, carriage of a single copy of the centromeric B haplotype may confer protection against severe malaria by preventing interactions between KIR2DL3 and HLA-C1 through preferential expression of KIR2DL2. However, larger studies of KIRs that take into account the genetic background of the population and the allelic diversity of both KIR and HLA class I molecules are needed to determine whether KIR receptors do in fact influence malaria disease progression. In this respect, it is disappointing that all of the recent genome-wide association studies of malaria susceptibility exclude KIR and/or HLA from their analyses (152, 153).

A major limitation of NK cell licensing as a possible explanation for the association between KIR AB heterozygosity and resistance to malaria is that licensing has only been shown to enhance NK cell cytotoxicity in situations where MHC class I expression is downregulated (i.e., missing self) [reviewed in Ref. (139)]. For activation *via* missing self, at least in terms of lack of MHC class I, to operate in malaria infections, the balance of NK cell receptor signaling would probably have to be altered by expression of a potent activating ligand. Moreover, it is not clear that licensed NK cells display a greater capacity for cytokine production (which is the more established role for NK cells in malaria infection) compared to unlicensed cells (154). Indeed, $CD56^{dim}$ NK cells (which express KIR) seem to produce lower levels of IFN-γ than $CD56^{bright}$ NK cells that lack KIR expression (106, 114), and in some viral infections unlicensed

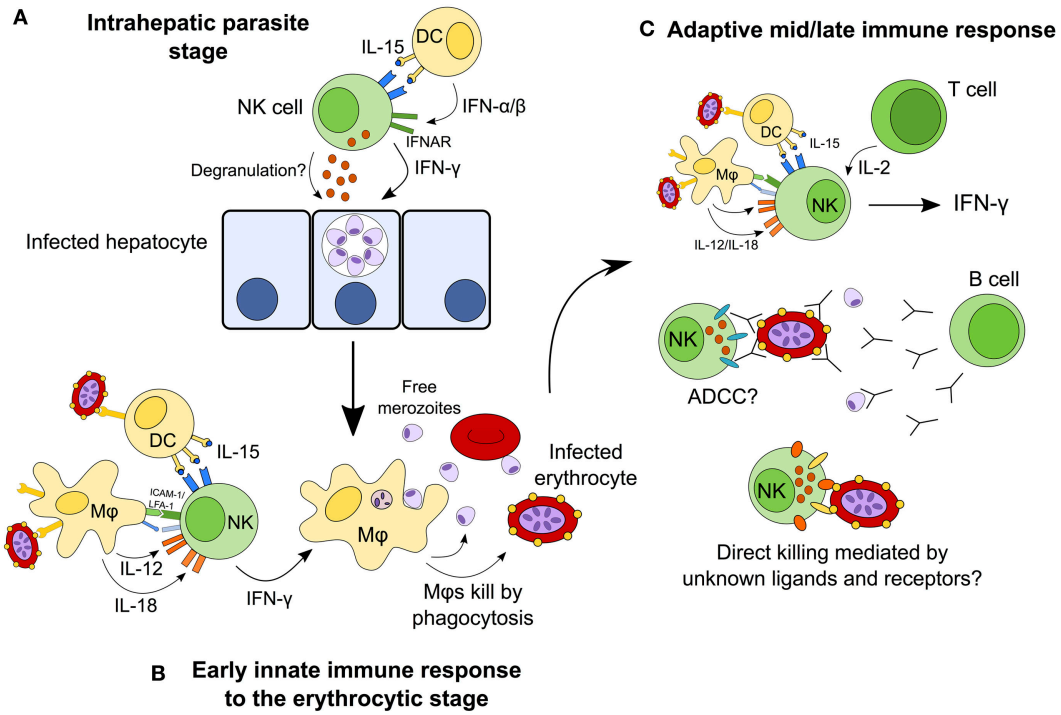


FIGURE 3 | Hypothesized model for the continuing role of natural killer (NK) cells during malaria infection. NK cells may destroy infected hepatocytes by perforin/granzyme-mediated cytotoxic killing or death receptor-induced apoptosis, or may kill the parasite within the hepatocyte *via* cytokine-mediated induction of toxic radicals **(A)**. During the early erythrocytic stage of infection, NK cells are activated by cytokines from macrophages and dendritic cells (DCs) and in turn release interferon gamma (IFN-γ) to activate macrophages that phagocytose infected erythrocytes **(B)**. Once the adaptive immune response has developed, T cells contribute interleukin (IL)-2 to enhance the ongoing NK cell response. In the presence of specific IgG antibodies, NK cells may now mediate parasite clearance and killing by antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells may also kill infected erythrocytes directly *via* formation of an immune synapse and release of cytolytic granules **(C)**.

cells are thought to be key producers of cytokines (155). Finally, in considering the potential role of KIR in controlling NK cell responses to malaria, more definitive evidence is required for direct, functional interactions between NK cells and infected erythrocytes, and it is also necessary to consider that genetic associations between malaria severity and KIR might be mediated by NK cell interactions with infected hepatocytes (which express MHC class I) during the pre-erythrocytic liver stage of infection.

CONCLUDING COMMENTS

Natural killer cells have traditionally been considered to contribute to the control of infection by producing IFN-γ and killing infected cells during the first hours and days of infection, before being superseded by the adaptive immune response. This narrow interpretation of NK cells as mediators of innate immunity has had to be re-evaluated in light of more recent studies implicating NK cells as effectors in the adaptive immune response (mediating antibody-dependent cellular cytotoxicity and responding to IL-2 from effector and effector memory $CD4^+$ T cells) (19, 24, 25) [reviewed in Ref. (9, 156)]. In the case of malaria, NK cells have been implicated in cytokine-mediated as well as cytotoxic activity against both erythrocyte and liver-stage parasites, and in both early and late stages of infection. Although the currently available data tend to support a primarily beneficial role for NK cells as an early source of a key cytokine (IFN-γ) and suggest that they might also contribute to controlling parasitemia by lysis of infected erythrocytes, these studies fall far short of convincingly demonstrating either a protective or deleterious role for NK cells in human malaria infection. **Figure 3** shows the different roles that NK cells may play throughout the course of malaria infection; future studies may confirm or refute these suggestions. An alternate hypothesis is that, in some people, NK cell cytokine production may contribute to immune pathology. If so, this may be a transient effect associated with particular stages of the development of antimalarial immunity.

Interferon gamma is produced by many lymphocyte populations at the various stages of malaria infections (including αβ $CD4^+$ T cells, γδ T cells, and NK T cells (112)), suggesting that the particular importance of NK cells as cytokine producing cells is during the very first few days of a blood-stage infection (63). The role of NK cells later in malaria infection, or upon secondary or subsequent infection, and in particular the importance of NK cell-mediated cytotoxicity, ADCC and NK

cell "memory," requires further investigation. As we learn more about how KIR and HLA genotypes influence NK cell function and licensing, and how NK cell phenotype and function change over the course of life, we may gain a better understanding of the role of NK cells throughout the course of malaria infections.

AUTHOR CONTRIBUTIONS

This review was written jointly by A-SW, SS, and EM.

FUNDING

This work was jointly funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat Agreement Grant number MR/P1000808/1. A-SW was supported by the Thorpe Legacy Studentship (LSHTM). SS is supported by the Biotechnology and Biological Sciences Research Council (grant number BB/J014567/1).

REFERENCES

1. Campbell KS, Hasegawa J. Natural killer cell biology: an update and future directions. *J Allergy Clin Immunol* (2013) 132(3):536–44. doi:10.1016/j.jaci.2013.07.006
2. Leibson PJ. Signal transduction during natural killer cell activation: inside the mind of a killer. *Immunity* (1997) 6(6):655–61. doi:10.1016/S1074-7613(00)80441-0
3. Lanier LL. NK cell recognition. *Annu Rev Immunol* (2005) 23:225–74. doi:10.1146/annurev.immunol.23.021704.115526
4. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol* (2008) 9(5):503–10. doi:10.1038/ni1582
5. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* (2001) 22(11):633–40. doi:10.1016/S1471-4906(01)02060-9
6. Hwang I, Scott JM, Kakarla T, Duriancik DM, Choi S, Cho C, et al. Activation mechanisms of natural killer cells during influenza virus infection. *PLoS One* (2012) 7(12):e51858. doi:10.1371/journal.pone.0051858
7. Fauriat C, Ivarsson MA, Ljunggren HG, Malmberg KJ, Michaelsson J. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* (2010) 115(6):1166–74. doi:10.1182/blood-2009-09-245746
8. Nielsen CM, Wolf AS, Goodier MR, Riley EM. Synergy between common gamma chain family cytokines and IL-18 potentiates innate and adaptive pathways of NK cell activation. *Front Immunol* (2016) 7:101. doi:10.3389/fimmu.2016.00101
9. Cerwenka A, Lanier LL. Natural killer cell memory in infection, inflammation and cancer. *Nat Rev Immunol* (2016) 16(2):112–23. doi:10.1038/nri.2015.9
10. Goodier MR, Rodriguez-Galan A, Lusa C, Nielsen CM, Darboe A, Moldoveanu AL, et al. Influenza vaccination generates cytokine-induced memory-like NK cells: impact of human cytomegalovirus infection. *J Immunol* (2016) 197(1):313–25. doi:10.4049/jimmunol.1502049
11. Reeves RK, Li H, Jost S, Blass E, Li H, Schafer JL, et al. Antigen-specific NK cell memory in rhesus macaques. *Nat Immunol* (2015) 16(9):927–32. doi:10.1038/ni.3227
12. Paust S, Gill HS, Wang BZ, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol* (2010) 11(12):1127–35. doi:10.1038/ni.1953
13. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* (2006) 7(5):507–16. doi:10.1038/ni1332
14. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* (2002) 296(5571):1323–6. doi:10.1126/science.1070884
15. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature* (2009) 457(7229):557–61. doi:10.1038/nature07665
16. Voigt V, Forbes CA, Tonkin JN, Degli-Esposti MA, Smith HR, Yokoyama WM, et al. Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells. *Proc Natl Acad Sci U S A* (2003) 100(23):13483–8. doi:10.1073/pnas.2233572100
17. Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM. Specific and nonspecific NK cell activation during virus infection. *Nat Immunol* (2001) 2(10):951–6. doi:10.1038/ni714
18. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* (1998) 391(6669):795–9. doi:10.1038/35869
19. Lee J, Zhang T, Hwang I, Kim A, Nitschke L, Kim M, et al. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. *Immunity* (2015) 42(3):431–42. doi:10.1016/j.immuni.2015.02.013
20. Schlums H, Cichocki F, Tesi B, Theorell J, Beziat V, Holmes TD, et al. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity* (2015) 42(3):443–56. doi:10.1016/j.immuni.2015.02.008
21. Lopez-Verges S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57(+)NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci U S A* (2011) 108(36):14725–32. doi:10.1073/pnas.1110900108
22. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* (2004) 104(12):3664–71. doi:10.1182/blood-2004-05-2058
23. Lopez-Botet M, Muntasell A, Vilches C. The CD94/NKG2C+ NK-cell subset on the edge of innate and adaptive immunity to human cytomegalovirus infection. *Semin Immunol* (2014) 26(2):145–51. doi:10.1016/j.smim.2014.03.002
24. Horowitz A, Behrens RH, Okell L, Fooks AR, Riley EM. NK cells as effectors of acquired immune responses: effector CD4+ T cell-dependent activation of NK cells following vaccination. *J Immunol* (2010) 185(5):2808–18. doi:10.4049/jimmunol.1000844
25. McCall MB, Roestenberg M, Ploemen I, Teirlinck A, Hopman J, de Mast Q, et al. Memory-like IFN-gamma response by NK cells following malaria infection reveals the crucial role of T cells in NK cell activation by *P. falciparum*. *Eur J Immunol* (2010) 40(12):3472–7. doi:10.1002/eji.201040587
26. Horowitz A, Hafalla JC, King E, Lusingu J, Dekker D, Leach A, et al. Antigen-specific IL-2 secretion correlates with NK cell responses after immunization of Tanzanian children with the RTS,S/AS01 malaria vaccine. *J Immunol* (2012) 188(10):5054–62. doi:10.4049/jimmunol.1102710
27. He XS, Draghi M, Mahmood K, Holmes TH, Kemble GW, Dekker CL, et al. T cell-dependent production of IFN-gamma by NK cells in response to influenza A virus. *J Clin Invest* (2004) 114(12):1812–9. doi:10.1172/JCI22797
28. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* (2005) 436(7051):709–13. doi:10.1038/nature03847
29. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* (2005) 5(3):201–14. doi:10.1038/nri1570
30. Kruse PH, Matta J, Ugolini S, Vivier E. Natural cytotoxicity receptors and their ligands. *Immunol Cell Biol* (2014) 92(3):221–9. doi:10.1038/icb.2013.98
31. Fehniger TA, Shah MH, Turner MJ, VanDeusen JB, Whitman SP, Cooper MA, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol* (1999) 162(8):4511–20.
32. Artavanis-Tsakonas K, Riley EM. Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. *J Immunol* (2002) 169(6):2956–63. doi:10.4049/jimmunol.169.6.2956

33. Aste-Amezaga M, D'Andrea A, Kubin M, Trinchieri G. Cooperation of natural killer cell stimulatory factor/interleukin-12 with other stimuli in the induction of cytokines and cytotoxic cell-associated molecules in human T and NK cells. *Cell Immunol* (1994) 156(2):480–92. doi:10.1006/cimm.1994.1192
34. Lieberman LA, Hunter CA. Regulatory pathways involved in the infection-induced production of IFN-gamma by NK cells. *Microbes Infect* (2002) 4(15):1531–8. doi:10.1016/S1286-4579(02)00036-9
35. Neves PC, Santos JR, Tubarao LN, Bonaldo MC, Galler R. Early IFN-gamma production after YF 17D vaccine virus immunization in mice and its association with adaptive immune responses. *PLoS One* (2013) 8(12):e81953. doi:10.1371/journal.pone.0081953
36. Haeberlein S, Sebald H, Bogdan C, Schleicher U. IL-18, but not IL-15, contributes to the IL-12-dependent induction of NK-cell effector functions by *Leishmania infantum* in vivo. *Eur J Immunol* (2010) 40(6):1708–17. doi:10.1002/eji.200939988
37. Lauwerys BR, Garot N, Renauld JC, Houssiau FA. Cytokine production and killer activity of NK/T-NK cells derived with IL-2, IL-15, or the combination of IL-12 and IL-18. *J Immunol* (2000) 165(4):1847–53. doi:10.4049/jimmunol.165.4.1847
38. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* (2007) 26(4):503–17. doi:10.1016/j.immuni.2007.03.006
39. Walther M, Tongren JE, Andrews L, Korbel D, King E, Fletcher H, et al. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* (2005) 23(3):287–96. doi:10.1016/j.immuni.2005.08.006
40. Prakash D, Fesel C, Jain R, Cazenave PA, Mishra GC, Pied S. Clusters of cytokines determine malaria severity in *Plasmodium falciparum*-infected patients from endemic areas of Central India. *J Infect Dis* (2006) 194(2):198–207. doi:10.1086/504720
41. Lapaque N, Walzer T, Meresse S, Vivier E, Trowsdale J. Interactions between human NK cells and macrophages in response to *Salmonella* infection. *J Immunol* (2009) 182(7):4339–48. doi:10.4049/jimmunol.0803329
42. Newman KC, Korbel DS, Hafalla JC, Riley EM. Cross-talk with myeloid accessory cells regulates human natural killer cell interferon-gamma responses to malaria. *PLoS Pathog* (2006) 2(12):e118. doi:10.1371/journal.ppat.0020118
43. Rosenberg R, Wirtz RA, Schneider I, Burge R. An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Trans R Soc Trop Med Hyg* (1990) 84(2):209–12. doi:10.1016/0035-9203(90)90258-G
44. Vaughan JA, Scheller LF, Wirtz RA, Azad AF. Infectivity of *Plasmodium berghei* sporozoites delivered by intravenous inoculation versus mosquito bite: implications for sporozoite vaccine trials. *Infect Immun* (1999) 67(8):4285–9.
45. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, et al. Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat Med* (2006) 12(2):220–4. doi:10.1038/nm1350
46. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* (2002) 419(6906):520–6. doi:10.1038/nature01107
47. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. *Nature* (2002) 415(6872):673–9. doi:10.1038/415673a
48. Boeuf PS, Loizon S, Awandare GA, Tetteh JK, Addae MM, Adjei GO, et al. Insights into deregulated TNF and IL-10 production in malaria: implications for understanding severe malarial anaemia. *Malar J* (2012) 11:253. doi:10.1186/1475-2875-11-253
49. Riley EM, Jakobsen PH, Allen SJ, Wheeler JG, Bennett S, Jepsen S, et al. Immune response to soluble exoantigens of *Plasmodium falciparum* may contribute to both pathogenesis and protection in clinical malaria: evidence from a longitudinal, prospective study of semi-immune African children. *Eur J Immunol* (1991) 21(4):1019–25. doi:10.1002/eji.1830210424
50. Rhee MS, Akanmori BD, Waterfall M, Riley EM. Changes in cytokine production associated with acquired immunity to *Plasmodium falciparum* malaria. *Clin Exp Immunol* (2001) 126(3):503–10. doi:10.1046/j.1365-2249.2001.01681.x
51. Dodoo D, Omer FM, Todd J, Akanmori BD, Koram KA, Riley EM. Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *Plasmodium falciparum* malaria. *J Infect Dis* (2002) 185(7):971–9. doi:10.1086/339408
52. World Health Organization, editor. *World Malaria Report 2016*. Geneva: World Health Organization (2016).
53. Calderaro A, Piccolo G, Gorrini C, Rossi S, Montecchini S, Dell'Anna ML, et al. Accurate identification of the six human *Plasmodium* spp. causing imported malaria, including *Plasmodium ovale* wallikeri and *Plasmodium knowlesi*. *Malar J* (2013) 12:321. doi:10.1186/1475-2875-12-321
54. Zuzarte-Luis V, Mota MM, Vigario AM. Malaria infections: what and how can mice teach us. *J Immunol Methods* (2014) 410:113–22. doi:10.1016/j.jim.2014.05.001
55. De Souza JB, Williamson KH, Otani T, Payfair JH. Early gamma interferon responses in lethal and nonlethal murine blood-stage malaria. *Infect Immun* (1997) 65(5):1593–8.
56. van der Heyde HC, Pepper B, Batchelder J, Cigel F, Weidanz WP. The time course of selected malarial infections in cytokine-deficient mice. *Exp Parasitol* (1997) 85(2):206–13. doi:10.1006/expr.1996.4132
57. Choudhury HR, Sheikh NA, Bancroft GJ, Katz DR, De Souza JB. Early nonspecific immune responses and immunity to blood-stage nonlethal *Plasmodium yoelii* malaria. *Infect Immun* (2000) 68(11):6127–32. doi:10.1128/IAI.68.11.6127-6132.2000
58. Stevenson MM, Riley EM. Innate immunity to malaria. *Nat Rev Immunol* (2004) 4(3):169–80. doi:10.1038/nri1311
59. Mitchell AJ, Hansen AM, Hee L, Ball HJ, Potter SM, Walker JC, et al. Early cytokine production is associated with protection from murine cerebral malaria. *Infect Immun* (2005) 73(9):5645–53. doi:10.1128/IAI.73.9.5645-5653.2005
60. Mota MM, Brown KN, Holder AA, Jarra W. Acute *Plasmodium chabaudi* chabaudi malaria infection induces antibodies which bind to the surfaces of parasitized erythrocytes and promote their phagocytosis by macrophages in vitro. *Infect Immun* (1998) 66(9):4080–6.
61. Su Z, Fortin A, Gros P, Stevenson MM. Opsonin-independent phagocytosis: an effector mechanism against acute blood-stage *Plasmodium chabaudi* AS infection. *J Infect Dis* (2002) 186(9):1321–9. doi:10.1086/344576
62. Fritsche G, Larcher C, Schennach H, Weiss G. Regulatory interactions between iron and nitric oxide metabolism for immune defense against *Plasmodium falciparum* infection. *J Infect Dis* (2001) 183(9):1388–94. doi:10.1086/319860
63. Horowitz A, Newman KC, Evans JH, Korbel DS, Davis DM, Riley EM. Cross-talk between T cells and NK cells generates rapid effector responses to *Plasmodium falciparum*-infected erythrocytes. *J Immunol* (2010) 184(11):6043–52. doi:10.4049/jimmunol.1000106
64. Stegmann KA, De Souza JB, Riley EM. IL-18-induced expression of high-affinity IL-2R on murine NK cells is essential for NK-cell IFN-gamma production during murine *Plasmodium yoelii* infection. *Eur J Immunol* (2015) 45(12):3431–40. doi:10.1002/eji.201546018
65. Mohan K, Moulin P, Stevenson MM. Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage *Plasmodium chabaudi* AS infection. *J Immunol* (1997) 159(10):4990–8.
66. Roland J, Soulard V, Sellier C, Drapier AM, Di Santo JP, Cazenave PA, et al. NK cell responses to *Plasmodium* infection and control of intrahepatic parasite development. *J Immunol* (2006) 177(2):1229–39. doi:10.4049/jimmunol.177.2.1229
67. Ing R, Stevenson MM. Dendritic cell and NK cell reciprocal cross talk promotes gamma interferon-dependent immunity to blood-stage *Plasmodium chabaudi* AS infection in mice. *Infect Immun* (2009) 77(2):770–82. doi:10.1128/IAI.00994-08
68. Ryg-Cornejo V, Nie CQ, Bernard NJ, Lundie RJ, Evans KJ, Crabb BS, et al. NK cells and conventional dendritic cells engage in reciprocal activation for the induction of inflammatory responses during *Plasmodium berghei* ANKA infection. *Immunobiology* (2013) 218(2):263–71. doi:10.1016/j.imbio.2012.05.018
69. Miller JL, Sack BK, Baldwin M, Vaughan AM, Kappe SH. Interferon-mediated innate immune responses against malaria parasite liver stages. *Cell Rep* (2014) 7(2):436–47. doi:10.1016/j.celrep.2014.03.018
70. Nguyen KB, Salazar-Mather TP, Dalod MY, Van Deusen JB, Wei XQ, Liew FY, et al. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol* (2002) 169(8):4279–87. doi:10.4049/jimmunol.169.8.4279

71. Yu X, Cai B, Wang M, Tan P, Ding X, Wu J, et al. Cross-regulation of two type I interferon signaling pathways in plasmacytoid dendritic cells controls anti-malaria immunity and host mortality. *Immunity* (2016) 45(5):1093–107. doi:10.1016/j.immuni.2016.10.001
72. Spaulding E, Fooksman D, Moore JM, Saidi A, Feintuch CM, Reizis B, et al. STING-licensed macrophages prime type I IFN production by plasmacytoid dendritic cells in the bone marrow during severe *Plasmodium yoelii* malaria. *PLoS Pathog* (2016) 12(10):e1005975. doi:10.1371/journal.ppat.1005975
73. Weidanz WP, LaFleur G, Brown A, Burns JM Jr, Gramaglia I, van der Heyde HC. Gammadelta T cells but not NK cells are essential for cell-mediated immunity against *Plasmodium chabaudi* malaria. *Infect Immun* (2010) 78(10):4331–40. doi:10.1128/IAI.00539-10
74. Ing R, Gros P, Stevenson MM. Interleukin-15 enhances innate and adaptive immune responses to blood-stage malaria infection in mice. *Infect Immun* (2005) 73(5):3172–7. doi:10.1128/IAI.73.5.3172-3177.2005
75. Leong JW, Chase JM, Romee R, Schneider SE, Sullivan RP, Cooper MA, et al. Preactivation with IL-12, IL-15, and IL-18 induces CD25 and a functional high-affinity IL-2 receptor on human cytokine-induced memory-like natural killer cells. *Biol Blood Marrow Transplant* (2014) 20(4):463–73. doi:10.1016/j.bbmt.2014.01.006
76. Gramzinski RA, Doolan DL, Sedegah M, Davis HL, Krieg AM, Hoffman SL. Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. *Infect Immun* (2001) 69(3):1643–9. doi:10.1128/IAI.69.3.1643-1649.2001
77. Stevenson MM, Tam MF, Wolf SF, Sher A. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. *J Immunol* (1995) 155(5):2545–56.
78. Su Z, Stevenson MM. IL-12 is required for antibody-mediated protective immunity against blood-stage *Plasmodium chabaudi* AS malaria infection in mice. *J Immunol* (2002) 168(3):1348–55. doi:10.4049/jimmunol.168.3.1348
79. Biron CA, Young HA, Kasaian MT. Interleukin 2-induced proliferation of murine natural killer cells in vivo. *J Exp Med* (1990) 171(1):173–88. doi:10.1084/jem.171.1.173
80. Lee SH, Fragoso MF, Biron CA. Cutting edge: a novel mechanism bridging innate and adaptive immunity: IL-12 induction of CD25 to form high-affinity IL-2 receptors on NK cells. *J Immunol* (2012) 189(6):2712–6. doi:10.4049/jimmunol.1201528
81. Lepenies B, Gaworski I, Tartz S, Langhorne J, Fleischer B, Jacobs T. CTLA-4 blockade differentially influences the outcome of non-lethal and lethal *Plasmodium yoelii* infections. *Microbes Infect* (2007) 9(6):687–94. doi:10.1016/j.micinf.2007.02.013
82. Villegas-Mendez A, Greig R, Shaw TN, de Souza JB, Gwyer Findlay E, Stumhofer JS, et al. IFN-gamma-producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T cell accumulation within the brain. *J Immunol* (2012) 189(2):968–79. doi:10.4049/jimmunol.1200688
83. Hansen DS, Ryg-Cornejo V, Ioannidis LJ, Chiu CY, Ly A, Nie CQ, et al. The contribution of natural killer complex loci to the development of experimental cerebral malaria. *PLoS One* (2014) 9(4):e93268. doi:10.1371/journal.pone.0093268
84. Grau GE, Heremans H, Piguet PF, Pointaire P, Lambert PH, Billiau A, et al. Monoclonal antibody against interferon gamma can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. *Proc Natl Acad Sci U S A* (1989) 86(14):5572–4. doi:10.1073/pnas.86.14.5572
85. Hansen DS, Bernard NJ, Nie CQ, Schofield L. NK cells stimulate recruitment of CXCR3+ T cells to the brain during *Plasmodium berghei*-mediated cerebral malaria. *J Immunol* (2007) 178(9):5779–88. doi:10.4049/jimmunol.178.9.5779
86. Li C, Corraliza I, Langhorne J. A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi* chabaudi infection in mice. *Infect Immun* (1999) 67(9):4435–42.
87. Li C, Sanni LA, Omer F, Riley E, Langhorne J. Pathology of *Plasmodium chabaudi* chabaudi infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor beta antibodies. *Infect Immun* (2003) 71(9):4850–6. doi:10.1128/IAI.71.9.4850-4856.2003
88. Omer FM, Riley EM. Transforming growth factor beta production is inversely correlated with severity of murine malaria infection. *J Exp Med* (1998) 188(1):39–48. doi:10.1084/jem.188.1.39
89. Couper KN, Blount DG, Wilson MS, Hafalla JC, Belkaid Y, Kamanaka M, et al. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog* (2008) 4(2):e1000004. doi:10.1371/journal.ppat.1000004
90. Bogdan C, Paik J, Vodovotz Y, Nathan C. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *J Biol Chem* (1992) 267(32):23301–8.
91. Hafalla JC, Claser C, Couper KN, Grau GE, Renia L, de Souza JB, et al. The CTLA-4 and PD-1/PD-L1 inhibitory pathways independently regulate host resistance to *Plasmodium*-induced acute immune pathology. *PLoS Pathog* (2012) 8(2):e1002504. doi:10.1371/journal.ppat.1002504
92. Tarrio ML, Lee SH, Fragoso MF, Sun HW, Kanno Y, O'Shea JJ, et al. Proliferation conditions promote intrinsic changes in NK cells for an IL-10 response. *J Immunol* (2014) 193(1):354–63. doi:10.4049/jimmunol.1302999
93. Perona-Wright G, Mohrs K, Szaba FM, Kummer LW, Madan R, Karp CL, et al. Systemic but not local infections elicit immunosuppressive IL-10 production by natural killer cells. *Cell Host Microbe* (2009) 6(6):503–12. doi:10.1016/j.chom.2009.11.003
94. Maroof A, Beattie L, Zubairi S, Svensson M, Stager S, Kaye PM. Posttranscriptional regulation of Il10 gene expression allows natural killer cells to express immunoregulatory function. *Immunity* (2008) 29(2):295–305. doi:10.1016/j.immuni.2008.06.012
95. Lee SH, Kim KS, Fodil-Cornu N, Vidal SM, Biron CA. Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. *J Exp Med* (2009) 206(10):2235–51. doi:10.1084/jem.20082387
96. McCall MB, Sauerwein RW. Interferon-gamma – central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. *J Leukoc Biol* (2010) 88(6):1131–43. doi:10.1189/jlb.0310137
97. Luty AJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, et al. Interferon-gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *J Infect Dis* (1999) 179(4):980–8. doi:10.1086/314689
98. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* (2009) 361(5):468–77. doi:10.1056/NEJMoa0805832
99. Roestenberg M, Teirlinck AC, McCall MB, Teelen K, Makamdop KN, Wiersma J, et al. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. *Lancet* (2011) 377(9779):1770–6. doi:10.1016/S0140-6736(11)60360-7
100. Walther M, Jeffries D, Finney OC, Njie M, Ebonyi A, Deininger S, et al. Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe *Plasmodium falciparum* malaria. *PLoS Pathog* (2009) 5(4):e1000364. doi:10.1371/journal.ppat.1000364
101. Jagannathan P, Eccles-James I, Bowen K, Nankya F, Auma A, Wamala S, et al. IFNgamma/IL-10 co-producing cells dominate the CD4 response to malaria in highly exposed children. *PLoS Pathog* (2014) 10(1):e1003864. doi:10.1371/journal.ppat.1003864
102. Ballet JJ, Druilhe P, Querleux MA, Schmitt C, Agrapart M. Parasite-derived mitogenic activity for human T cells in *Plasmodium falciparum* continuous cultures. *Infect Immun* (1981) 33(3):758–62.
103. Riley EM, Jepsen S, Andersson G, Otoo LN, Greenwood BM. Cell-mediated immune responses to *Plasmodium falciparum* antigens in adult Gambians. *Clin Exp Immunol* (1988) 71(3):377–82.
104. Dick S, Waterfall M, Currie J, Maddy A, Riley E. Naive human alpha beta T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of interferon-gamma. *Immunology* (1996) 88(3):412–20. doi:10.1046/j.1365-2567.1996.d01-661.x
105. Waterfall M, Black A, Riley E. Gammadelta+ T cells preferentially respond to live rather than killed malaria parasites. *Infect Immun* (1998) 66(5):2393–8.
106. Artavanis-Tsakonas K, Eleme K, McQueen KL, Cheng NW, Parham P, Davis DM, et al. Activation of a subset of human NK cells upon contact with *Plasmodium falciparum*-infected erythrocytes. *J Immunol* (2003) 171(10):5396–405. doi:10.4049/jimmunol.171.10.5396

107. Rampling T, Ewer KJ, Bowyer G, Bliss CM, Edwards NJ, Wright D, et al. Safety and high level efficacy of the combination malaria vaccine regimen of RTS,S/AS01B with chimpanzee adenovirus 63 and modified vaccinia Ankara vectored vaccines expressing ME-TRAP. *J Infect Dis* (2016) 214(5):772–81. doi:10.1093/infdis/jiw244
108. King T, Lamb T. Interferon-gamma: the Jekyll and hyde of malaria. *PLoS Pathog* (2015) 11(10):e1005118. doi:10.1371/journal.ppat.1005118
109. Ojo-Amaize EA, Salimonu LS, Williams AI, Akinwolere OA, Shabo R, Alm GV, et al. Positive correlation between degree of parasitemia, interferon titers, and natural killer cell activity in *Plasmodium falciparum*-infected children. *J Immunol* (1981) 127(6):2296–300.
110. Agudelo O, Bueno J, Villa A, Maestre A. High IFN-gamma and TNF production by peripheral NK cells of Colombian patients with different clinical presentation of *Plasmodium falciparum*. *Malar J* (2012) 11:38. doi:10.1186/1475-2875-11-38
111. Hermsen CC, Konijnenberg Y, Mulder L, Loe C, van Deuren M, van der Meer JW, et al. Circulating concentrations of soluble granzyme A and B increase during natural and experimental *Plasmodium falciparum* infections. *Clin Exp Immunol* (2003) 132(3):467–72. doi:10.1046/j.1365-2249.2003.02160.x
112. Teirlinck AC, McCall MB, Roestenberg M, Scholzen A, Woestenenk R, de Mast Q, et al. Longevity and composition of cellular immune responses following experimental *Plasmodium falciparum* malaria infection in humans. *PLoS Pathog* (2011) 7(12):e1002389. doi:10.1371/journal.ppat.1002389
113. Korbel DS, Newman KC, Almeida CR, Davis DM, Riley EM. Heterogeneous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. *J Immunol* (2005) 175(11):7466–73. doi:10.4049/jimmunol.175.11.7466
114. Korbel DS, Norman PJ, Newman KC, Horowitz A, Gendzekhadze K, Parham P, et al. Killer Ig-like receptor (KIR) genotype predicts the capacity of human KIR-positive CD56dim NK cells to respond to pathogen-associated signals. *J Immunol* (2009) 182(10):6426–34. doi:10.4049/jimmunol.0804224
115. Filtjens J, Foquet L, Taveirne S, Van Ammel E, Vanhees M, Van Acker A, et al. Contribution of the Ly49E natural killer receptor in the immune response to *Plasmodium berghei* infection and control of hepatic parasite development. *PLoS One* (2014) 9(1):e87463. doi:10.1371/journal.pone.0087463
116. Hansen DS, Evans KJ, D'Ombrain MC, Bernard NJ, Sexton AC, Buckingham L, et al. The natural killer complex regulates severe malarial pathogenesis and influences acquired immune responses to *Plasmodium berghei* ANKA. *Infect Immun* (2005) 73(4):2288–97. doi:10.1128/IAI.73.4.2288-2297.2005
117. Baratin M, Roetynck S, Lepolard C, Falk C, Sawadogo S, Uematsu S, et al. Natural killer cell and macrophage cooperation in MyD88-dependent innate responses to *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* (2005) 102(41):14747–52. doi:10.1073/pnas.0507355102
118. Baratin M, Roetynck S, Pouvelle B, Lemmers C, Viebig NK, Johansson S, et al. Dissection of the role of PfEMP1 and ICAM-1 in the sensing of *Plasmodium falciparum*-infected erythrocytes by natural killer cells. *PLoS One* (2007) 2(2):e228. doi:10.1371/journal.pone.0000228
119. Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat Rev Immunol* (2007) 7(4):279–91. doi:10.1038/nri2057
120. Gowda NM, Wu X, Kumar S, Febbraio M, Gowda DC. CD36 contributes to malaria parasite-induced pro-inflammatory cytokine production and NK and T cell activation by dendritic cells. *PLoS One* (2013) 8(10):e77604. doi:10.1371/journal.pone.0077604
121. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, et al. Sex differences in the toll-like receptor-mediated response of plasmacytoid dendritic cells to HIV-1. *Nat Med* (2009) 15(8):955–9. doi:10.1038/nm.2004
122. Seixas E, Cross C, Quin S, Langhorne J. Direct activation of dendritic cells by the malaria parasite, *Plasmodium chabaudi* chabaudi. *Eur J Immunol* (2001) 31(10):2970–8. doi:10.1002/1521-4141(2001010)31:10<60;2970::AID-IMMU2970>62;3.0.CO;2-S
123. Castillo EF, Schluns KS. Regulating the immune system via IL-15 transpresentation. *Cytokine* (2012) 59(3):479–90. doi:10.1016/j.cyto.2012.06.017
124. Sun JC, Madera S, Bezman NA, Beilke JN, Kaplan MH, Lanier LL. Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J Exp Med* (2012) 209(5):947–54. doi:10.1084/jem.20111760
125. Bejon P, Keating S, Mwacharo J, Kai OK, Dunachie S, Walther M, et al. Early gamma interferon and interleukin-2 responses to vaccination predict the late resting memory in malaria-naive and malaria-exposed individuals. *Infect Immun* (2006) 74(11):6331–8. doi:10.1128/IAI.00774-06
126. Orago AS, Facer CA. Cytotoxicity of human natural killer (NK) cell subsets for *Plasmodium falciparum* erythrocytic schizonts: stimulation by cytokines and inhibition by neomycin. *Clin Exp Immunol* (1991) 86(1):22–9. doi:10.1111/j.1365-2249.1991.tb05768.x
127. Talman AM, Blagborough AM, Sinden RE. A *Plasmodium falciparum* strain expressing GFP throughout the parasite's life-cycle. *PLoS One* (2010) 5(2):e9156. doi:10.1371/journal.pone.0009156
128. Rak GD, Mace EM, Banerjee PP, Svitkina T, Orange JS. Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol* (2011) 9(9):e1001151. doi:10.1371/journal.pbio.1001151
129. Chen Q, Amaladoss A, Ye W, Liu M, Dummler S, Kong F, et al. Human natural killer cells control *Plasmodium falciparum* infection by eliminating infected red blood cells. *Proc Natl Acad Sci U S A* (2014) 111(4):1479–84. doi:10.1073/pnas.1323318111
130. Maier AG, Cooke BM, Cowman AF, Tilley L. Malaria parasite proteins that remodel the host erythrocyte. *Nat Rev Microbiol* (2009) 7(5):341–54. doi:10.1038/nrmicro2110
131. Smith JD, Craig AG, Kriek N, Hudson-Taylor D, Kyes S, Fagan T, et al. Identification of a *Plasmodium falciparum* intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. *Proc Natl Acad Sci U S A* (2000) 97(4):1766–71. doi:10.1073/pnas.040545897
132. Baruch DI, Ma XC, Singh HB, Bi X, Pasloske BL, Howard RJ. Identification of a region of PfEMP1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36: conserved function with variant sequence. *Blood* (1997) 90(9):3766–75.
133. Fried M, Duffy PE. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* (1996) 272(5267):1502–4. doi:10.1126/science.272.5267.1502
134. Smith JD. The role of PfEMP1 adhesion domain classification in *Plasmodium falciparum* pathogenesis research. *Mol Biochem Parasitol* (2014) 195(2):82–7. doi:10.1016/j.molbiopara.2014.07.006
135. Mavoungou E, Held J, Mewono L, Kremsner PG. A Duffy binding-like domain is involved in the NKp30-mediated recognition of *Plasmodium falciparum*-parasitized erythrocytes by natural killer cells. *J Infect Dis* (2007) 195(10):1521–31. doi:10.1086/515579
136. Böttger E, Multhoff G, Kun JF, Esen M. *Plasmodium falciparum*-infected erythrocytes induce granzyme B by NK cells through expression of host-Hsp70. *PLoS One* (2012) 7(3):e33774. doi:10.1371/journal.pone.0033774
137. Nielsen CM, White MJ, Bottomley C, Lusa C, Rodriguez-Galan A, Turner SE, et al. Impaired NK cell responses to pertussis and H1N1 influenza vaccine antigens in human cytomegalovirus-infected individuals. *J Immunol* (2015) 194(10):4657–67. doi:10.4049/jimmunol.1403080
138. Nielsen CM, White MJ, Goodier MR, Riley EM. Functional significance of CD57 expression on human NK cells and relevance to disease. *Front Immunol* (2013) 4:422. doi:10.3389/fimmu.2013.00422
139. Hoglund P, Brodin P. Current perspectives of natural killer cell education by MHC class I molecules. *Nat Rev Immunol* (2010) 10(10):724–34. doi:10.1038/nri2835
140. Horowitz A, Strauss-Albee DM, Leipold M, Kubo J, Nemat-Gorgani N, Dogan OC, et al. Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med* (2013) 5(208):208ra145. doi:10.1126/scitranslmed.3006702
141. Pyo CW, Guethlein LA, Vu Q, Wang R, Abi-Rached L, Norman PJ, et al. Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS One* (2010) 5(12):e15115. doi:10.1371/journal.pone.0015115
142. Middleton D, Gonzelez F. The extensive polymorphism of KIR genes. *Immunology* (2010) 129(1):8–19. doi:10.1111/j.1365-2567.2009.03208.x

143. Parham P, Norman PJ, Abi-Rached L, Guethlein LA. Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. *Philos Trans R Soc Lond B Biol Sci* (2012) 367(1590):800–11. doi:10.1098/rstb.2011.0266
144. Norman PJ, Abi-Rached L, Gendzekhadze K, Hammond JA, Moesta AK, Sharma D, et al. Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. *Genome Res* (2009) 19(5):757–69. doi:10.1101/gr.085738.108
145. Martin MP, Bashirova A, Traherne J, Trowsdale J, Carrington M. Cutting edge: expansion of the KIR locus by unequal crossing over. *J Immunol* (2003) 171(5):2192–5. doi:10.4049/jimmunol.171.5.2192
146. Olaniyan SA, Amodu OK, Yindom LM, Conway DJ, Aka P, Bakare AA, et al. Killer-cell immunoglobulin-like receptors and falciparum malaria in southwest Nigeria. *Hum Immunol* (2014) 75(8):816–21. doi:10.1016/j.humimm.2014.06.002
147. Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunol Rev* (2006) 214:143–54. doi:10.1111/j.1600-065X.2006.00458.x
148. Yindom LM, Forbes R, Aka P, Janha O, Jeffries D, Jallow M, et al. Killer-cell immunoglobulin-like receptors and malaria caused by *Plasmodium falciparum* in The Gambia. *Tissue Antigens* (2012) 79(2):104–13. doi:10.1111/j.1399-0039.2011.01818.x
149. Ivarsson MA, Michaelsson J, Fauriat C. Activating killer cell Ig-like receptors in health and disease. *Front Immunol* (2014) 5:184. doi:10.3389/fimmu.2014.00184
150. Hirayasu K, Ohashi J, Kashiwase K, Hananantachai H, Naka I, Ogawa A, et al. Significant association of KIR2DL3-HLA-C1 combination with cerebral malaria and implications for co-evolution of KIR and HLA. *PLoS Pathog* (2012) 8(3):e1002565. doi:10.1371/journal.ppat.1002565
151. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol* (2008) 180(6):3969–79. doi:10.4049/jimmunol.180.6.3969
152. Malaria Genomic Epidemiology Network, Band G, Rockett KA, Spencer CC, Kwiatkowski DP. A novel locus of resistance to severe malaria in a region of ancient balancing selection. *Nature* (2015) 526(7572):253–7. doi:10.1038/nature15390
153. Shelton JM, Corran P, Risley P, Silva N, Hubbart C, Jeffreys A, et al. Genetic determinants of anti-malarial acquired immunity in a large multi-centre study. *Malar J* (2015) 14:333. doi:10.1186/s12936-015-0833-x
154. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* (2005) 105(11):4416–23. doi:10.1182/blood-2004-08-3156
155. Orr MT, Murphy WJ, Lanier LL. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol* (2010) 11(4):321–7. doi:10.1038/ni.1849
156. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science* (2011) 331(6013):44–9. doi:10.1126/science.1198687

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 11 January 2017
doi: 10.3389/fimmu.2016.00692

Granule-Dependent Natural Killer Cell Cytotoxicity to Fungal Pathogens

*Henry Ogbomo[1,2,3] and Christopher H. Mody[1,2,4]**

[1] The Calvin, Phoebe and Joan Snyder Institute for Chronic Diseases, University of Calgary, Calgary, AB, Canada, [2] Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, AB, Canada, [3] Department of Physiology and Pharmacology, University of Calgary, Calgary, AB, Canada, [4] Department of Internal Medicine, University of Calgary, Calgary, AB, Canada

Natural killer (NK) cells kill or inhibit the growth of a number of fungi including *Cryptococcus*, *Candida*, *Aspergillus*, *Rhizopus*, and *Paracoccidioides*. Although many fungi are not dangerous, invasive fungal pathogens, such as *Cryptococcus neoformans*, cause life-threatening disease in individuals with impaired cell-mediated immunity. While there are similarities to cell-mediated killing of tumor cells, there are also important differences. Similar to tumor killing, NK cells directly kill fungi in a receptor-mediated and cytotoxic granule-dependent manner. Unlike tumor cell killing where multiple NK cell-activating receptors cooperate and signal events that mediate cytotoxicity, only the NKp30 receptor has been described to mediate signaling events that trigger the NK cell to mobilize its cytolytic payload to the site of interaction with *C. neoformans* and *Candida albicans*, subsequently leading to granule exocytosis and fungal killing. More recently, the NKp46 receptor was reported to bind *Candida glabrata* adhesins Epa1, 6, and 7 and directly mediate fungal clearance. A number of unanswered questions remain. For example, is only one NK cell-activating receptor sufficient for signaling leading to fungal killing? Are the signaling pathways activated by fungi similar to those activated by tumor cells during NK cell killing? How do the cytolytic granules traffic to the site of interaction with fungi, and how does this process compare with tumor killing? Recent insights into receptor use, intracellular signaling and cytolytic granule trafficking during NK cell-mediated fungal killing will be compared to tumor killing, and the implications for therapeutic approaches will be discussed.

Keywords: fungal cytotoxicity, tumor cytotoxicity, cytotoxic granules, fungal cytotoxic receptors, fungal cytotoxicity signaling pathways

OPEN ACCESS

Edited by:
Vincent Vieillard, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Frank Momburg, German Cancer Research Center (HZ), Germany
Sophie Caillat-Zucman, French Institute of Health and Medical Research, France

****Correspondence:***
Christopher H. Mody
cmody@ucalgary.ca

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *14 November 2016*
Accepted: *28 December 2016*
Published: *11 January 2017*

Citation:
Ogbomo H and Mody CH (2017) Granule-Dependent Natural Killer Cell Cytotoxicity to Fungal Pathogens. Front. Immunol. 7:692. doi: 10.3389/fimmu.2016.00692

INTRODUCTION

In the mid-1970s, two independent groups discovered natural killer (NK) cells when they described their ability to lyse tumor cells without prior exposure (1, 2). It is now well established that NK cells are also effective cytotoxic lymphocytes against fungi (3), in addition to their ability to lyse tumors and virus-infected tumor cells (4). NK cell-mediated cytotoxicity is a complex process that involves receptor-mediated binding and signaling, synapse formation, granule polarization, and granule release (5). For tumors, NK cell cytotoxicity is regulated by activating and inhibitory receptors that are expressed on its cell surface. Ligation of these receptors with their cognate ligands triggers downstream signaling events, and a balance in both activating and inhibiting signals tightly

controls NK cell function. While there are some similarities with respect to NK cell-mediated killing of tumor cells or fungi, some important differences exist. For example, in tumor cell killing, NK cells release perforin and granzyme B as effector molecules, with the perforin-forming pores in the tumor cell membrane, thereby allowing entrance of granzyme B to activate caspases and induce target cell death (6). In the case of fungi killing, there is no reported use of granzyme B. Instead, NK cells release perforin to directly kill *Cryptococcus neoformans* and *Candida albicans* (7, 8), or granulysin to directly kill *Paracoccidioides brasiliensis* (9), or IFN-gamma to directly damage *Aspergillus fumigatus* (10). NK cells also indirectly mediated fungal elimination by secreting IFN-gamma to either mediate fungicidal activity of murine peritoneal exudate cells against *C. neoformans* (11) or mediate phagocytosis of *C. albicans* by splenic macrophages (12) or secrete GM-CSF to promote neutrophil phagocytosis of *C. albicans* (13). It has also been shown that the receptor and signaling pathway used for cryptococcal killing differs from that used for tumor killing (14, 15). This review will focus on the recent insights into activating receptor-mediated signaling and granule trafficking during NK cell killing of fungi compared to that of tumor cells.

RECEPTORS USED BY NK CELLS DURING KILLING

During tumor cytotoxicity, NK cells use a large number of activating receptors to mediate granule-dependent killing. Such receptors include the natural cytotoxic receptors (NCRs) such as NKp30, NKp44, and NKp46, as well as NKG2D, DNAM-1, 2B4, CD2, NKp80, CD48 and Ly9 (CD229), LFA-1, and CD16 (5, 16–28). Interestingly, no activating receptor was found to be sufficient in inducing degranulation, except when used in combination with other receptors (5, 17, 19). It is possible that synergy among NK receptors could be required to mediate fungal cytotoxicity as it is in tumor cytotoxicity.

In the context of fungal cytotoxicity, only two NK cell-activating receptors, NKp30 and NKp46, have been identified. The NKp30 receptor was identified as a fungal cytotoxic receptor when antibodies that were generated against an NK cell line, YT, inhibited fungal killing by NK cells. The NKp30 receptor directly recognized and mediated NK cell killing of *C. neoformans* and *C. albicans* (7) and antibody blocking or siRNA knockdown of NKp30 expression reduced fungal binding and killing (7). More recently, the NKp46 receptor was discovered to directly recognize and mediate killing of *Candida glabrata* (29). NKp46 was identified as a fungal cytotoxic receptor when soluble NKp46–IgG1 fusion construct specifically bounded multiple fungal adhesins, Epa1, 6, and 7, expressed on *C. glabrata* and mediated killing of *C. glabrata* (29). In addition, mice deficient in NCR1 (mouse ortholog of NKp46) could not mediate clearance of systemic *C. glabrata* infection (29). Since several fungi including *Cryptococcus*, *Candida*, *Aspergillus*, and *Coccidioides* express adhesins (30), it is interesting to speculate that other fungal adhesins could be recognized by NK cell receptors for fungal cytotoxicity.

SIGNALING PATHWAY ACTIVATED IN NK CELLS DURING KILLING

Signaling through NK cell-activating receptors triggers cytotoxicity (6). While the molecular pathways that are associated with NK cell killing of tumor target cells have been elucidated [**Figure 1**; (6, 31)], the pathways associated with NK cell antifungal activity are still being elucidated (**Figure 2**). In tumor killing, the inhibition of phosphatidylinositol 3-kinase (PI3K) in NK cells blocked p21-activated kinase 1 (PAK1), MAPK kinase (MEK), and extracellular signal regulated kinase (Erk) activation and interfered with cytotoxic granule movement toward the target cells, thereby suppressing NK cytotoxicity (32). Hence, NK cell antitumor signaling follows the sequential activation of PI3K → Rac1 → PAK1 → MEK → Erk (6, 32). A Vav1 → PLCγ2 → Erk sequence has also been reported to mediate cytotoxicity (31, 33) and Vav1, which is a guanine exchange factor, activated Rac1 by catalyzing GDP/GTP exchange on Rac1 (34). Similar to tumor killing, fungal killing, demonstrated using *Cryptococcus*, depended on PI3K → Erk signaling (35). However, unlike tumor killing, PLCγ was not required for cryptococcal killing (15). Further, in the context of tumor killing, and depending on the activating receptor involved, Src family kinases (SFKs) either directly activated PI3K, leading to Rac and Erk activation, or recruited Vav1 to activate Rac (**Figure 1**). While the NCRs signal through the PI3K/Rac/Erk axis (6), DNAM-1 and 2B4 signal through the Vav1/Rac/Erk axis (36), and NKG2D transmits its signal through both axes (31, 33, 36–40). Similar to tumor killing, two SFKs, Fyn and Lyn, redundantly mediated NK cell anticryptococcal activity by activating PI3K and Erk1/2 (41). Also, SFK was required to form NK cell-cryptococcal conjugates (15), and NKp30 was required for NK cell–fungal conjugate formation and PI3K–Erk signaling (7), making it likely that NKp30 activated SFK. Unlike in tumor killing where Rac is activated by SFK and is downstream of PI3K,

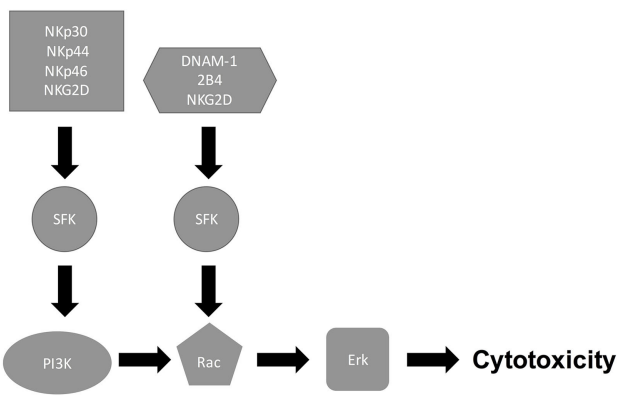


FIGURE 1 | Natural killer (NK) cell/tumor cytotoxicity pathway. Following receptor–ligand interaction, NK cell receptors initiate multiple signaling cascades. While the natural cytotoxic receptors (NKp30, NKp44, and NKp46) signal through Src family kinase (SFK) to activate PI3K/Rac/Erk pathway, DNAM-1 and 2B4 signal SFK to activate Rac/Erk pathway, thereby leading to cytotoxicity.

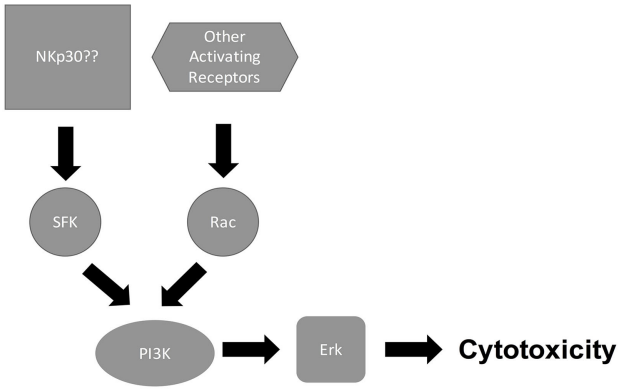


FIGURE 2 | Natural killer (NK) cell/*Cryptococcus* cytotoxicity pathway. Following recognition of cryptococcal capsule and cell wall component by NK cell-activating receptors such as NKp30, receptor binding signals both Src family kinase (SFK) and Rac to activate PI3K/Erk, leading to cytotoxicity.

both Rac and SFK were found not to activate each other, but both were essential for PI3K activation in NK cell-mediated killing of *Cryptococcus* (15). In fact, Rac was found to be upstream of PI3K and was required for the activation of the PI3K → Erk signaling pathway during NK cell antifungal activity (15), thereby suggesting two separate pathways for PI3K activation (**Figure 2**). This interesting finding that Rac is upstream of PI3K needs to be confirmed by others. Ultimately, in tumor and cryptococcal killing, all pathways converged at Erk to mediate granule polarization and exocytosis, resulting in target cell death. The signaling pathway activated by the other known activating receptor for fungi cytotoxicity, NKp46, remains to be identified, and the signaling pathway involved in rearming of fungal cytotoxicity also remains to be investigated (42).

Tumor cells express several activating ligands on their surface to ligate NK cell-activating receptors, thereby resulting in multiple activating signaling pathways that contribute to cytotoxicity. For example, NKG2D binds the ULBP ligands, DNAM-1 binds CD112 and CD155, 2B4 binds CD48, and NKp30 binds B7-H6 (43). To date, the only known activating fungal ligands are three adhesins, Epa1, 6, and 7, of *C. glabrata*, which bind to NKp46 (29). Thus, whether there is coordination of signaling from multiple activating receptors expressed on NK cells to mediate fungi cytotoxicity remains an unanswered question.

CYTOTOXIC GRANULE TRAFFICKING

In response to tumor cells, coordinated signaling through different NK cell receptors and signaling molecules leads to polarization of cytotoxic granules toward the NK immune synapse (NKIS) formed with the target cell (44). The polarization of cytotoxic granules to the NKIS has been described in a sequential order. Upon conjugate formation with the target cell, the engagement of LFA-1 led to Vav1 activation (45). This in turn led to the polymerization and recruitment of filamentous actin (F-actin) to the NKIS (46). Dynein, a minus-end directed motor, rapidly moved granules along microtubules to converge on the microtubule-organizing center (MTOC) (47), followed by the polarization of the MTOC, together with the converged granules, to the NKIS (48). This process was mediated by kinesin-1, a plus-end motor that moves granules in the opposite direction, away from the MTOC (48). Although it is not clear how a kinesin would mediate MTOC movement to the NK cell synapse, in T cell cytotoxicity, the distal microtubule was tethered at the immune synapse and the MTOC was reeled in to the synapse by a dynein motor (49). Also, in T cell-mediated killing, microtubules linked the MTOC to the target contact site and the MTOC was progressively pulled to the contact site by a microtubule sliding mechanism (50). The MTOC movement resulted from the vector sum of tension on multiple microtubules (50). Following polarization, the lytic granules associated with myosin IIA, which enabled their interaction with F-actin and final transit through the actin-rich synapse to join the NK cell membrane (51). The contents of the cytolytic granules were then secreted directly toward the target cell through a pervasive F-actin network at the NKIS (52). While SFK signal mediated the rapid convergence of cytolytic granules to the MTOC without the involvement of PI3K, MEK, or PLCγ (53), PI3K–Erk signal was required for the polarization of the MTOC and converged cytolytic granules to the NKIS (32, 44).

We are only beginning to understand the mechanisms by which cytotoxic granules traffic during fungal killing. Cytotoxic granule trafficking during NK cell cytotoxicity of *Cryptococcus* was different from that of tumors, especially because NK cells did not require LFA-1 in the process, even though the β2 chain of LFA-1 bound to cryptococcal capsular components GXM and GalXM (14). Instead, NK cells used NKp30 to bind *Cryptococcus* and *C. albicans*, mediate microbial synapse formation, and signal PI3K–Erk to release perforin granules (7). Also the SFKs, Fyn and Lyn, redundantly mediated NK cell anticryptococcal activity by activating PI3K and Erk, which in turn polarized perforin-containing granules to the synapse (41). Furthermore, MTOC polarization toward the binding site with *Cryptococcus* was required for cryptococcal killing (54). It remains unknown whether a dynein is required for convergence of granules to the MTOC or whether a kinesin is required for polarization in response to fungi.

IMPLICATION FOR THERAPEUTIC APPROACHES

Understanding the receptors used and signaling pathways activated during NK cell function may lead to therapeutic opportunities. For example, compared to healthy adults, NK cells from HIV-infected patients had diminished expression of NKp30 (7), defective binding, reduced perforin content, defective perforin-containing granule polarization (55), reduced perforin release in response to *Cryptococcus*, and reduced cryptococcal killing (7, 55). Interestingly, treatment of NK cells from HIV-infected patients with IL-12 reversed these multiple defects (7, 55). Since a percentage of HIV-infected patients are subclinically infected with *C. neoformans* (56), treatment

with IL-12, or similar agent, could reduce or eliminate this complication. Another example is the development of pulmonary cryptococcosis and cryptococcal meningitis in patients with Crohn's disease or autoimmune hepatitis that were treated with the purine analog, azathioprine (57, 58). Azathioprine prevented Rac1 activation by blocking GTP binding to Rac1 (59, 60), and Rac1 activation in NK cells is required for NK cell cytotoxicity of tumors (32) and *Cryptococcus* (15). Thus, the increased susceptibility to *Cryptococcus* in patients with Crohn's disease and autoimmune hepatitis that are treated with azathioprine may in part be due to the defective NK cell function resulting from azathioprine-induced blockade of the Rac1 → PI3K → Erk cytotoxicity pathway.

CONCLUDING REMARKS

Fungi, like tumors, are susceptible to NK cell killing. While NK cells use multiple receptors to recognize and kill tumor cells (16), they use NKp30 to recognize and kill *C. neoformans* and *C. albicans* (7), and use NKp46 to kill *C. glabrata* (29). The paradigm in tumor cytotoxicity is that NK cells use multiple activating receptors to recognize tumor targets and the multiple NK cell-activating receptors cooperate to mediate tumor cytotoxicity. Interestingly, several fungi express multiple pattern-associated molecular patterns including adhesins (30), and NK cells used NKp46 to recognize the fungal adhesins Epa1, 6, and 7, which were expressed on *C. glabrata* (30). Further studies are needed to delineate other NK-activating receptors that could mediate fungal killing and to investigate whether cooperative recognition by multiple NK cell-activating receptors is required to mediate fungal cytotoxicity.

AUTHOR CONTRIBUTIONS

All the authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was supported by grants from the Canadian Institute for Health Research (CIHR 365812 to CM), and the Jessie Bowden Lloyd Professorship in Immunology (CM).

REFERENCES

1. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* (1975) 5:112–7. doi:10.1002/eji.1830050208
2. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* (1975) 16:216–29. doi:10.1002/ijc.2910160205
3. Schmidt S, Zimmermann SY, Tramsen L, Koehl U, Lehrnbecher T. Natural killer cells and antifungal host response. *Clin Vaccine Immunol* (2013) 20:452–8. doi:10.1128/CVI.00606-12
4. Moretta A, Marcenaro E, Parolini S, Ferlazzo G, Moretta L. NK cells at the interface between innate and adaptive immunity. *Cell Death Differ* (2008) 15:226–33. doi:10.1038/sj.cdd.4402170
5. Bryceson YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med* (2005) 202:1001–12. doi:10.1084/jem.20051143
6. Djeu JY, Jiang K, Wei S. A view to a kill: signals triggering cytotoxicity. *Clin Cancer Res* (2002) 8:636–40.
7. Li SS, Kyei SK, Timm-McCann M, Ogbomo H, Jones GJ, Shi M, et al. The NK receptor NKp30 mediates direct fungal recognition and killing and is diminished in NK cells from HIV-infected patients. *Cell Host Microbe* (2013) 14:387–97. doi:10.1016/j.chom.2013.09.007
8. Voigt J, Hunniger K, Bouzani M, Jacobsen ID, Barz D, Hube B, et al. Human natural killer cells acting as phagocytes against *Candida albicans* and mounting an inflammatory response that modulates neutrophil antifungal activity. *J Infect Dis* (2014) 209:616–26. doi:10.1093/infdis/jit574
9. Longhi LN, da Silva RM, Fornazim MC, Spago MC, de Oliveira RT, Nowill AE, et al. Phenotypic and functional characterization of NK cells in human immune response against the dimorphic fungus *Paracoccidioides brasiliensis*. *J Immunol* (2012) 189:935–45. doi:10.4049/jimmunol.1102563
10. Bouzani M, Ok M, McCormick A, Ebel F, Kurzai O, Morton CO, et al. Human NK cells display important antifungal activity against *Aspergillus fumigatus*, which is directly mediated by IFN-gamma release. *J Immunol* (2011) 187:1369–76. doi:10.4049/jimmunol.1003593
11. Zhang T, Kawakami K, Qureshi MH, Okamura H, Kurimoto M, Saito A. Interleukin-12 (IL-12) and IL-18 synergistically induce the fungicidal activity of murine peritoneal exudate cells against *Cryptococcus neoformans* through production of gamma interferon by natural killer cells. *Infect Immun* (1997) 65:3594–9.
12. Algarra I, Ortega E, Serrano MJ, Alvarez de Cienfuegos G, Gaforio JJ. Suppression of splenic macrophage *Candida albicans* phagocytosis following in vivo depletion of natural killer cells in immunocompetent BALB/c mice and T-cell-deficient nude mice. *FEMS Immunol Med Microbiol* (2002) 33:159–63. doi:10.1111/j.1574-695X.2002.tb00586.x
13. Bar E, Whitney PG, Moor K, Reis e Sousa C, LeibundGut-Landmann S. IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells. *Immunity* (2014) 40:117–27. doi:10.1016/j.immuni.2013.12.002
14. Jones GJ, Wiseman JC, Marr KJ, Wei S, Djeu JY, Mody CH. In contrast to anti-tumor activity, YT cell and primary NK cell cytotoxicity for *Cryptococcus neoformans* bypasses LFA-1. *Int Immunol* (2009) 21:423–32. doi:10.1093/intimm/dxp010
15. Xiang RF, Stack D, Huston SM, Li SS, Ogbomo H, Kyei SK, et al. Ras-related C3 botulinum toxin substrate (Rac) and Src family kinases (SFK) are proximal and essential for phosphatidylinositol 3-kinase (PI3K) activation in natural killer (NK) cell-mediated direct cytotoxicity against *Cryptococcus neoformans*. *J Biol Chem* (2016) 291:6912–22. doi:10.1074/jbc.M115.681544
16. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* (2001) 19:197–223. doi:10.1146/annurev.immunol.19.1.197
17. Bryceson YT, Ljunggren HG, Long EO. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. *Blood* (2009) 114:2657–66. doi:10.1182/blood-2009-01-201632
18. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev* (2006) 214:73–91. doi:10.1111/j.1600-065X.2006.00457.x
19. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* (2006) 107:159–66. doi:10.1182/blood-2005-04-1351
20. Castriconi R, Dondero A, Corrias MV, Lanino E, Pende D, Moretta L, et al. Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer Res* (2004) 64:9180–4. doi:10.1158/0008-5472.CAN-04-2682

21. Glasner A, Ghadially H, Gur C, Stanietsky N, Tsukerman P, Enk J, et al. Recognition and prevention of tumor metastasis by the NK receptor NKp46/NCR1. *J Immunol* (2012) 188:2509–15. doi:10.4049/jimmunol.1102461
22. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* (2008) 28:571–80. doi:10.1016/j.immuni.2008.02.016
23. Halfteck GG, Elboim M, Gur C, Achdout H, Ghadially H, Mandelboim O. Enhanced in vivo growth of lymphoma tumors in the absence of the NK-activating receptor NKp46/NCR1. *J Immunol* (2009) 182:2221–30. doi:10.4049/jimmunol.0801878
24. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J Exp Med* (2008) 205:2959–64. doi:10.1084/jem.20081611
25. Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, et al. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *Eur J Immunol* (2001) 31:1076–86. doi:10.1002/1521-4141(200104)31:4<1076::AID-IMMU1076>3.0.CO;2-Y
26. Sivori S, Parolini S, Marcenaro E, Castriconi R, Pende D, Millo R, et al. Involvement of natural cytotoxicity receptors in human natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines. *J Neuroimmunol* (2000) 107:220–5. doi:10.1016/S0165-5728(00)00221-6
27. Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM, Hayakawa Y. NKG2D function protects the host from tumor initiation. *J Exp Med* (2005) 202:583–8. doi:10.1084/jem.20050994
28. Alari-Pahissa E, Grandclement C, Jeevan-Raj B, Leclercq G, Veillette A, Held W. Activation by SLAM family receptors contributes to NK cell mediated "missing-self" recognition. *PLoS One* (2016) 11:e0153236. doi:10.1371/journal.pone.0153236
29. Vitenshtein A, Charpak-Amikam Y, Yamin R, Bauman Y, Isaacson B, Stein N, et al. NK cell recognition of *Candida glabrata* through binding of NKp46 and NCR1 to fungal ligands Epa1, Epa6, and Epa7. *Cell Host Microbe* (2016) 20:527–34. doi:10.1016/j.chom.2016.09.008
30. de Groot PW, Bader O, de Boer AD, Weig M, Chauhan N. Adhesins in human fungal pathogens: glue with plenty of stick. *Eukaryot Cell* (2013) 12:470–81. doi:10.1128/EC.00364-12
31. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol* (2013) 31:227–58. doi:10.1146/annurev-immunol-020711-075005
32. Jiang K, Zhong B, Gilvary DL, Corliss BC, Hong-Geller E, Wei S, et al. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. *Nat Immunol* (2000) 1:419–25. doi:10.1038/80859
33. Kim HS, Das A, Gross CC, Bryceson YT, Long EO. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity* (2010) 32:175–86. doi:10.1016/j.immuni.2010.02.004
34. Crespo P, Schuebel KE, Ostrom AA, Gutkind JS, Bustelo XR. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature* (1997) 385:169–72. doi:10.1038/385169a0
35. Wiseman JC, Ma LL, Marr KJ, Jones GJ, Mody CH. Perforin-dependent cryptococcal microbicidal activity in NK cells requires PI3K-dependent ERK1/2 signaling. *J Immunol* (2007) 178:6456–64. doi:10.4049/jimmunol.178.10.6456
36. Xiong P, Sang HW, Zhu M. Critical roles of co-activation receptor DNAX accessory molecule-1 in natural killer cell immunity. *Immunology* (2015) 146:369–78. doi:10.1111/imm.12516
37. Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol* (2003) 4:557–64. doi:10.1038/ni929
38. Gilfillan S, Ho EL, Cella M, Yokoyama WM, Colonna M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol* (2002) 3:1150–5. doi:10.1038/ni857
39. Graham DB, Cella M, Giurisato E, Fujikawa K, Miletic AV, Kloeppel T, et al. Vav1 controls DAP10-mediated natural cytotoxicity by regulating actin and microtubule dynamics. *J Immunol* (2006) 177:2349–55. doi:10.4049/jimmunol.177.4.2349
40. Upshaw JL, Arneson LN, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat Immunol* (2006) 7:524–32. doi:10.1038/ni1325
41. Oykhman P, Timm-McCann M, Xiang RF, Islam A, Li SS, Stack D, et al. Requirement and redundancy of the Src family kinases Fyn and Lyn in perforin-dependent killing of *Cryptococcus neoformans* by NK cells. *Infect Immun* (2013) 81:3912–22. doi:10.1128/IAI.00533-13
42. Marr KJ, Jones GJ, Zheng C, Huston SM, Timm-McCann M, Islam A, et al. *Cryptococcus neoformans* directly stimulates perforin production and rearms NK cells for enhanced anticryptococcal microbicidal activity. *Infect Immun* (2009) 77:2436–46. doi:10.1128/IAI.01232-08
43. Chester C, Fritsch K, Kohrt HE. Natural killer cell immunomodulation: targeting activating, inhibitory, and co-stimulatory receptor signaling for cancer immunotherapy. *Front Immunol* (2015) 6:601. doi:10.3389/fimmu.2015.00601
44. Chen X, Trivedi PP, Ge B, Krzewski K, Strominger JL. Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. *Proc Natl Acad Sci U S A* (2007) 104:6329–34. doi:10.1073/pnas.0611655104
45. Riteau B, Barber DF, Long EO. Vav1 phosphorylation is induced by beta2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization. *J Exp Med* (2003) 198:469–74. doi:10.1084/jem.20021995
46. Watzl C, Long EO. Signal transduction during activation and inhibition of natural killer cells. *Curr Protoc Immunol* (2010) Chapter 11:Unit 11 9B. doi:10.1002/0471142735.im1109bs90
47. Mentlik AN, Sanborn KB, Holzbaur EL, Orange JS. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Mol Biol Cell* (2010) 21:2241–56. doi:10.1091/mbc.E09-11-0930
48. Tuli A, Thiery J, James AM, Michelet X, Sharma M, Garg S, et al. Arf-like GTPase Arl8b regulates lytic granule polarization and natural killer cell-mediated cytotoxicity. *Mol Biol Cell* (2013) 24:3721–35. doi:10.1091/mbc.E13-05-0259
49. Yi J, Wu X, Chung AH, Chen JK, Kapoor TM, Hammer JA. Centrosome repositioning in T cells is biphasic and driven by microtubule end-on capture-shrinkage. *J Cell Biol* (2013) 202:779–92. doi:10.1083/jcb.201301004
50. Kuhn JR, Poenie M. Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity* (2002) 16:111–21. doi:10.1016/S1074-7613(02)00262-5
51. Sanborn KB, Rak GD, Maru SY, Demers K, Difeo A, Martignetti JA, et al. Myosin IIA associates with NK cell lytic granules to enable their interaction with F-actin and function at the immunological synapse. *J Immunol* (2009) 182:6969–84. doi:10.4049/jimmunol.0804337
52. Rak GD, Mace EM, Banerjee PP, Svitkina T, Orange JS. Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol* (2011) 9:e1001151. doi:10.1371/journal.pbio.1001151
53. James AM, Hsu HT, Dongre P, Uzel G, Mace EM, Banerjee PP, et al. Rapid activation receptor- or IL-2-induced lytic granule convergence in human natural killer cells requires Src, but not downstream signaling. *Blood* (2013) 121:2627–37. doi:10.1182/blood-2012-06-437012
54. Hidore MR, Mislan TW, Murphy JW. Responses of murine natural killer cells to binding of the fungal target *Cryptococcus neoformans*. *Infect Immun* (1991) 59:1489–99.
55. Kyei SK, Ogbomo H, Li S, Timm-McCann M, Xiang RF, Huston SM, et al. Mechanisms by which interleukin-12 corrects defective NK cell anticryptococcal activity in HIV-infected patients. *MBio* (2016) 7. doi:10.1128/mBio.00878-16
56. Sawadogo S, Makumbi B, Purfield A, Ndjavera C, Mutandi G, Maher A, et al. Estimated prevalence of *Cryptococcus* antigenemia (CrAg) among HIV-infected adults with advanced immunosuppression in Namibia justifies routine screening and preemptive treatment. *PLoS One* (2016) 11:e0161830. doi:10.1371/journal.pone.0161830
57. Fraison JB, Guilpain P, Schiffmann A, Veyrac M, Le Moing V, Rispail P, et al. Pulmonary cryptococcosis in a patient with Crohn's disease treated with prednisone, azathioprine and adalimumab: exposure to chicken manure

as a source of contamination. *J Crohns Colitis* (2013) 7:e11–4. doi:10.1016/j.crohns.2012.04.016

58. Sethi NK, Torgovnick J, Sethi PK. Cryptococcal meningitis after imuran (azathioprine) therapy for autoimmune hepatitis. *Eur J Gastroenterol Hepatol* (2007) 19:913–4. doi:10.1097/MEG.0b013e3282cf4ed9
59. Neurath M. Thiopurines in IBD: what is their mechanism of action? *Gastroenterol Hepatol (N Y)* (2010) 6:435–6.
60. Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, et al. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J Clin Invest* (2003) 111:1133–45. doi:10.1172/JCI16432

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 21 March 2017
doi: 10.3389/fimmu.2017.00315

Role of Natural Killer Cells in HIV-Associated Malignancies

*Fabio E. Leal[1], Thomas A. Premeaux[2], Mohamed Abdel-Mohsen[3,4†] and Lishomwa C. Ndhlovu[2]**

[1] Programa de Oncovirologia, Instituto Nacional de Cancer, Rio de Janeiro, Brazil, [2] Department of Tropical Medicine, Hawaii Center for AIDS, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI, USA, [3] Blood Systems Research Institute, San Francisco, CA, USA, [4] University of California, San Francisco, CA, USA

Now in its fourth decade, the burden of HIV disease still persists, despite significant milestone achievements in HIV prevention, diagnosis, treatment, care, and support. Even with long-term use of currently available antiretroviral therapies (ARTs), eradication of HIV remains elusive and now poses a unique set of challenges for the HIV-infected individual. The occurrence of HIV-associated non-AIDS-related comorbidities outside the scope of AIDS-defining illnesses, in particular non-AIDS-defining cancers, is much greater than the age-matched uninfected population. The underlying mechanism is now recognized in part to be related to the immune dysregulated and inflammatory status characteristic of HIV infection that persists despite ART. Natural killer (NK) cells are multifunctional effector immune cells that play a critical role in shaping the innate immune responses to viral infections and cancer. NK cells can modulate the adaptive immune response *via* their role in dendritic cell (DC) maturation, removal of immature tolerogenic DCs, and their ability to produce immunoregulatory cytokines. NK cells are therefore poised as attractive therapeutic targets that can be harnessed to control or clear both HIV and HIV-associated malignancies. To date, features of the tumor microenvironment and the evolution of NK-cell function among individuals with HIV-related malignancies remain unclear and may be distinct from malignancies observed in uninfected persons. This review intends to uncouple anti-HIV and antitumor NK-cell features that can be manipulated to halt the evolution of HIV disease and HIV-associated malignancies and serve as potential preventative and curative immunotherapeutic options.

Keywords: HIV, cancer, natural killer cells, ADCC, antiretroviral therapy, lymphoma, eradication and control

OPEN ACCESS

Edited by:
Sandra Laurence Lopez-Verges, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama

Reviewed by:
Roland Jacobs, Hannover Medical School, Germany
Amedeo Amedei, University of Florence, Italy

****Correspondence:***
Lishomwa C. Ndhlovu
lndhlovu@hawaii.edu

†Present address:
Mohamed Abdel-Mohsen, The Wistar Institute, Philadelphia, PA, USA

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *16 December 2016*
Accepted: *06 March 2017*
Published: *21 March 2017*

Citation:
Leal FE, Premeaux TA, Abdel-Mohsen M and Ndhlovu LC (2017) Role of Natural Killer Cells in HIV-Associated Malignancies. Front. Immunol. 8:315. doi: 10.3389/fimmu.2017.00315

INTRODUCTION

Since the introduction of antiretroviral therapy (ART), life expectancy of people living with HIV (PLHIV) has notably improved, and the gap between the uninfected population ranging from 60 to 90%, of normal life expectancy is narrowing in regions of the world among those with access to ART (1). Before the availability of ART, immune suppression-related complications represented the predominant cause of mortality among HIV-infected individuals. Incidence rates of non-Hodgkin lymphoma (NHL) and Kaposi sarcoma (KS) were more than 100 times higher in the pre-ART era and were classified, together with cervical cancer, as AIDS-defining cancers (ADCs) (2). Overtime, as ART became the standard of care, prolonged use has lead to a remarkable improvement in immune status, dramatically reducing ADC rates (ratio of ART to no-ART) by 0.61 per year (3).

In the United States, despite the sharp decline in the incidence of ADCs, increased risk of developing specific types of NHL such as Burkitt lymphoma and classical Hodgkin lymphoma (HL) has evolved (4, 5). A meta-analysis of standardized incidence ratios from 18 studies showed that infection-related cancers such as anal, liver and HL, as well as smoking-related cancers such as lung, kidney, and oral cancers, had an increased incidence among the HIV population despite being on ART, with lymphomas being the most frequent type of cancer observed (6). A separate comprehensive epidemiological study showed that the PLHIV also had significantly higher rates of these now termed "non-AIDS defining cancers" (NADCs) when compared to matched HIV-negative individuals, suggesting the likelihood of other etiologies besides an increase in life expectancy as predisposing the HIV population to develop these types of cancers more frequently (7). In fact, NADCs have now become one of the leading causes of mortality among HIV-infected individuals (8, 9). Surprisingly, however, the incidences of breast and prostate cancers have significantly declined in HIV-infected persons, suggesting that HIV infection, ART, or other viral–host interactions have differential impacts on cancer risk in this population (10, 11). The increased incidence of NADC despite viral suppression and CD4 T-cell recovery in the era of ART raises important key mechanistic questions in the oncogenesis of NADC. The direct oncogenic effect of HIV, HIV-induced immunodeficiency, and chronic inflammation, as well as ART toxicities are some of the plausible mechanisms that are being investigated (12). Complete immune recovery after prolonged ART is variable and underscores that harnessing specific components of the host immune response may play a vital role in preventing NADC.

Chronic immune activation and immune senescence contribute to immune dysfunction in chronic HIV infection and partially persist even after CD4 T-cell count recovery and viral suppression by ART (13). Such processes lead to immune exhaustion/senescence, thereby facilitating reactivation of other latent viral infections, such as Epstein–Barr virus (EBV). Besides HIV, all ADCs and the majority of NADCs appear to be associated with several chronic viral infections (12), justifying a new way to categorize HIV malignancies into infection-related and non-infectious-related cancers. KS is intimately associated with HHV-8 infection (14), and cervical cancer is almost always caused by HPV infection (15). B-cell lymphoproliferative disorders are frequently associated with EBV infection, and such association is even more common in HIV-infected individuals, ranging from 60 to 100% (5, 16). Viral co-infections are present in NADC. The incidence of hepatocellular carcinoma has progressively increased among HIV-infected persons in the last decade (17). Merkel cell carcinoma, recently reported to be associated with Merkel cell virus, has a 20-fold increased risk among HIV-infected individuals (18). The potential direct effects of HIV in modulating oncogenes are under investigation, but how HIV impacts the oncogenic potential of other chronic viral infections is unclear (19–21). Persistent immune alterations may play a critical role in the oncogenic process in this population and deserve special attention, particularly in the context of co-infections.

NATURAL KILLER (NK) CELLS: A CRITICAL IMMUNE PLAYER IN ANTITUMOR AND ANTI-HIV IMMUNITY

Since the discovery of NK cells 40 years ago, a plethora of research has uncovered their phenotypic and functional capacity against virally infected and tumor cells (22–25). NK cells are CD3− multifunctional effector lymphocytes defined based on levels of CD56 and CD16 expression (26), the majority (>90% of NK cells) in the peripheral blood being CD56dim and predominantly cytotoxic upon activation, thereby releasing pro-apoptotic cytoplasmic granules composed of granzymes and perforins. CD56dim NK cells can also induce cytolysis *via* induction of Fas/FasL-dependent or TRAIL-dependent apoptotic signals. In addition, a minority of NK cells express the FcγRIIIA receptor (CD16) that binds to the constant (Fc) domain of IgG antibodies that can bind to viral antigens expressed on the surface of infected cells. This antibody conjugation of NK-cell and antibody-coated target cell, strongly mediating NK-cell activation, is known as antibody-dependent cell-mediated cytotoxicity (ADCC) (27). A distinct subset of CD56bright cytokine-producing NK cells with a limited cytotoxic capacity is more abundantly present in lymph nodes (28). By producing IFN-γ, TNF-α, IL-10, and chemokines, this NK subset predominantly modulates other subsets of lymphocytes, thereby regulating dendritic cell maturation, differentiation of helper T cells, and B- and T-cell-specific immune responses (29, 30).

To understand the NK-cell effector functions, it is paramount to take into consideration the balance between activating and inhibitory signals (31) that drive NK-cell cytotoxicity. NK-cell activation relies on stimulatory signals capable of overcoming the steady inhibitory state that is maintained by signaling through inhibitory receptors. Self-recognition of MHC-I proteins through C-type lectin receptor NKG2A and inhibitory killer cell immunoglobulin-like receptors (KIRs) represent the physiological interaction between NK and target cells. The absence of recognition of "self" by inhibitory receptors characterizes the "missing-self" phenomenon and lowers the activating threshold. NK cells become more susceptible to activation, especially if activating molecules are expressed in infected or transformed target cells and recognized by activating receptors, characterizing the altered-self phenomenon. Activating C-type lectin receptor NKG2D recognizes the altered self-state of infected or transformed cells and triggers NK-cell cytolytic activity. Other surface molecules, such as natural cytotoxic receptors Nkp30, Nkp44, and Nkp46, and activating KIRs also contribute to NK-cell activation process and are critical to determine whether NK cells will be activated to target infected or transformed cells (27, 31).

Both HIV infection and oncogenesis lead to a downregulation of surface MHC-I expression as a way to avoid T-cell recognition but in turn renders target cells more susceptible to NK-cell-mediated cytolysis. However, HIV has developed immune evasion mechanisms *via* the viral protein Nef, thereby leading to preferential downregulation of HLA-A and -B, and preserving expression of HLA-C and -E (32). Therefore, HIV prevents NK activation as well as CTL recognition of infected cells. Besides

interfering with self-recognition, HIV infection and cancer can induce expression of stress signaling molecules, in particular MHC class I polypeptide-related sequence A/B (MICA/MICB). More importantly, HIV leads to persistent activation and consequently T cell and NK-cell immune exhaustion. Despite viral suppression and normal CD4 T-cell counts in the majority of HIV-infected persons on ART, NK-cell phenotype and functionality are not fully restored, suggesting that these individuals may be more susceptible to long-term comorbidities associated with immune dysfunction, such as HIV-related malignancies (33).

THE INTERPLAY BETWEEN THE TUMOR MICROENVIRONMENT AND NK-CELL IMMUNITY

The process by which the immune system can promote or suppress tumor growth and development is based on animal models and data from cancer patients and has evolved to define the concept of cancer immunoediting (34). Tumor immunoediting is comprised of three phases: elimination, equilibrium, and escape. The elimination phase is when immune cells target cancer cells that succeeded in overcoming intrinsic tumor suppressor mechanisms. If tumor elimination is only partially achieved, a state of equilibrium between malignant cells and the immune system ensues. Tumor cells can become dormant or accumulate mutations, while the immune system continues to exert selective pressure, thereby controlling tumor progress temporarily or eventually eliminating the cancer cells. If elimination does not occur, tumor cell variants resistant to the existent immune response eventually give rise to tumor progression, thereby initiating the escape phase and characterizing failure of tumor immune control. The contribution of NK cells in cancer immunoediting and clinical outcomes is now being appreciated (35).

Natural killer cells have proved to be critical for the eradication and inhibition of metastasis of cancer cells *in vivo* (36). Perforin protein (pfp) and/or IFN-γ knockout (KO) mice predominately develop B-cell lymphomas, especially after 1 year of age (older animals) with a combination of pfp and IFN-γ KO inducing an early onset of lymphoma, suggesting a synergistic immunosurveillance effect (37). Late age development of B-cell lymphoma and lung adenocarcinoma were also observed in TRAIL KO mice (38) and pfp KO mice (37), respectively. These findings support a role for NK-cell immunosurveillance of B-cell lymphomas as well as epithelial malignancies, through a combination of NK-cell-mediated cytotoxic activity, IFN-γ secretion, and TRAIL killing pathways. In humans, NK cells are particularly relevant in the prevention of tumor development. An 11-year follow-up of the general population for cancer incidence showed an association between reduced NK-cell cytotoxicity and increased risk of cancer (39). It has been postulated that NK cells are critical to the prevention of cancer development (elimination and equilibrium); however, once the tumor microenvironment is established (escape), suppressor factors such as TGF-β and IL-10 are induced and negative inhibitory receptors, such as the T-cell immunoglobulin-and-mucin-domain-containing molecule-3 receptor (TIM-3) on NK cells, that maintain an NK-cell anergic state (40) are upregulated. The induced aberrant expression of HLA-G (membrane-bound and soluble) and increased shedding of MICA (sMICA) seen in tumor cells (41, 42) can further suppress NK-cell antitumor immune responses. HLA-G interaction with ILT2 and CD94/NKG2A results in the inhibition of NK-cell cytotoxicity, IFN-γ secretion, and chemotaxis (43), while sMICA–NKG2D binding impairs NK-cell tumor-specific cytotoxicity, NKG2D expression, and homeostatic maintenance (42).

As a result of these immune deregulated events, NK-cell-associated suppressor factors are currently being considered as immunotherapeutic targets. TIM-3, for instance, is an immunoregulatory checkpoint expressed by most lymphocyte subtypes with critical and complex implications in cytotoxic NK cells (44, 45). Increased TIM-3 expression on NK cells has been shown as a marker of poorer prognosis in lung adenocarcinoma and other types of cancer and correlates with reduced NK-cell cytotoxicity. Blockade of TIM-3 is capable of restoring IFN-γ secretion and cytotoxicity of NK cells in lung cancer (46). Recent studies by Fowke et al. have shown that low expression of various inhibitory molecules on NK cells were associated with HIV viral control (47). Despite the complexity of the immune suppressive strategy of the tumor microenvironment, targeting these inhibitory checkpoint receptors shows potential to restore NK-cell functionality in the control or clearance of solid tumors (48). Currently, several trials are underway assessing the impact of Tim-3 blockade in cancer patients (http://ClinicalTrials.gov: NCT02817633; NCT02608268). However, such therapeutic tools are still in their infancy in the context of HIV-associated ADC or NADCs. Given the success of immunotherapy targeting the inhibitory receptors PD-1 and CTLA-4 against several malignancies (49), evaluation of the impact of NK-cell function following immune checkpoint blockade may have relevance in the setting of HIV and may serve a dual purpose in both HIV eradication and tumor clearance. Combining immunotherapy and NK-cell-based therapies is another potential targeted strategy and warrants further investigation in individuals with HIV-associated malignancies (50–53).

NK-CELL IMMUNE CONTROL OF HIV INFECTION DURING ART

HIV infection induces significant phenotypic changes and negatively impacts NK-cell cytotoxicity (54). Cytotoxic NK cells in aviremic HIV donors have impaired ADCC that is associated with a reduced expression of FcRIIIA, an activated phenotype represented by increased expression of CD38 and HLA-DR. Furthermore, NK cells are rarely found in lymph nodes, an important site of both HIV replication and B-cell transformation (55). Critical activating receptors important in cancer immunosurveillance such as NKp30 and NKp46 are downregulated (56) in HIV infection, and expansion of dysfunctional CD56− NK-cell subsets (57) persists even after cART.

Non-neutralizing anti-HIV-1 antibodies can mediate protection through ADCC in assays of HIV candidate vaccines in non-human primate models of HIV infection. Several studies have suggested that HIV-specific ADCC responses may be contributing to partial control of HIV viremia during acute infection. The early initial interest in the utility of NK-mediated

ADCC *via* HIV-specific antibodies was to enhance the development of an HIV vaccine and novel therapies to suppress HIV replication. Interestingly, in the RV144 HIV vaccine Thai trial, the robust ADCC responses observed were associated with modest protective efficacy (58). It would be intriguing to determine the effects of NK-mediated ADCC in the recently launched HVTN 702 study that builds on the success of the RV144 trial in support of this correlate. In addition, recent studies suggest that cytokine stimulation can enhance direct NK cytotoxicity and NK-mediated ADCC of autologous HIV-infected CD4+ T cells (59–61). Therefore, modulating NK activity is a potential strategy to develop novel immunotherapies to prevent and possibly lead to the eradication of HIV.

INFLUENCE OF NK-CELL IMMUNITY IN HIV-ASSOCIATED MALIGNANCIES

It is becoming apparent that NK cells may also contribute to tumorigenesis. The potential impact of such alterations in HIV malignancies is illustrated in **Figure 1**. Overexpression of activation markers on NK cells and spontaneous degranulation occurring during HIV infection may directly contribute to tumor development (33). Analysis of NK cells from patients with lymphoma demonstrated decreased levels of activating receptors in those with HIV compared to uninfected patients, suggesting that these cells might be less efficient to target cancer cells (62). NK cells can also present proangiogenic activity in the tumor microenvironment in a similar way to decidual NK (dNK) cells early on in pregnancy. In fact, in non-small cell lung cancer, the majority of tumor-infiltrating NK cells have a dNK-cell phenotype: CD56bright, CD9+, perforin low, and high production of vascular endothelial growth factor (VEGF) (63). Hypoxia and TGF-β secreted by tumor cells has a known immunomodulatory impact in the tumor microenvironment and induces VEGF secretion (64). *In vitro* exposure to TGF-β and hypoxia led to conversion of CD56dim NK cells into dNK-like cells (65). HIV infection leads to increased levels of TGF-β by monocytes (66) and T cells (67), suggesting that TGF-β may play a more prominent role in tumorigenesis during HIV infection.

The combination of (1) reduced expression of activating receptors and increased inhibitory receptors (e.g., TIM-3), (2) reduced ADCC, (3) reduced secretion of TNF-α and IFN-γ, and (4) development of pro-cancer features such as persistent activation, spontaneous degranulation, and production of VEGF suggests that NK cells may directly be associated with the increased cancer risk in the setting of ART-treated HIV infection. It is fair to speculate that targeted immunotherapies reversing specific NK cells deficits may be relevant for many HIV-related malignancies.

NK-CELL-BASED IMMUNOTHERAPIES TARGETING HIV AND HIV-ASSOCIATED MALIGNANCIES

With the exception of the Berlin patient (75), and given the continued resurgence of virus in HIV-infected persons in various eradication approaches (76–78), it is clear that elimination of all latent HIV reservoirs is going to be critical to successfully achieve ART-free sustained HIV control or remission. Innovative approaches that are extrapolated from these studies and cases have lead to renewed interest in determining ways to bolster the host immune response and/or manipulate HIV target cells to render them refractory to infection. Since HIV and cancers have evolved sophisticated modalities to escape the host immune defense mechanisms, enhancing NK-cell function may serve as a promising tool as part of a multifaceted approach in the elimination of HIV as well as HIV-associated malignancies.

Recently, there has been renewed interest in harnessing HIV-specific ADCC responses as an HIV cure strategy. A monoclonal antibody (mAb) targeting the CD4-binding site on the HIV envelope spike (3BNC117) may have the potential to guide host immune effector cells to accelerate the clearance of HIV-1-infected cells by an FcγR-dependent mechanism (79, 80). In addition, Byrareddy et al. recently reported that a rhesusized mAb against α4β7 mediated sustained control of SIV infection in the absence of ART in non-human primates. This remarkable response was associated with increased cytokine-synthesizing NK cells (81). These studies, and others, highlight the potential of using mAbs through modulation of NK-cell-mediated activity as an exciting therapeutic tool to achieve sustained HIV remission and be beneficial in the context of HIV-associated tumors.

Natural killer-cell-based antitumor immunotherapeutic strategies targeting NK-cell activity have shown some promise in the oncology field (**Table 1**). Infusion of allogeneic or autologous NK cells has, to some degree, been successful in tumor clearance. Recently, single-chain variable fragment recombinant reagents, such as bispecific and trispecific killer cell engagers, are being considered as novel immunomodulators to enhance NK-cell function, antigen specificity, and *in vivo* expansion of these infused cells; however, these reagents need to be fully evaluated for clinical use (82–91). The efficiency of ADCC-mediated NK-cell responses is dependent on several factors from the mAbs themselves, NK-cell, and target cell status and also to the glycosylation state and the expression of glycosylation-specific ligands of both the NK-cell and target cells (92, 93). These features can be modified to enhance antiviral and immunomodulatory functions and/or the ability of the target cell to trigger or evade immunological recognition.

Several gene-editing technologies have been explored to genetically reprogram NK cells to optimize their persistence, expansion, migration, and cytotoxic capacity to improve the antitumor efficacy of primary human NK cells *in vivo*. With many challenges associated with most of these technologies, CRISPR/Cas9 nuclease system offers a new promising tool to gene-edit NK cells to improve their utility as a novel cell-based cancer immunotherapy strategy (142). Finally, latency reversal agents (LRAs) are being explored as a part of a "shock" approach to reverse cellular HIV latency and expose HIV reservoirs to immune-mediated clearance. Garrido et al. recently studied the impact of LRAs on the function of primary NK cells *ex vivo* and showed a heterogeneous mixed effect of different LRAs on antiviral activity, cytotoxicity, cytokine secretion, phenotype, and viability (143). Therefore,

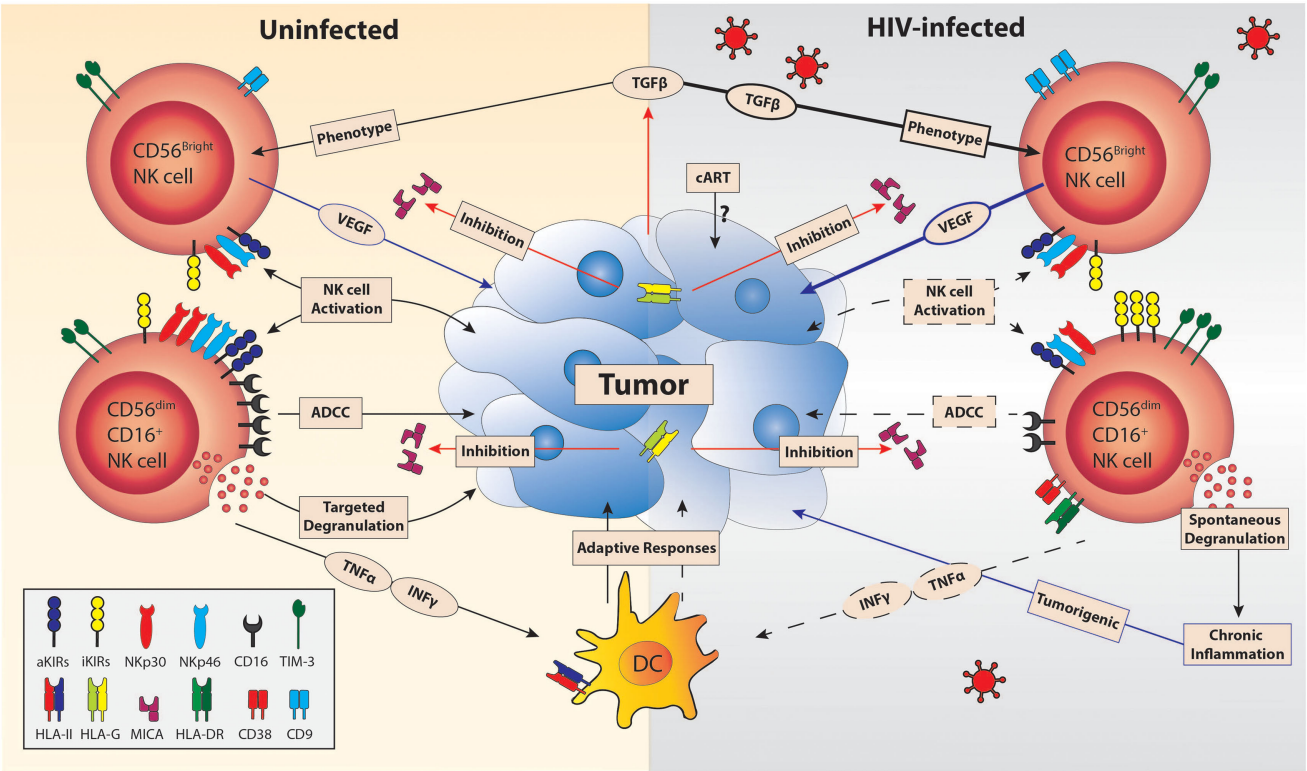


FIGURE 1 | Compounded effect of HIV infection on natural killer (NK) cell antitumor responses. The tumor microenvironment constrains NK-cell functionality through the expression of tumor-derived transforming growth factor β (TGF-β) (68), shedding of MICA, and HLA-G (69). The limitations of antitumor mechanisms by NK cells are exacerbated in HIV infection. HIV infection reduces the surface expression of activation receptors (aKIRs, NKp30, and NKp46) and CD16 (56) while upregulating the expression of inhibitory receptors (iKIRs). The net result of the influence of HIV on NK-cell receptor expression further impairs NK-cell activation by cancer cell interaction and decreases tumor-directed antibody-dependent cell-mediated cytotoxicity (ADCC) responses (70). HIV infection decreases INF-γ and TNF-α production by NK cells despite HIV viral suppression by cART (71, 72), which will limit dendritic cell (DC) maturation and thus prevent efficient tumor-directed adaptive responses (73). The increased plasma TGF-β and loss of cell-specific degranulation of NK cells seen in HIV infection could lead to tumorigenicity *via* contributing to increased frequency of vascular endothelial growth factor (VEGF)-producing intratumoral NK cells (63) and occurrence of chronic inflammation, respectively (74). Furthermore, the affect of cART on tumor activity is yet to be explored. Blue lines represent responses that promote tumor growth, while responses that inhibit NK-cell function are indicated in red. Decreases are indicated by dashed lines and increases by bolded lines.

TABLE 1 | Therapeutic strategies utilizing natural killer (NK) cells in cancer immunotherapy that should be evaluated in the eradication of both HIV and HIV-associated malignancies.

Strategy	Summary	Limitations	Reference
Allogeneic NK-cell-based immunotherapy	Freshly isolated or IL-2-stimulated NK-donor lymphocyte infusion	Requires further optimization to avoid graft-versus-host disease and to enhance efficiency	(94–103)
Autologous NK-cell-based immunotherapy	Activating endogenous NK cells and promoting their proliferation and function in patients using pro-inflammatory cytokine stimulation, or bispecific killer cell engagers (BiKEs) and trispecific killer cell engagers (TriKEs)	Low cytotoxic potential and possible side effects when using high doses of cytokines. BiKEs and TriKEs need to be fully evaluated for clinical use	(82–91, 104–106)
ADCC-based immunotherapy	Tumor-targeting monoclonal antibodies (e.g., anti-CD20, anti-HER-2, anti-GD2, anti-EGFR, and anti-GD2) or bispecific antibodies to induce antibody-dependent cell-mediated cytotoxicity (ADCC)	Requires tumor antigen-specific antibodies	(79, 107–111)
Immune checkpoint inhibitors-based immunotherapy	Blockade of NK-cell surface inhibitory receptors by specific antibodies (e.g., anti-PD-1, anti-NKG2A, anti-KIRs, anti-TIM-3, and anti-CTLA-4) in order to induce NK cells cytolytic activity	Possible side effects	(44, 112–118)
Genetically reprogrammed NK cells	Genetic modification of NK cells to induce the expression of activating receptors, silencing inhibitory receptors, inducing cytokine production, or genetic transferring of chimeric antigen receptors	Methods need further optimization	(119–141)

it will be important to comprehensively evaluate the impact of potential HIV curative strategies and elucidate the possible effect on NK cells against HIV-associated cancers.

CONCLUSION

To achieve ART-free sustained HIV remission, it is likely that a multidisciplinary approach that includes a combination of immunotherapies and novel targeted approaches that will boost both the adaptive and innate immunity will be necessary. From this review, we posit that NK cells remain a very attractive therapeutic target to develop curative interventions for both HIV and HIV-associated malignancies.

AUTHOR CONTRIBUTIONS

LN and MA-M conceived and designed the review; LN, MA-M, and FL wrote the paper; and TP contributed to editing the review and designed and developed the figure.

FUNDING

MA-M receives support from the National Institutes of Health, National Institute of Allergy and Infectious Diseases (NIH/NIAID) grant number R21AI129636 and LN receives support from NIH/NIAHD grant numbers P30 AI027757 and R21 AI122393.

REFERENCES

1. Wandeler G, Johnson LF, Egger M. Trends in life expectancy of HIV-positive adults on antiretroviral therapy across the globe: comparisons with general population. *Curr Opin HIV AIDS* (2016) 11(5):492–500. doi:10.1097/COH.0000000000000298
2. Goedert JJ, Cote TR, Virgo P, Scoppa SM, Kingma DW, Gail MH, et al. Spectrum of AIDS-associated malignant disorders. *Lancet* (1998) 351(9119):1833–9. doi:10.1016/S0140-6736(97)09028-4
3. Chao C, Leyden WA, Xu L, Horberg MA, Klein D, Towner WJ, et al. Exposure to antiretroviral therapy and risk of cancer in HIV-infected persons. *AIDS* (2012) 26(17):2223–31. doi:10.1097/QAD.0b013e32835935b3
4. Biggar RJ, Chaturvedi AK, Goedert JJ, Engels EA; HIV/AIDS Cancer Match Study. AIDS-related cancer and severity of immunosuppression in persons with AIDS. *J Natl Cancer Inst* (2007) 99(12):962–72. doi:10.1093/jnci/djm010
5. Carbone A, Volpi CC, Gualeni AV, Gloghini A. Epstein-Barr virus associated lymphomas in people with HIV. *Curr Opin HIV AIDS* (2017) 12(1):39–46. doi:10.1097/COH.0000000000000333
6. Shiels MS, Cole SR, Kirk GD, Poole C. A meta-analysis of the incidence of non-AIDS cancers in HIV-infected individuals. *J Acquir Immune Defic Syndr* (2009) 52(5):611–22. doi:10.1097/QAI.0b013e3181b327ca
7. Robbins HA, Pfeiffer RM, Shiels MS, Li J, Hall HI, Engels EA. Excess cancers among HIV-infected people in the United States. *J Natl Cancer Inst* (2015) 107(4):dju503. doi:10.1093/jnci/dju503
8. Zucchetto A, Virdone S, Taborelli M, Grande E, Camoni L, Pappagallo M, et al. Non-AIDS-defining cancer mortality: emerging patterns in the late HAART era. *J Acquir Immune Defic Syndr* (2016) 73(2):190–6. doi:10.1097/QAI.0000000000001033
9. Rodger AJ, Lodwick R, Schechter M, Deeks S, Amin J, Gilson R, et al. Mortality in well controlled HIV in the continuous antiretroviral therapy arms of the SMART and ESPRIT trials compared with the general population. *AIDS* (2013) 27(6):973–9. doi:10.1097/QAD.0b013e32835cae9c
10. Engels EA, Biggar RJ, Hall HI, Cross H, Crutchfield A, Finch JL, et al. Cancer risk in people infected with human immunodeficiency virus in the United States. *Int J Cancer* (2008) 123(1):187–94. doi:10.1002/ijc.23487
11. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet* (2007) 370(9581):59–67. doi:10.1016/S0140-6736(07)61050-2
12. Borges AH, Dubrow R, Silverberg MJ. Factors contributing to risk for cancer among HIV-infected individuals, and evidence that earlier combination antiretroviral therapy will alter this risk. *Curr Opin HIV AIDS* (2014) 9(1):34–40. doi:10.1097/COH.0000000000000025
13. Nasi M, De Biasi S, Gibellini L, Bianchini E, Pecorini S, Bacca V, et al. Ageing and inflammation in patients with HIV infection. *Clin Exp Immunol* (2016) 187(1):44–52. doi:10.1111/cei.12814
14. Dittmer DP, Damania B. Kaposi sarcoma-associated herpesvirus: immunobiology, oncogenesis, and therapy. *J Clin Invest* (2016) 126(9):3165–75. doi:10.1172/JCI84418
15. Senapati R, Senapati NN, Dwibedi B. Molecular mechanisms of HPV mediated neoplastic progression. *Infect Agent Cancer* (2016) 11:59. doi:10.1186/s13027-016-0107-4
16. Carbone A, Cesarman E, Spina M, Gloghini A, Schulz TF. HIV-associated lymphomas and gamma-herpesviruses. *Blood* (2009) 113(6):1213–24. doi:10.1182/blood-2008-09-180315
17. Merchante N, Merino E, Lopez-Aldeguer J, Jover F, Delgado-Fernandez M, Galindo MJ, et al. Increasing incidence of hepatocellular carcinoma in HIV-infected patients in Spain. *Clin Infect Dis* (2013) 56(1):143–50. doi:10.1093/cid/cis777
18. Wieland U, Kreuter A. Merkel cell polyomavirus infection and Merkel cell carcinoma in HIV-positive individuals. *Curr Opin Oncol* (2011) 23(5):488–93. doi:10.1097/CCO.0b013e3283495a5b
19. De Paoli P, Carbone A. Microenvironmental abnormalities induced by viral cooperation: impact on lymphomagenesis. *Semin Cancer Biol* (2015) 34:70–80. doi:10.1016/j.semcancer.2015.03.009
20. Dolcetti R, Gloghini A, Caruso A, Carbone A. A lymphomagenic role for HIV beyond immune suppression? *Blood* (2016) 127(11):1403–9. doi:10.1182/blood-2015-11-681411
21. Wang L, Li G, Yao ZQ, Moorman JP, Ning S. microRNA regulation of viral immunity, latency, and carcinogenesis of selected tumor viruses and HIV. *Rev Med Virol* (2015) 25(5):320–41. doi:10.1002/rmv.1850
22. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int J Cancer* (1975) 16(2):230–9. doi:10.1002/ijc.2910160205
23. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* (1975) 16(2):216–29. doi:10.1002/ijc.2910160205
24. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* (1975) 5(2):117–21. doi:10.1002/eji.1830050208
25. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* (1975) 5(2):112–7. doi:10.1002/eji.1830050208
26. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* (2001) 22(11):633–40. doi:10.1016/S1471-4906(01)02060-9
27. Wu J, Lanier LL. Natural killer cells and cancer. *Adv Cancer Res* (2003) 90:127–56. doi:10.1016/S0065-230X(03)90004-2
28. Ferlazzo G, Thomas D, Lin SL, Goodman K, Morandi B, Muller WA, et al. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *J Immunol* (2004) 172(3):1455–62. doi:10.4049/jimmunol.172.3.1455
29. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* (2004) 5(12):1260–5. doi:10.1038/ni1138

30. Mocikat R, Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U, et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity* (2003) 19(4):561–9. doi:10.1016/S1074-7613(03)00264-4
31. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. *Nat Rev Immunol* (2001) 1(1):41–9. doi:10.1038/35095564
32. Specht A, DeGottardi MQ, Schindler M, Hahn B, Evans DT, Kirchhoff F. Selective downmodulation of HLA-A and -B by Nef alleles from different groups of primate lentiviruses. *Virology* (2008) 373(1):229–37. doi:10.1016/j.virol.2007.11.019
33. Lichtfuss GF, Cheng WJ, Farsakoglu Y, Paukovics G, Rajasuriar R, Velayudham P, et al. Virologically suppressed HIV patients show activation of NK cells and persistent innate immune activation. *J Immunol* (2012) 189(3):1491–9. doi:10.4049/jimmunol.1200458
34. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* (2004) 22:329–60. doi:10.1146/annurev.immunol.22.012703.104803
35. Tu MM, Mahmoud AB, Makrigiannis AP. Licensed and unlicensed NK cells: differential roles in cancer and viral control. *Front Immunol* (2016) 7:166. doi:10.3389/fimmu.2016.00166
36. Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci U S A* (2000) 97(6):2731–6. doi:10.1073/pnas.050588297
37. Street SE, Trapani JA, MacGregor D, Smyth MJ. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med* (2002) 196(1):129–34. doi:10.1084/jem.20020063
38. Zerafa N, Westwood JA, Cretney E, Mitchell S, Waring P, Iezzi M, et al. Cutting edge: TRAIL deficiency accelerates hematological malignancies. *J Immunol* (2005) 175(9):5586–90. doi:10.4049/jimmunol.175.9.5586
39. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* (2000) 356(9244):1795–9. doi:10.1016/S0140-6736(00)03231-1
40. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* (2012) 12(4):298–306. doi:10.1038/nrc3245
41. Curigliano G, Criscitiello C, Gelao L, Goldhirsch A. Molecular pathways: human leukocyte antigen G (HLA-G). *Clin Cancer Res* (2013) 19(20):5564–71. doi:10.1158/1078-0432.CCR-12-3697
42. Wu J. NKG2D ligands in cancer immunotherapy: target or not? *Austin J Clin Immunol* (2014) 1(1):2.
43. Lin A, Yan WH. HLA-G expression in cancers: roles in immune evasion, metastasis and target for therapy. *Mol Med* (2015) 21(1):782–91. doi:10.2119/molmed.2015.00083
44. Ndhlovu LC, Lopez-Verges S, Barbour JD, Jones RB, Jha AR, Long BR, et al. Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. *Blood* (2012) 119(16):3734–43. doi:10.1182/blood-2011-11-392951
45. Han G, Chen G, Shen B, Li Y. Tim-3: an activation marker and activation limiter of innate immune cells. *Front Immunol* (2013) 4:449. doi:10.3389/fimmu.2013.00449
46. Xu L, Huang Y, Tan L, Yu W, Chen D, Lu C, et al. Increased Tim-3 expression in peripheral NK cells predicts a poorer prognosis and Tim-3 blockade improves NK-cell-mediated cytotoxicity in human lung adenocarcinoma. *Int Immunopharmacol* (2015) 29(2):635–41. doi:10.1016/j.intimp.2015.09.017
47. Taborda NA, Hernandez JC, Lajoie J, Juno JA, Kimani J, Rugeles MT, et al. Short communication: low expression of activation and inhibitory molecules on NK cells and CD4(+) T cells is associated with viral control. *AIDS Res Hum Retroviruses* (2015) 31(6):636–40. doi:10.1089/AID.2014.0325
48. da Silva IP, Gallois A, Jimenez-Baranda S, Khan S, Anderson AC, Kuchroo VK, et al. Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. *Cancer Immunol Res* (2014) 2(5):410–22. doi:10.1158/2326-6066.CIR-13-0171
49. Swart M, Verbrugge I, Beltman JB. Combination approaches with immune-checkpoint blockade in cancer therapy. *Front Oncol* (2016) 6:233. doi:10.3389/fonc.2016.00233
50. Dahlberg CI, Sarhan D, Chrobok M, Duru AD, Alici E. Natural killer cell-based therapies targeting cancer: possible strategies to gain and sustain anti-tumor activity. *Front Immunol* (2015) 6:605. doi:10.3389/fimmu.2015.00605
51. Jones RB, Mueller S, O'Connor R, Rimpel K, Sloan DD, Karel D, et al. A subset of latency-reversing agents expose HIV-infected resting CD4+ T-cells to recognition by cytotoxic T-lymphocytes. *PLoS Pathog* (2016) 12(4):e1005545. doi:10.1371/journal.ppat.1005545
52. Rosario M, Liu B, Kong L, Collins LI, Schneider SE, Chen X, et al. The IL-15-based ALT-803 complex enhances FcgammaRIIIa-triggered NK-cell responses and in vivo clearance of B cell lymphomas. *Clin Cancer Res* (2016) 22(3):596–608. doi:10.1158/1078-0432.CCR-15-1419
53. Seay K, Church C, Zheng JH, Deneroff K, Ochsenbauer C, Kappes JC, et al. In vivo activation of human NK cells by treatment with an interleukin-15 superagonist potently inhibits acute in vivo HIV-1 infection in humanized mice. *J Virol* (2015) 89(12):6264–74. doi:10.1128/JVI.00563-15
54. Scully E, Alter G. NK cells in HIV disease. *Curr HIV/AIDS Rep* (2016) 13(2):85–94. doi:10.1007/s11904-016-0310-3
55. Luteijn R, Sciaranghella G, van Lunzen J, Nolting A, Dugast AS, Ghebremichael MS, et al. Early viral replication in lymph nodes provides HIV with a means by which to escape NK-cell-mediated control. *Eur J Immunol* (2011) 41(9):2729–40. doi:10.1002/eji.201040886
56. Zhou J, Amran FS, Kramski M, Angelovich TA, Elliott J, Hearps AC, et al. An NK-cell population lacking FcRgamma is expanded in chronically infected HIV patients. *J Immunol* (2015) 194(10):4688–97. doi:10.4049/jimmunol.1402448
57. Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, et al. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci U S A* (2005) 102(8):2886–91. doi:10.1073/pnas.0409872102
58. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med* (2012) 366(14):1275–86. doi:10.1056/NEJMoa1113425
59. Tomescu C, Chehimi J, Maino VC, Montaner LJ. NK-cell lysis of HIV-1-infected autologous CD4 primary T cells: requirement for IFN-mediated NK activation by plasmacytoid dendritic cells. *J Immunol* (2007) 179(4):2097–104. doi:10.4049/jimmunol.179.4.2097
60. Tomescu C, Mavilio D, Montaner LJ. Lysis of HIV-1-infected autologous CD4+ primary T cells by interferon-alpha-activated NK cells requires NKp46 and NKG2D. *AIDS* (2015) 29(14):1767–73. doi:10.1097/QAD.0000000000000777
61. Tomescu C, Tebas P, Montaner LJ. IFN-alpha augments NK-mediated antibody-dependent cellular cytotoxicity (ADCC) of HIV-1 infected autologous CD4+ T cells regardless of MHC-I downregulation. *AIDS* (2017) 31(5):613–22. doi:10.1097/QAD.0000000000001380
62. Mercier-Bataille D, Sanchez C, Baier C, Le Treut T, Mounier N, Mokhtari S, et al. Expression of activating receptors on natural killer cells from AIDS-related lymphoma patients. *AIDS Res Ther* (2014) 11:38. doi:10.1186/1742-6405-11-38
63. Bruno A, Focaccetti C, Pagani A, Imperatori AS, Spagnoletti M, Rotolo N, et al. The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer. *Neoplasia* (2013) 15(2):133–42. doi:10.1593/neo.121758
64. Li Z, Zhang LJ, Zhang HR, Tian GF, Tian J, Mao XL, et al. Tumor-derived transforming growth factor-beta is critical for tumor progression and evasion from immune surveillance. *Asian Pac J Cancer Prev* (2014) 15(13):5181–6. doi:10.7314/APJCP.2014.15.13.5181
65. Cerdeira AS, Rajakumar A, Royle CM, Lo A, Husain Z, Thadhani RI, et al. Conversion of peripheral blood NK cells to a decidual NK-like phenotype by a cocktail of defined factors. *J Immunol* (2013) 190(8):3939–48. doi:10.4049/jimmunol.1202582
66. Gibellini D, Zauli G, Re MC, Milani D, Furlini G, Caramelli E, et al. Recombinant human immunodeficiency virus type-1 (HIV-1) Tat protein sequentially up-regulates IL-6 and TGF-beta 1 mRNA expression and protein synthesis in peripheral blood monocytes. *Br J Haematol* (1994) 88(2):261–7. doi:10.1111/j.1365-2141.1994.tb05016.x

67. Sawaya BE, Thatikunta P, Denisova L, Brady J, Khalili K, Amini S. Regulation of TNFalpha and TGFbeta-1 gene transcription by HIV-1 Tat in CNS cells. *J Neuroimmunol* (1998) 87(1–2):33–42. doi:10.1016/S0165-5728(98)00044-7
68. Gigante M, Gesualdo L, Ranieri E. TGF-beta: a master switch in tumor immunity. *Curr Pharm Des* (2012) 18(27):4126–34. doi:10.2174/138161212802430378
69. Bruno A, Ferlazzo G, Albini A, Noonan DM. A think tank of TINK/TANKs: tumor-infiltrating/tumor-associated natural killer cells in tumor progression and angiogenesis. *J Natl Cancer Inst* (2014) 106(8):dju200. doi:10.1093/jnci/dju200
70. Liu Q, Sun Y, Rihn S, Nolting A, Tsoukas PN, Jost S, et al. Matrix metalloprotease inhibitors restore impaired NK-cell-mediated antibody-dependent cellular cytotoxicity in human immunodeficiency virus type 1 infection. *J Virol* (2009) 83(17):8705–12. doi:10.1128/JVI.02666-08
71. Azzoni L, Papasavvas E, Chehimi J, Kostman JR, Mounzer K, Ondercin J, et al. Sustained impairment of IFN-gamma secretion in suppressed HIV-infected patients despite mature NK-cell recovery: evidence for a defective reconstitution of innate immunity. *J Immunol* (2002) 168(11):5764–70. doi:10.4049/jimmunol.168.11.5764
72. Goodier MR, Imami N, Moyle G, Gazzard B, Gotch F. Loss of the CD56hiCD16- NK-cell subset and NK-cell interferon-gamma production during antiretroviral therapy for HIV-1: partial recovery by human growth hormone. *Clin Exp Immunol* (2003) 134(3):470–6. doi:10.1111/j.1365-2249.2003.02329.x
73. Chehimi J, Campbell DE, Azzoni L, Bacheller D, Papasavvas E, Jerandi G, et al. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. *J Immunol* (2002) 168(9):4796–801. doi:10.4049/jimmunol.168.9.4796
74. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. *J Pathol* (2008) 214(2):231–41. doi:10.1002/path.2276
75. Hutter G, Nowak D, Mossner M, Ganepola S, Mussig A, Allers K, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* (2009) 360(7):692–8. doi:10.1056/NEJMoa0802905
76. Ananworanich J, Robb ML. The transient HIV remission in the Mississippi baby: why is this good news? *J Int AIDS Soc* (2014) 17(1):19859. doi:10.7448/IAS.17.1.19859
77. Butler KM, Gavin P, Coughlan S, Rochford A, Donagh SM, Cunningham O, et al. Rapid viral rebound after 4 years of suppressive therapy in a sero-negative HIV-1 infected infant treated from birth. *Pediatr Infect Dis J* (2015) 34:235–40. doi:10.1097/INF.0000000000000570
78. Henrich TJ, Hanhauser E, Marty FM, Sirignano MN, Keating S, Lee TH, et al. Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: report of 2 cases. *Ann Intern Med* (2014) 161(5):319–27. doi:10.7326/M14-1027
79. Veluchamy JP, Spanholtz J, Tordoir M, Thijssen VL, Heideman DA, Verheul HM, et al. Combination of NK cells and cetuximab to enhance anti-tumor responses in RAS mutant metastatic colorectal cancer. *PLoS One* (2016) 11(6):e0157830. doi:10.1371/journal.pone.0157830
80. Schoofs T, Klein F, Braunschweig M, Kreider EF, Feldmann A, Nogueira L, et al. HIV-1 therapy with monoclonal antibody 3BNC117 elicits host immune responses against HIV-1. *Science* (2016) 352(6288):997–1001. doi:10.1126/science.aaf0972
81. Byrareddy SN, Arthos J, Cicala C, Villinger F, Ortiz KT, Little D, et al. Sustained virologic control in SIV+ macaques after antiretroviral and alpha-4beta7 antibody therapy. *Science* (2016) 354(6309):197–202. doi:10.1126/science.aag1276
82. Felices M, Lenvik TR, Davis ZB, Miller JS, Vallera DA. Generation of BiKEs and TriKEs to improve NK-cell-mediated targeting of tumor cells. *Methods Mol Biol* (2016) 1441:333–46. doi:10.1007/978-1-4939-3684-7_28
83. Gleason MK, Ross JA, Warlick ED, Lund TC, Verneris MR, Wiernik A, et al. CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets. *Blood* (2014) 123(19):3016–26. doi:10.1182/blood-2013-10-533398
84. Gleason MK, Verneris MR, Todhunter DA, Zhang B, McCullar V, Zhou SX, et al. Bispecific and trispecific killer cell engagers directly activate human NK cells through CD16 signaling and induce cytotoxicity and cytokine production. *Mol Cancer Ther* (2012) 11(12):2674–84. doi:10.1158/1535-7163.MCT-12-0692
85. Schmohl JU, Gleason MK, Dougherty PR, Miller JS, Vallera DA. Heterodimeric bispecific single chain variable fragments (scFv) killer engagers (BiKEs) enhance NK-cell activity against CD133+ colorectal cancer cells. *Target Oncol* (2016) 11(3):353–61. doi:10.1007/s11523-015-0391-8
86. Tay SS, Carol H, Biro M. TriKEs and BiKEs join CARs on the cancer immunotherapy highway. *Hum Vaccin Immunother* (2016) 12(11):2790–6. doi:10.1080/21645515.2016.1198455
87. Vallera DA, Felices M, McElmurry R, McCullar V, Zhou X, Schmohl JU, et al. IL15 trispecific killer engagers (TriKE) make natural killer cells specific to CD33+ targets while also inducing persistence, in vivo expansion, and enhanced function. *Clin Cancer Res* (2016) 22(14):3440–50. doi:10.1158/1078-0432.CCR-15-2710
88. Vallera DA, Zhang B, Gleason MK, Oh S, Weiner LM, Kaufman DS, et al. Heterodimeric bispecific single-chain variable-fragment antibodies against EpCAM and CD16 induce effective antibody-dependent cellular cytotoxicity against human carcinoma cells. *Cancer Biother Radiopharm* (2013) 28(4):274–82. doi:10.1089/cbr.2012.1329
89. Reiners KS, Kessler J, Sauer M, Rothe A, Hansen HP, Reusch U, et al. Rescue of impaired NK-cell activity in Hodgkin lymphoma with bispecific antibodies in vitro and in patients. *Mol Ther* (2013) 21(4):895–903. doi:10.1038/mt.2013.14
90. Singer H, Kellner C, Lanig H, Aigner M, Stockmeyer B, Oduncu F, et al. Effective elimination of acute myeloid leukemic cells by recombinant bispecific antibody derivatives directed against CD33 and CD16. *J Immunother* (2010) 33(6):599–608. doi:10.1097/CJI.0b013e3181dda225
91. Wiernik A, Foley B, Zhang B, Verneris MR, Warlick E, Gleason MK, et al. Targeting natural killer cells to acute myeloid leukemia in vitro with a CD16 x 33 bispecific killer cell engager and ADAM17 inhibition. *Clin Cancer Res* (2013) 19(14):3844–55. doi:10.1158/1078-0432.CCR-13-0505
92. Hudak JE, Canham SM, Bertozzi CR. Glycocalyx engineering reveals a Siglec-based mechanism for NK-cell immunoevasion. *Nat Chem Biol* (2014) 10(1):69–75. doi:10.1038/nchembio.1388
93. Xiao H, Woods EC, Vukojicic P, Bertozzi CR. Precision glycocalyx editing as a strategy for cancer immunotherapy. *Proc Natl Acad Sci U S A* (2016) 113(37):10304–9. doi:10.1073/pnas.1608069113
94. Arpinati M, Curti A. Immunotherapy in acute myeloid leukemia. *Immunotherapy* (2014) 6(1):95–106. doi:10.2217/imt.13.152
95. Bachanova V, Burns LJ, McKenna DH, Curtsinger J, Panoskaltsis-Mortari A, Lindgren BR, et al. Allogeneic natural killer cells for refractory lymphoma. *Cancer Immunol Immunother* (2010) 59(11):1739–44. doi:10.1007/s00262-010-0896-z
96. Brehm C, Huenecke S, Esser R, Kloess S, Quaiser A, Betz S, et al. Interleukin-2-stimulated natural killer cells are less susceptible to mycophenolate mofetil than non-activated NK cells: possible consequences for immunotherapy. *Cancer Immunol Immunother* (2014) 63(8):821–33. doi:10.1007/s00262-014-1556-5
97. Brehm C, Huenecke S, Quaiser A, Esser R, Bremm M, Kloess S, et al. IL-2 stimulated but not unstimulated NK cells induce selective disappearance of peripheral blood cells: concomitant results to a phase I/II study. *PLoS One* (2011) 6(11):e27351. doi:10.1371/journal.pone.0027351
98. Geller MA, Ivy JJ, Ghebre R, Downs LS Jr, Judson PL, Carson LF, et al. A phase II trial of carboplatin and docetaxel followed by radiotherapy given in a "Sandwich" method for stage III, IV, and recurrent endometrial cancer. *Gynecol Oncol* (2011) 121(1):112–7. doi:10.1016/j.ygyno.2010.12.338
99. Kloess S, Huenecke S, Piechulek D, Esser R, Koch J, Brehm C, et al. IL-2-activated haploidentical NK cells restore NKG2D-mediated NK-cell cytotoxicity in neuroblastoma patients by scavenging of plasma MICA. *Eur J Immunol* (2010) 40(11):3255–67. doi:10.1002/eji.201040568
100. Kottaridis PD, North J, Tsirogianni M, Marden C, Samuel ER, Jide-Banwo S, et al. Two-stage priming of allogeneic natural killer cells for the treatment of patients with acute myeloid leukemia: a phase I trial. *PLoS One* (2015) 10(6):e0123416. doi:10.1371/journal.pone.0123416
101. Penack O, Gentilini C, Fischer L, Asemissen AM, Scheibenbogen C, Thiel E, et al. CD56dimCD16neg cells are responsible for natural cytotoxicity against tumor targets. *Leukemia* (2005) 19(5):835–40. doi:10.1038/sj.leu.2403704

102. Stern M, Passweg JR, Meyer-Monard S, Esser R, Tonn T, Soerensen J, et al. Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers. *Bone Marrow Transplant* (2013) 48(3):433–8. doi:10.1038/bmt.2012.162
103. Ullrich E, Koch J, Cerwenka A, Steinle A. New prospects on the NKG2D/NKG2DL system for oncology. *Oncoimmunology* (2013) 2(10):e26097. doi:10.4161/onci.26097
104. Gunther S, Ostheimer C, Stangl S, Specht HM, Mozes P, Jesinghaus M, et al. Correlation of Hsp70 serum levels with gross tumor volume and composition of lymphocyte subpopulations in patients with squamous cell and adeno non-small cell lung cancer. *Front Immunol* (2015) 6:556. doi:10.3389/fimmu.2015.00556
105. Krause SW, Gastpar R, Andreesen R, Gross C, Ullrich H, Thonigs G, et al. Treatment of colon and lung cancer patients with ex vivo heat shock protein 70-peptide-activated, autologous natural killer cells: a clinical phase i trial. *Clin Cancer Res* (2004) 10(11):3699–707. doi:10.1158/1078-0432.CCR-03-0683
106. Specht HM, Ahrens N, Blankenstein C, Duell T, Fietkau R, Gaipl US, et al. Heat shock protein 70 (Hsp70) peptide activated natural killer (NK) cells for the treatment of patients with non-small cell lung cancer (NSCLC) after radiochemotherapy (RCTx) – from preclinical studies to a clinical phase II trial. *Front Immunol* (2015) 6:162. doi:10.3389/fimmu.2015.00162
107. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* (2002) 99(3):754–8. doi:10.1182/blood.V99.3.754
108. Goede V, Fischer K, Busch R, Engelke A, Eichhorst B, Wendtner CM, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *N Engl J Med* (2014) 370(12):1101–10. doi:10.1056/NEJMoa1313984
109. Junttila TT, Parsons K, Olsson C, Lu Y, Xin Y, Theriault J, et al. Superior in vivo efficacy of afucosylated trastuzumab in the treatment of HER2-amplified breast cancer. *Cancer Res* (2010) 70(11):4481–9. doi:10.1158/0008-5472.CAN-09-3704
110. Messersmith WA, Ahnen DJ. Targeting EGFR in colorectal cancer. *N Engl J Med* (2008) 359(17):1834–6. doi:10.1056/NEJMe0806778
111. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med* (2010) 363(14):1324–34. doi:10.1056/NEJMoa0911123
112. Benson DM Jr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116(13):2286–94. doi:10.1182/blood-2010-02-271874
113. Benson DM Jr, Cohen AD, Jagannath S, Munshi NC, Spitzer G, Hofmeister CC, et al. A phase I trial of the anti-KIR antibody IPH2101 and lenalidomide in patients with relapsed/refractory multiple myeloma. *Clin Cancer Res* (2015) 21(18):4055–61. doi:10.1158/1078-0432.CCR-15-0304
114. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat Immunol* (2014) 15(5):431–8. doi:10.1038/ni.2850
115. Gleason MK, Lenvik TR, McCullar V, Felices M, O'Brien MS, Cooley SA, et al. Tim-3 is an inducible human natural killer cell receptor that enhances interferon gamma production in response to galectin-9. *Blood* (2012) 119(13):3064–72. doi:10.1182/blood-2011-06-360321
116. Guo Y, Feng X, Jiang Y, Shi X, Xing X, Liu X, et al. PD1 blockade enhances cytotoxicity of in vitro expanded natural killer cells towards myeloma cells. *Oncotarget* (2016) 7(30):48360–74. doi:10.18632/oncotarget.10235
117. Vey N, Bourhis JH, Boissel N, Bordessoule D, Prebet T, Charbonnier A, et al. A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission. *Blood* (2012) 120(22):4317–23. doi:10.1182/blood-2012-06-437558
118. Westin JR, Chu F, Zhang M, Fayad LE, Kwak LW, Fowler N, et al. Safety and activity of PD1 blockade by pidilizumab in combination with rituximab in patients with relapsed follicular lymphoma: a single group, open-label, phase 2 trial. *Lancet Oncol* (2014) 15(1):69–77. doi:10.1016/S1470-2045(13)70551-5
119. Boissel L, Betancur M, Lu W, Wels WS, Marino T, Van Etten RA, et al. Comparison of mRNA and lentiviral based transfection of natural killer cells with chimeric antigen receptors recognizing lymphoid antigens. *Leuk Lymphoma* (2012) 53(5):958–65. doi:10.3109/10428194.2011.634048
120. Boissel L, Betancur-Boissel M, Lu W, Krause DS, Van Etten RA, Wels WS, et al. Retargeting NK-92 cells by means of CD19- and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity. *Oncoimmunology* (2013) 2(10):e26527. doi:10.4161/onci.26527
121. Chen X, Han J, Chu J, Zhang L, Zhang J, Chen C, et al. A combinational therapy of EGFR-CAR NK cells and oncolytic herpes simplex virus 1 for breast cancer brain metastases. *Oncotarget* (2016) 7(19):27764–77. doi:10.18632/oncotarget.8526
122. Chu J, Deng Y, Benson DM, He S, Hughes T, Zhang J, et al. CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance in vitro and in vivo antitumor activity against human multiple myeloma. *Leukemia* (2014) 28(4):917–27. doi:10.1038/leu.2013.279
123. Esser R, Muller T, Stefes D, Kloess S, Seidel D, Gillies SD, et al. NK cells engineered to express a GD2 -specific antigen receptor display built-in ADCC-like activity against tumour cells of neuroectodermal origin. *J Cell Mol Med* (2012) 16(3):569–81. doi:10.1111/j.1582-4934.2011.01343.x
124. Genssler S, Burger MC, Zhang C, Oelsner S, Mildenberger I, Wagner M, et al. Dual targeting of glioblastoma with chimeric antigen receptor-engineered natural killer cells overcomes heterogeneity of target antigen expression and enhances antitumor activity and survival. *Oncoimmunology* (2016) 5(4):e1119354. doi:10.1080/2162402X.2015.1119354
125. Han J, Chu J, Keung Chan W, Zhang J, Wang Y, Cohen JB, et al. CAR-engineered NK cells targeting wild-type EGFR and EGFRvIII enhance killing of glioblastoma and patient-derived glioblastoma stem cells. *Sci Rep* (2015) 5:11483. doi:10.1038/srep11483
126. Konstantinidis KV, Alici E, Aints A, Christensson B, Ljunggren HG, Dilber MS. Targeting IL-2 to the endoplasmic reticulum confines autocrine growth stimulation to NK-92 cells. *Exp Hematol* (2005) 33(2):159–64. doi:10.1016/j.exphem.2004.11.003
127. Liu H, Yang B, Sun T, Lin L, Hu Y, Deng M, et al. Specific growth inhibition of ErbB2 expressing human breast cancer cells by genetically modified NK92 cells. *Oncol Rep* (2015) 33(1):95–102. doi:10.3892/or.2014.3548
128. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* (2005) 105(8):3051–7. doi:10.1182/blood-2004-07-2974
129. Muller T, Uherek C, Maki G, Chow KU, Schimpf A, Klingemann HG, et al. Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. *Cancer Immunol Immunother* (2008) 57(3):411–23. doi:10.1007/s00262-007-0383-3
130. Nagashima S, Mailliard R, Kashii Y, Reichert TE, Herberman RB, Robbins P, et al. Stable transduction of the interleukin-2 gene into human natural killer cell lines and their phenotypic and functional characterization in vitro and in vivo. *Blood* (1998) 91(10):3850–61.
131. Ni Z, Knorr DA, Kaufman DS. Hematopoietic and nature killer cell development from human pluripotent stem cells. *Methods Mol Biol* (2013) 1029:33–41. doi:10.1007/978-1-62703-478-4_3
132. Oelsner S, Wagner J, Friede ME, Pfirrmann V, Genssler S, Rettinger E, et al. Chimeric antigen receptor-engineered cytokine-induced killer cells overcome treatment resistance of pre-B-cell acute lymphoblastic leukemia and enhance survival. *Int J Cancer* (2016) 139(8):1799–809. doi:10.1002/ijc.30217
133. Romanski A, Uherek C, Bug G, Seifried E, Klingemann H, Wels WS, et al. CD19-CAR engineered NK-92 cells are sufficient to overcome NK-cell resistance in B-cell malignancies. *J Cell Mol Med* (2016) 20(7):1287–94. doi:10.1111/jcmm.12810
134. Schirrmann T, Pecher G. Human natural killer cell line modified with a chimeric immunoglobulin T-cell receptor gene leads to tumor growth

inhibition in vivo. *Cancer Gene Ther* (2002) 9(4):390–8. doi:10.1038/sj.cgt.7700453
135. Schonfeld K, Sahm C, Zhang C, Naundorf S, Brendel C, Odendahl M, et al. Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor. *Mol Ther* (2015) 23(2):330–8. doi:10.1038/mt.2014.219
136. Tam YK, Maki G, Miyagawa B, Hennemann B, Tonn T, Klingemann HG. Characterization of genetically altered, interleukin 2-independent natural killer cell lines suitable for adoptive cellular immunotherapy. *Hum Gene Ther* (1999) 10(8):1359–73. doi:10.1089/10430349950018030
137. Uherek C, Tonn T, Uherek B, Becker S, Schnierle B, Klingemann HG, et al. Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. *Blood* (2002) 100(4):1265–73.
138. Woll PS, Grzywacz B, Tian X, Marcus RK, Knorr DA, Verneris MR, et al. Human embryonic stem cells differentiate into a homogeneous population of natural killer cells with potent in vivo antitumoractivity.*Blood*(2009)113(24):6094–101.doi:10.1182/blood-2008-06-165225
139. Yang B, Liu H, Shi W, Wang Z, Sun S, Zhang G, et al. Blocking transforming growth factor-beta signaling pathway augments antitumor effect of adoptive NK-92 cell therapy. *Int Immunopharmacol* (2013) 17(2):198–204. doi:10.1016/j.intimp.2013.06.003
140. Zhang J, Sun R, Wei H, Zhang J, Tian Z. Characterization of interleukin-15 gene-modified human natural killer cells: implications for adoptive cellular immunotherapy. *Haematologica* (2004) 89(3):338–47.
141. Zhang J, Sun R, Wei H, Zhang J, Tian Z. Characterization of stem cell factor gene-modified human natural killer cell line, NK-92 cells: implication in NK-cell-based adoptive cellular immunotherapy. *Oncol Rep* (2004) 11(5):1097–106. doi:10.3892/or.11.5.1097
142. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* (2014) 32(4):347–55. doi:10.1038/nbt.2842
143. Garrido C, Spivak AM, Soriano-Sarabia N, Checkley MA, Barker E, Karn J, et al. HIV latency-reversing agents have diverse effects on natural killer cell function. *Front Immunol* (2016) 7:356. doi:10.3389/fimmu.2016.00356

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 January 2017
doi: 10.3389/fimmu.2016.00687

MICA Expression Is Regulated by Cell Adhesion and Contact in a FAK/Src-Dependent Manner

Gerald Moncayo†, Da Lin, Michael T. McCarthy, Aleksandra A. Watson† and Christopher A. O'Callaghan**

Henry Wellcome Building for Molecular Physiology, University of Oxford, Oxford, UK

MICA is a major ligand for the NKG2D immune receptor, which plays a key role in activating natural killer (NK) cells and cytotoxic T cells. We analyzed NKG2D ligand expression on a range of cell types and could demonstrate that MICA expression levels were closely linked to cellular growth mode. While the expression of other NKG2D ligands was largely independent of cell growth mode, MICA expression was mainly found on cells cultured as adherent cells. In addition, MICA surface expression was reduced through increase in cell–cell contact or loss of cell–matrix adherence. Furthermore, we found that the reduction in MICA expression was modulated by focal adhesion kinase (FAK)/Src signaling and associated with increased susceptibility to NK cell-mediated killing. While the mechanisms of tumor immune evasion are not fully understood, the reduction of MICA expression following loss of attachment poises a potential way by which metastasizing tumor cells avoid immune detection. The role of FAK/Src in this process indicates a potential therapeutic approach to modulate MICA expression and immune recognition of tumor cells during metastasis.

Keywords: MICA, NKG2D, FAK/Src, adhesion, contact, metastasis

OPEN ACCESS

Edited by:
Sandra Laurence Lopez-Verges, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama

Reviewed by:
Nadia Guerra, Imperial College London, UK
Kamalakannan Rajasekaran, Genentech Foundation, USA
Courtney Crane, University of Washington, USA

****Correspondence:***
Gerald Moncayo
gmoncayo@indicasat.org.pa;
Christopher A. O'Callaghan
chris.ocallaghan@ndm.ox.ac.uk

†Present address:
Gerald Moncayo, Instituto de Investigaciones Científicas Y Servicios de Alta Tecnología (INDICASAT-AIP), Clayton, Ciudad del Saber, Panama;
Aleksandra A. Watson, Department of Biochemistry, University of Cambridge, Cambridge, UK

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *24 September 2016*
Accepted: *23 December 2016*
Published: *19 January 2017*

Citation:
Moncayo G, Lin D, McCarthy MT, Watson AA and O'Callaghan CA (2017) MICA Expression Is Regulated by Cell Adhesion and Contact in a FAK/Src-Dependent Manner. Front. Immunol. 7:687. doi: 10.3389/fimmu.2016.00687

BULLET POINT SUMMARY

1. MICA was predominantly expressed on adherent cell lines, and this expression was reduced in the absence of cell-surface adherence.
2. MICA downregulation with loss of adherence was modulated through FAK/Src.
3. Cell–cell contact, influenced by extracellular calcium availability, regulated MICA expression.
4. Cellular growth mode modulated NK cell-mediated cytotoxicity, therefore affecting NK cell recognition of metastatic cells.

INTRODUCTION

Tumors are complex networks of cells that can interact with the extracellular matrix and neighboring normal tissues (1). Alterations in cell–cell and cell–matrix interactions are classic phenomena that occur during cancer development and affect disease progression (2). As adherent cancer-derived cell lines lose contact with their underlying surface, they tend to aggregate into three-dimensional tumor-like masses that recapitulate some features of *in vivo* tumor growth (3). These "spheroids" reflect aspects of solid tumors in morphology, compact organization, growth dynamics, capacity to

develop a necrotic core, proliferation in the periphery, induction of hypoxia, and increased resistance to chemo- and radiotherapy (4, 5). Spheroids are therefore useful *in vitro* models of cancers for a range of studies (4).

Integrins are major cell–matrix adhesion receptors (6). During adhesion and migration, integrins activate a range of signal transduction molecules, such as focal adhesion kinase (FAK) and the Rous sarcoma oncogene family (Src) (6, 7). FAK and Src signal through PI3K/Akt(PKB)/GSK-3/mTOR (8) and the Ras/Raf-1/ERK pathways (9), and their expression is often deregulated in cancers. Cell–cell adhesion is mediated by proteins including cadherins, immunoglobulin superfamily proteins, EGF family members, C-type lectins, and proteins containing leucine-rich repeats (6, 10). Cell–cell and cell–matrix adhesion receptors participate in intracellular communication linked to the cytoskeleton, affecting cell shape and polarity, cytoplasmic organization, cell motility, intracellular signal transduction, cancer progression, and metastasis (6).

Natural killer (NK), cytotoxic T cells, and gamma-delta T cells are critical cellular effectors of the immune system, which can recognize and kill virus-infected and tumor-transformed cells and can also release chemokines and cytokines, such as tumor necrosis factor-alpha (TNF-α) (11). NK cell activity is modulated by signaling through a complex balance of ligand–receptor interactions (12). Inhibitory receptors recognize a range of ligands including MHC class I molecules and thereby hinder cytotoxicity against normal self-tissues (13).

NKG2D is a key activating receptor expressed on NK cells, cytotoxic T cells, and gamma-delta T cells, which recognizes a variety of ligands including MHC class I-related chain (MIC)-A and -B (14) and the UL16 binding proteins (ULBPs) (15). A wide range of stresses have been shown to modulate the expression of these ligands, including viral infection, oxidative stress (16), heat shock (17), TNF-α (18), metalloproteases that regulate the release of the soluble forms (19), DNA damage, and cell cycle modulators (20). The surface expression of these ligands must be closely regulated to avoid an inappropriate immune assault on otherwise healthy cells. Conversely, if tumors or transformed cells do not express these ligands, this will facilitate their escape from recognition.

This study demonstrates that MICA, a key activating ligand for NKG2D, is mainly expressed on adherent cells and that this expression is reduced upon loss of surface attachment and increased cell–cell contact, underscoring the importance of the FAK/Src signaling pathway in modulating MICA expression. Reduced MICA expression upon loss of attachment or increased cell–cell contact results in reduced susceptibility to NK cell killing, suggesting a mechanism whereby metastasizing tumor cells may evade immune recognition.

RESULTS

MICA Is Mainly Expressed in Adherent Cell Lines

A range of human cell lines of different (mainly cancer-derived) origins which were cultured adherently or in suspension were screened by flow cytometry for MICA surface expression. Most adherent cell types tested expressed moderate to high levels of MICA (**Figure 1A**; Figure S1A in Supplementary Material), including two primary adherent non-cancer cell types growing as monolayers (fibroblasts and normal human astrocytes). In contrast, MICA surface expression was absent or low in most of the suspension cell lines tested (**Figure 1B**; Figure S1B in Supplementary Material). This was not the case for other NKG2D ligands as ULBPs were often found in suspension cell lines, while MICB was not always expressed in adherent cells (**Figure 1C**).

MICA Expression Is Reduced in the Absence of Cell-Surface Adherence

When adherent tumor cell lines are cultured as monolayers, a small proportion of cells detach from the adherent monolayer (Figure S1C in Supplementary Material). To examine whether loss of surface attachment showed a correlation with a reduction in MICA expression, we analyzed MICA expression on adherent cells and their non-adherent counterparts which had detached and were floating in suspension. Up to 60% of cells found in each suspension sample were alive as assessed by propidium iodide staining, and these live cells expressed lower levels of MICA on their surface compared to their adherent counterparts in the same media across a range of cell types (**Figure 2A**).

Cells which are normally adherent can be forced to grow in suspension by culture over agarose-coated plates, to which they cannot adhere. Under these conditions, they may form multicellular masses called spheroids (Figure S1D in Supplementary Material) (4). To investigate whether loss of surface adhesion would modulate MICA surface expression, cells were cultured as spheroids and MICA expression was compared to the equivalent surface adherent cells. Cells cultured under adherent conditions expressed high levels of surface MICA, but when surface adherence was prevented by culture in agarose-coated plates, MICA expression was reduced substantially (**Figure 2B**).

Culture in Suspension Also Alters HLA-A, B, C and MICB, ULBP2, and ULBP3 Expression

As NKG2D has several human ligands, we examined whether growth in suspension affected the surface expression levels of the other NKG2D ligands on U2OS and HeLa cells (Figure S2 in Supplementary Material). Forcing HeLa cells to grow in suspension by culture on agarose resulted in lower levels of expression of ULBP2 and ULBP3 compared to the levels seen on adherent cells, although the effect was less striking than that observed for MICA. MICB and ULBP1 surface expression levels were very low, but even this expression was reduced in suspension cells (Figure S2A in Supplementary Material). As a control, cell-surface expression of the non-NKG2D ligands HLA-A, B, C was assessed on adherent and suspension cultured cells. After growth in suspension, HLA-A, B, C expression was lower than when grown in an adherent fashion but was still very high compared to NKG2D ligands. The same was the case with U2OS cells, which do not express MICB and ULBP1 (Figure S2B in Supplementary Material). In summary, several cell lines expressed lower levels of NKG2D

ligands when grown in suspension compared to when cultured as an adherent monolayer.

MICA Downregulation with Loss of Adherence Is Not Caused by Hypoxia, Apoptosis, or Metalloprotease Action

Hypoxic environments may be found in the center of spheroids (21), but neither hypoxia nor 24 h of hypoxia followed by 24 h of reoxygenation affected MICA surface expression (Figure S3A in Supplementary Material). Similar results were observed following a full hypoxic stimulus with anoxia (0.1% oxygen) in adherent U2OS, HeLa, and 786-O cells (Figure S3B in Supplementary Material). Furthermore, although 786-O cells are deficient in HIF-1a, which is the key transcription factor in the hypoxic response, these cells still demonstrate reduced MICA expression when grown as spheroids compared to when grown as adherent cells. This pattern was unaffected by hypoxia. Therefore, we found no evidence that hypoxia triggered a reduction in MICA expression in adherent cells.

Loss of adherence can induce cell death through mechanisms involving p53, ATM, and caspase activation. In the experiments detailed above, the analysis was restricted to viable cells as identified by propidium iodide staining. To investigate whether the induction of apoptosis was responsible for the reduction in MICA expression, spheroids were assessed after culture with the p53 inhibitor pifithrin alpha or the caspase inhibitor z-vad (Figure S3C in Supplementary Material). Treatment with these inhibitors did not prevent the downregulation of MICA expression observed on spheroids. This suggests that the downregulation of MICA expression on spheroids is not due to the induction of apoptosis.

Treatment of cells with the metalloprotease inhibitor GM6001 is known to increase MICA expression at the cell surface (22). To test if the downregulation of MICA observed in spheroids was due to metalloprotease shedding of MICA, spheroids and confluent adherent cells were treated with the broad spectrum metalloprotease inhibitor GM6001 during culture. GM6001 had no effect on surface MICA expression of spheroids (Figure S3D in Supplementary Material) suggesting that the observed reduction in MICA was not due to metalloprotease action.

Cell–Cell Contact Downregulates MICA Expression

It has previously been reported that MICA is downregulated on normal fibroblasts upon cell contact (23). We were interested to investigate whether this would also be the case in tumor cell lines

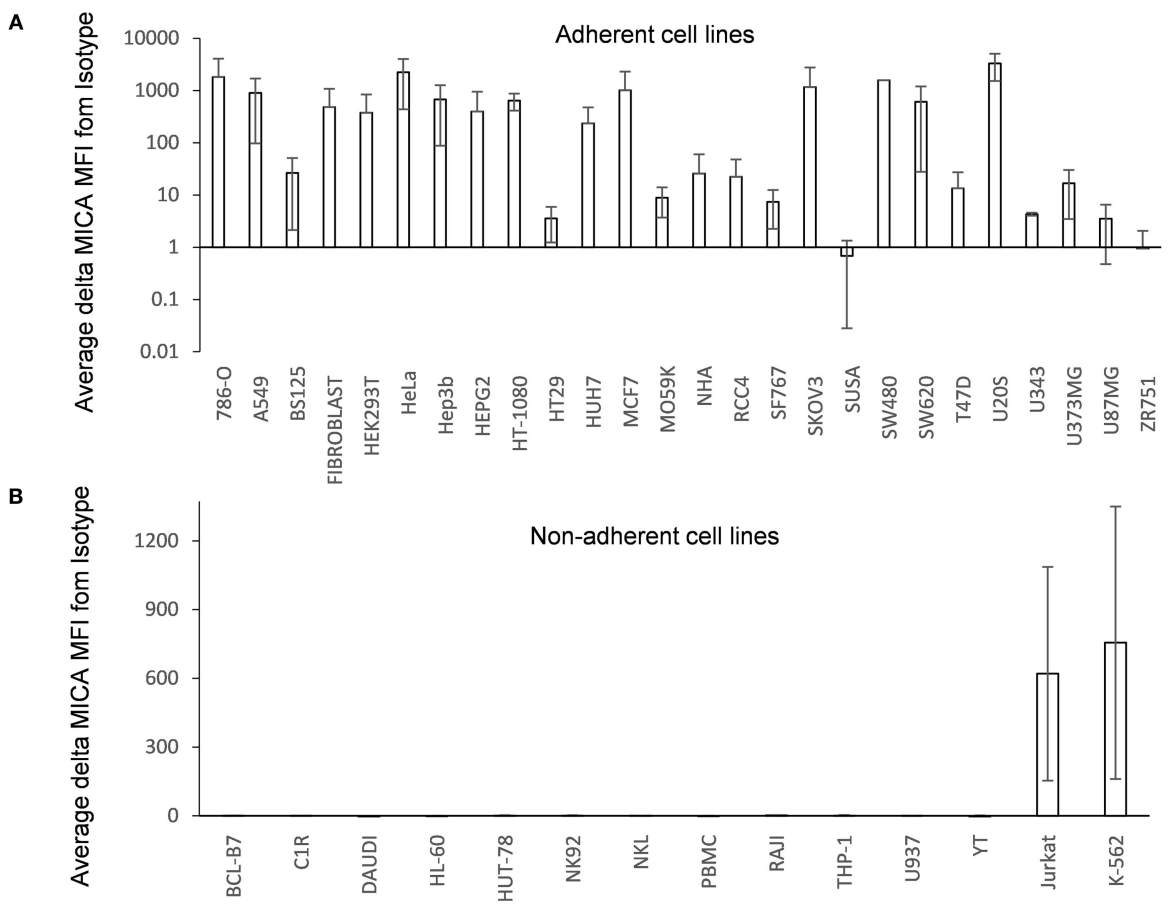


FIGURE 1 | Continued

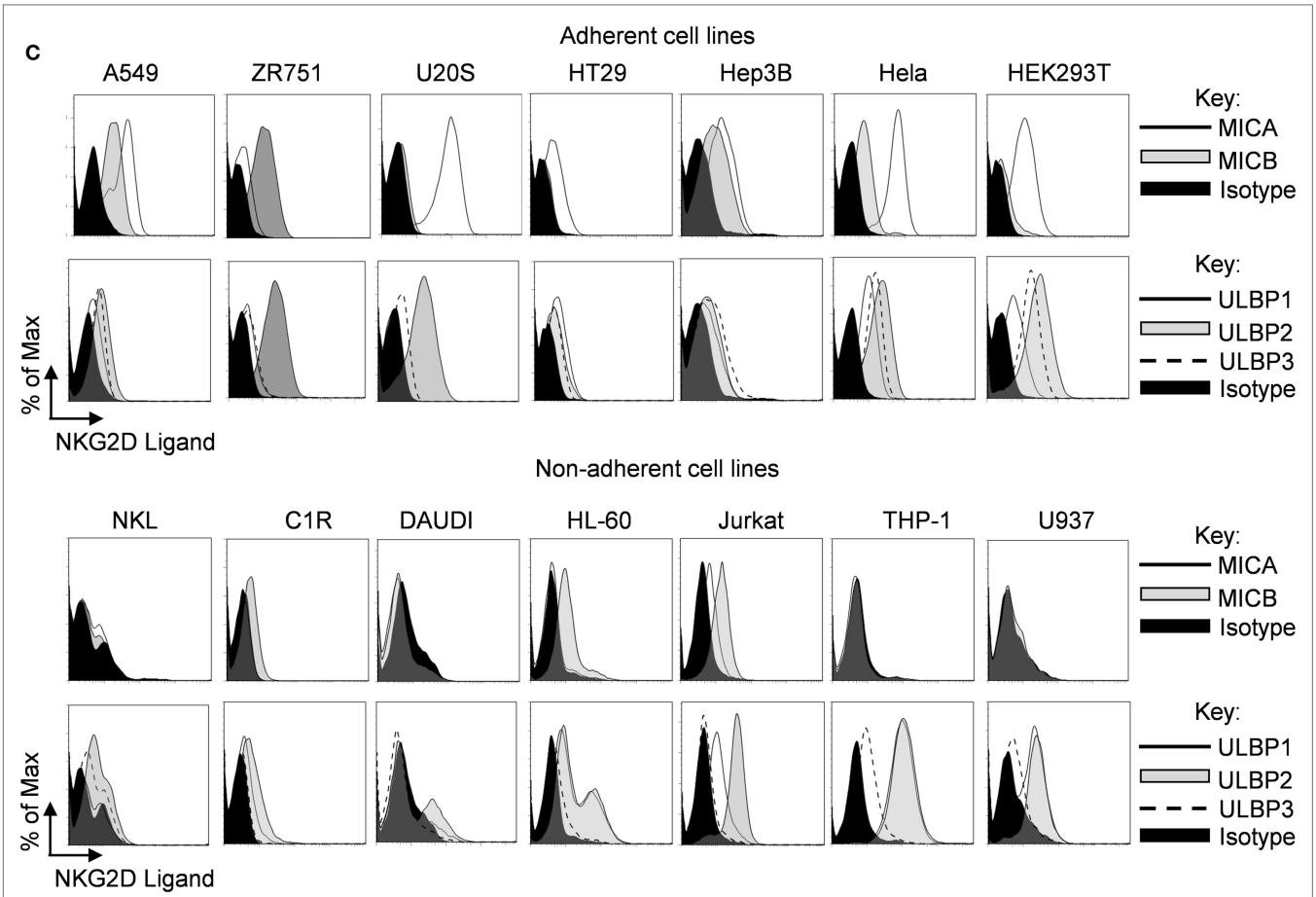


FIGURE 1 | **MICA surface expression on adherent and suspension cells**. **(A)** Adherent and **(B)** non-adherent human cell lines were screened for MICA expression using the anti-MICA monoclonal antibody 2C10. Surface expression was analyzed by flow cytometry. The mean delta MFI as MFI (MICA) − MFI (ISOTYPE) is displayed in the table. Error bars represent the SD of $n > 3$ independent experiments. **(C)** Adherent and suspension cell lines were cultured under normal conditions and stained for MICA or MICB or UL16 binding proteins 1–3 or with an isotype control antibody. Dead cells were excluded using propidium iodide staining. $n = 3$ independent experiments.

and what would be the mechanism for this regulation. Spheroids differ from adherent monolayers not only in their loss of cell–matrix interactions but also in their increased cell–cell interactions. Adherent cells were cultured at increasing cell density, and MICA expression was measured by flow cytometry (**Figure 3A**). Additionally, cell density regulated MICA mRNA expression was also demonstrated by semiquantitative RT-PCR (**Figure 3B**).

Furthermore, we wished to investigate whether this reduction of MICA surface expression was due to increased adherent cell–cell contact or was an indirect consequence of the addition of more cells, for example, by reducing the availability of nutrients. Therefore, CFSE-labeled U2OS cells were cocultured under non-confluent (10%) conditions with non-adherent, non-activated U937 monocytes or with U937 monocytes that had been activated with 20 nM PMA for 24 h to render them adherent. Coculture with activated adherent U937 monocytes reduced MICA expression on U2OS cells, while coculture with non-activated non-adherent monocytes had no effect on MICA expression (**Figure 3C**). Furthermore, coculture with adherent HeLa, HT29, and HEK293T cells also reduced MICA expression (**Figure 3D**), while culture with non-adherent DAUDI, K-562, and Jurkat cells had no effect on MICA expression (**Figure 3E**). Together, these results indicate that increased cell–cell contact or cell density of adherent cells is correlated with reduced MICA surface expression on a range of cancer cell lines and indicates that the reduction in MICA expression observed at high cell densities is not simply due to factors such as the reduced availability of nutrients. Therefore, we conclude that, in addition to causing loss of adherence, cell–cell contact also inhibits MICA expression.

MICA Expression Is Influenced by Extracellular Calcium

Calcium-dependent cellular adhesion molecules are essential to cell contact mechanisms (24). To investigate the nature of the cell–cell interactions that regulate MICA expression, spheroids were treated with the calcium chelating agent EDTA. Treatment with up to 5 mM EDTA increased MICA expression on spheroids

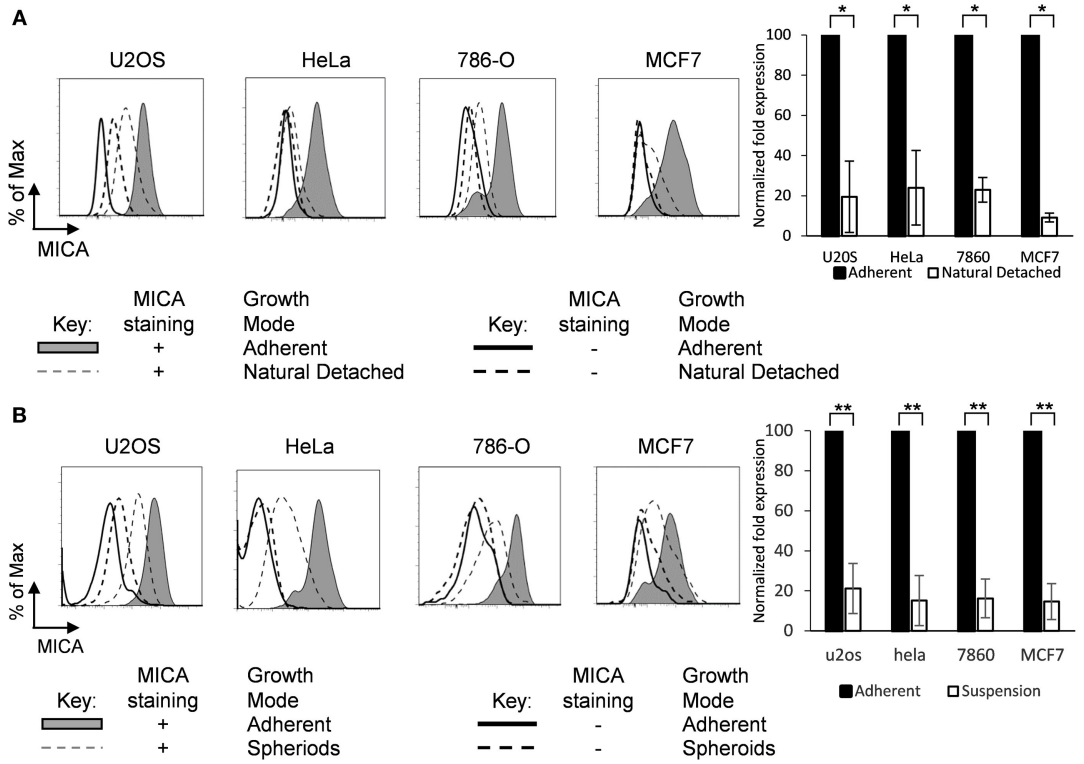


FIGURE 2 | **MICA is expressed on adherent cells and loss of adhesion downregulates surface expression**. **(A)** Adherent cells (shaded gray histogram) and the cells which had detached from the surface within the cultures ("natural detached," dashed gray line) were stained for MICA or with an isotype control antibody. **(B)** Adherent cell lines were cultured over agarose-coated plates for 5 days to force formation of non-adherent spheroids (dashed gray line) or were cultured under non-confluent adherent conditions (shaded gray histogram) and stained for MICA surface expression (isotype control—black line for adherent cells or dashed black line for non-adherent cells). *n* = 3 independent experiments.

(**Figure 4A**). Interestingly, we observed a concentration-dependent adhesion and contact regulation of MICA expression in adherent cells (**Figures 4B,C**). At 5 mM EDTA, cells detached from the surface and MICA levels were reduced. At the lower concentration of 1–2 mM EDTA treatment, where calcium-dependent cell–cell contact was partially disrupted but cells remained attached to the surface, we observed an increase in the levels of MICA expression. In addition, the more confluent cells are, the less contact area each cell can have with the underlying surface (see **Figure 4C** untreated vs. 2mM EDTA). Overall, these findings suggest that calcium-dependent cell–matrix adherence promotes MICA expression, while calcium-dependent cell–cell contact inhibits MICA expression.

MICA Protein Expression Levels Are Reduced after Confluency and Forced Suspension Correlating with FAK Activation

Focal adhesion kinase and Src have been shown to be essential for cell survival following loss of surface attachment (25). To test if FAK was activated after loss of adhesion or increased cell contact, U2OS cells were grown either un-confluent, over-confluent or under force suspension for 5 days and cell extracts taken. At day 5, MICA levels were clearly downregulated both at high confluency and at forced suspension (**Figure 5A**). However, at this time point, FAK phosphorylation levels were clearly reduced. In contrast, p21 levels were found increased in confluent and forced suspension treatments. We therefore elected to look at earlier timepoints. At 1 day after confluency and forced suspension, we observed that FAK phosphorylation levels were indeed increased (**Figure 5B**).

FAK/Src Downregulates MICA Expression

We then sought to determine whether modulating FAK activation would regulate MICA surface expression. Treatment of spheroids with the FAK/Src inhibitors tyrphostin AG82 or PP2 increased MICA expression (**Figure 6A**). MICA expression was also increased by treatment of spheroids with manumycin, an inhibitor of Ras, which is downstream of FAK (**Figure 6A**). We then investigated whether FAK expression could influence the surface expression of MICA. Overexpression of FRNK, a dominant negative FAK mutant, had only a small effect on MICA expression in non-confluent adherent cells, but overexpression of FAK reduced surface levels of MICA (**Figure 6B**). Transfected cells were also resuspended over agarose for 2 days, and then MICA expression was analyzed. Here, we could see that the FRNK transfected cells had higher MICA expression compared to mock-treated suspension cells. These results indicated that inhibition of FAK/

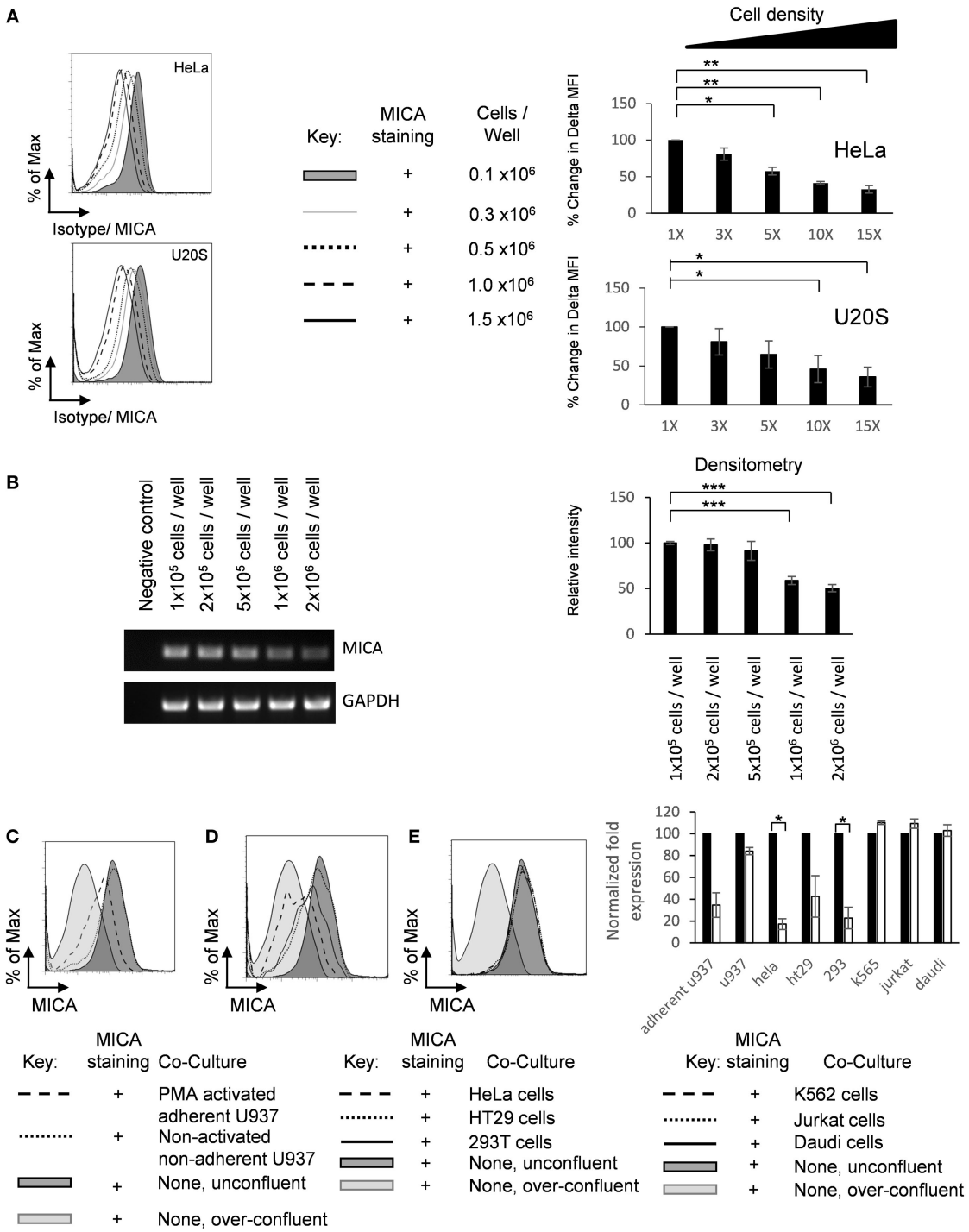


FIGURE 3 | **Adherent cell–cell contact downregulates MICA expression**. **(A)** HeLa and U2OS cells were seeded into six-well plate at indicated cell density, cultured for 72 h, and analyzed for surface MICA expression by flow cytometry $n = 3$. **(B)** U2OS cells were plated at increasing densities and MICA mRNA analyzed compared to a GAPDH control. Normalized quantification is shown in the bar chart. $n = 3$. **(C)** U2OS cells were stained with CFSE and plated at 10% confluence (shaded dark gray histogram), over-confluent (shaded light gray histogram), or cocultured with either PMA-activated adherent U937 cells (dashed histogram) or non-adherent U937 cells (dotted histogram). **(D)** These cells were also grown in coculture with adherent HeLa cells (dashed histogram), adherent HT29 cells (dotted black histogram), or adherent HEK293T cells (solid histogram). **(E)** U2OS cells were also grown with suspension cells K-562 cells (dashed histogram), Jurkat cells (dotted histogram), or Daudi cells (solid histogram). Forty-eight hours later, CFSE-stained U2OS cells were analyzed for MICA expression. $n = 3$ independent experiments.

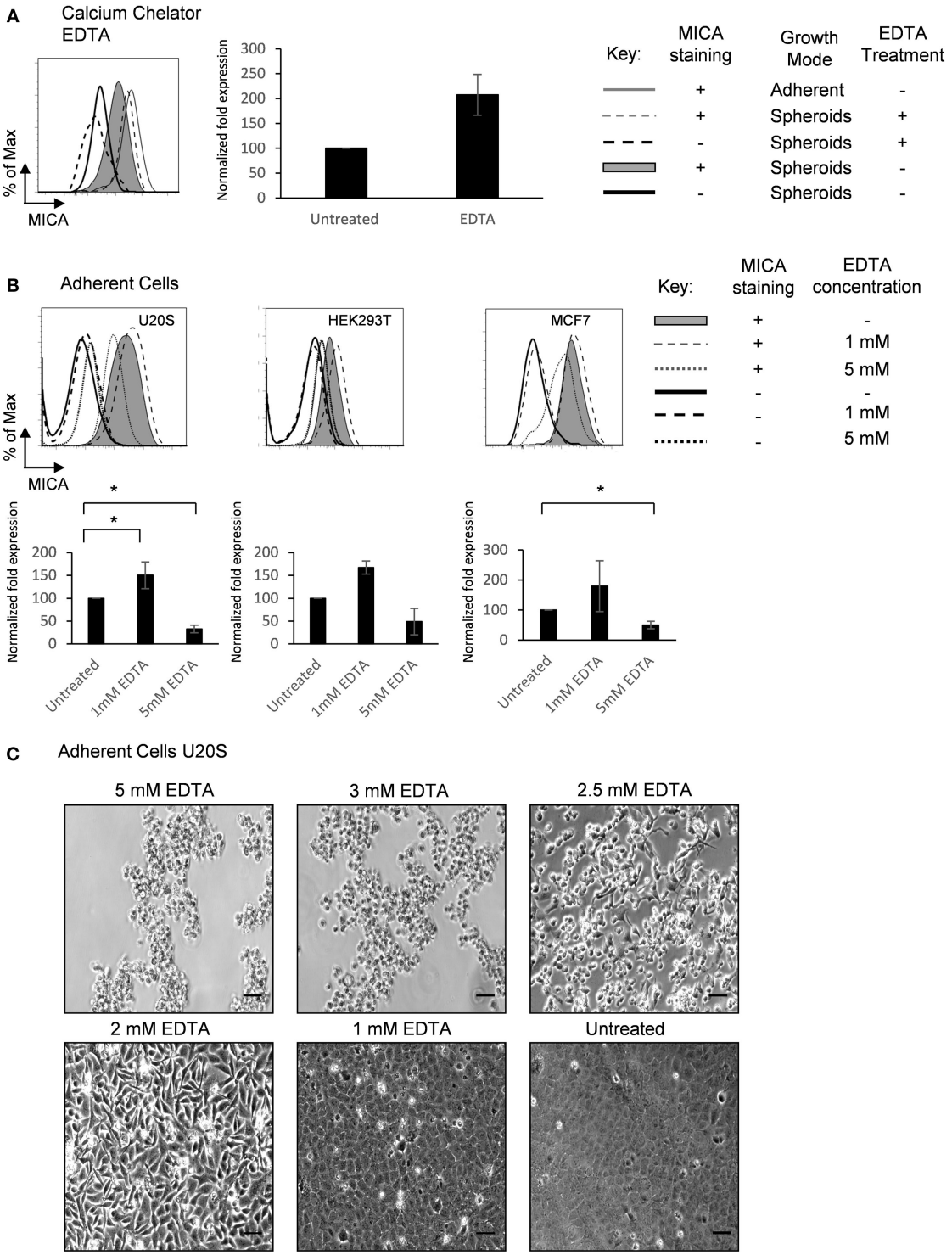


FIGURE 4 | **Extracellular calcium availability modulates MICA surface expression**. **(A)** U2OS cells were cultured either adherently (gray line) or in suspension as spheroids in the presence (dashed gray line; isotype control—dashed black line) or absence of 5 mM EDTA (shaded gray histogram; isotype control—black line) and MICA surface expression analyzed. $n = 4$. **(B)** Adherent U2OS, HEK293T, and MCF7 cells were either left untreated (shaded gray histogram; isotype control—black line) or treated with 1 mM (dashed gray line; isotype control—dashed black line) or 5 mM EDTA (dotted gray line; isotype control—dotted black line) and MICA surface expression analyzed. $n = 4$. **(C)** Images of adherent U2OS cells treated with 5, 3, 2.5, 2, and 1 mM EDTA for 48 h or left untreated. Bar represents 50 μm. $n = 3$.

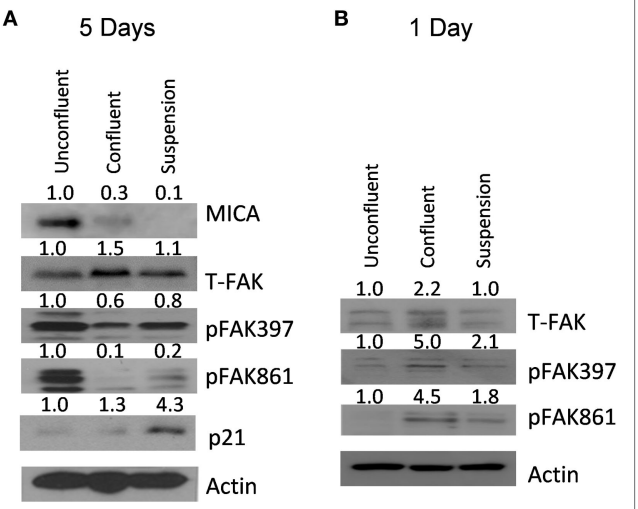


FIGURE 5 | MICA protein expression levels are reduced after confluency and forced suspension correlating with focal adhesion kinase (FAK) activation. **(A)** U2OS cells were either grown subconfluently, confluently or as spheroids for 5 days and MICA, p21, beta-actin, FAK, and FAK tyr 397 and tyr 861 phosphorylation measured by western blot. **(B)** U2OS cells were either grown subconfluently, confluently or as spheroids for 1 day and beta-actin, FAK, and FAK tyr 397 and tyr 861 phosphorylation measured by western blot.

Src signaling leads to increased levels of MICA in spheroids, suggesting that FAK/Src can play a role in regulating MICA expression. Adherent cells transfected with the kinase deficient mutant FAKY397F also showed a slight upregulation in MICA expression (**Figure 6C**). Additionally, knockdown of FAK with shRNA led to upregulation of MICA expression compared to control scramble shRNA (**Figure 6D**). FAK and shRNA expression was monitored through EGFP expression on an IRES of the plasmids. To further investigate whether FAK activation could reduce MICA expression on adherent cells, cells were cultured with the FAK activators acrylamide (26), colchicine, and lysophosphatidic acid (LPA) (27). MICA expression was reduced following treatments with these FAK activators (**Figure 6E**). Treatment of adherent cells with the FAK/Src inhibitor tyrphostin AG82 and PF 573228 upregulated MICA surface expression (**Figure 6F**). Together, these data show that FAK/Src can downregulate MICA expression in adherent cells.

Cellular Growth Mode Influences NK Cell-Mediated Cytotoxicity

Next, we tested whether the different levels of MICA expression observed on spheroids, confluent adherent cells, and non-confluent adherent cells were functionally relevant and whether they influenced susceptibility to NK cell-mediated killing. Spheroids, confluent, and non-confluent adherent cells were incubated with NK cells in a chromium-release killing assay. The non-confluent adherent cells with the highest MICA expression were more susceptible to NK cell killing than confluent adherent cells. Suspension spheroids were killed less readily than the confluent adherent cells (**Figure 7A**). Treatment with anti-NKG2D antibody significantly reduced the susceptibility of non-confluent cells to NK cell-mediated killing (**Figure 7B**). In line with the observed reduction in MICA expression, treatment with the NKG2D blocking antibody had no significant impact on spheroid susceptibility. MICA expression therefore correlates with susceptibility to NK cell killing.

To confirm whether NK cell susceptibility was modulated by FAK/Src activity, adherent cells were treated with the FAK/Src inhibitor AG82 to increase MICA expression and these cells were then exposed to NK cells. AG82-treated U2OS and HeLa cells were more susceptible to NK cell-mediated killing than mock-treated cells (**Figures 7C,D**). Therefore, FAK/Src signaling inhibition results in increased susceptibility to NK cell killing.

In Vivo MICA Expression of Human in Mice Xenograft

All previous experiments were done *in vitro* with well-established cell lines, but we were interested to see if what we saw *in vitro* could be translated *in vivo*. For this, we selected a cell line, SW620 adenocarcinoma, derived from a metastatic lymph node tumor (28). We first confirmed that this cell line could also downregulate MICA expression after loss of adhesion (**Figure 8A**). We then stained an SW620 in nude mice xenograft for MICA expression. Here, we could see that the surface of the tumor had a stronger MICA expression than at the core (**Figure 8B**), recapitulating the *in vitro* results. Therefore, our observation may be especially important, as treatments that upregulate MICA expression in spheroids may upregulate MICA expression *in vivo* where tumor MICA expression is often negligible.

DISCUSSION

MICA is an important ligand for the immune receptor NKG2D, which plays an activating role in NK and T cells. This study demonstrates that MICA expression is strongly influenced by cell-surface adhesion and cell–cell contact and this has implications for understanding the immunopathogenesis of cancer metastasis.

We initially observed that MICA was expressed on almost all adherent cell lines, but expression was low or absent on cell lines which normally grow in suspension. One of the MICA expressing suspension cell lines was K-562, which expresses the BCR-ABL fusion oncoprotein which has been reported to induce MICA expression directly (29). MICB expression in contrast was less often found on adherent cell lines and on more suspension cell lines like HL-60. ULBP2 was found in almost all cell lines regardless of whether they were adherent or not. While adherent cells were shown to express high levels of MICA, MICA levels were considerably reduced on cells that had detached from the surface under the same culture conditions. Forcing cells to grow in suspension as spheroids through culture over agarose led to a downregulation of MICA expression.

Focal adhesion kinase and Src play key roles in cell–cell contact and have been shown to be essential for growth in suspension by inhibition of anoikis, which is death resulting from loss of matrix attachment (25, 30). We investigated whether the

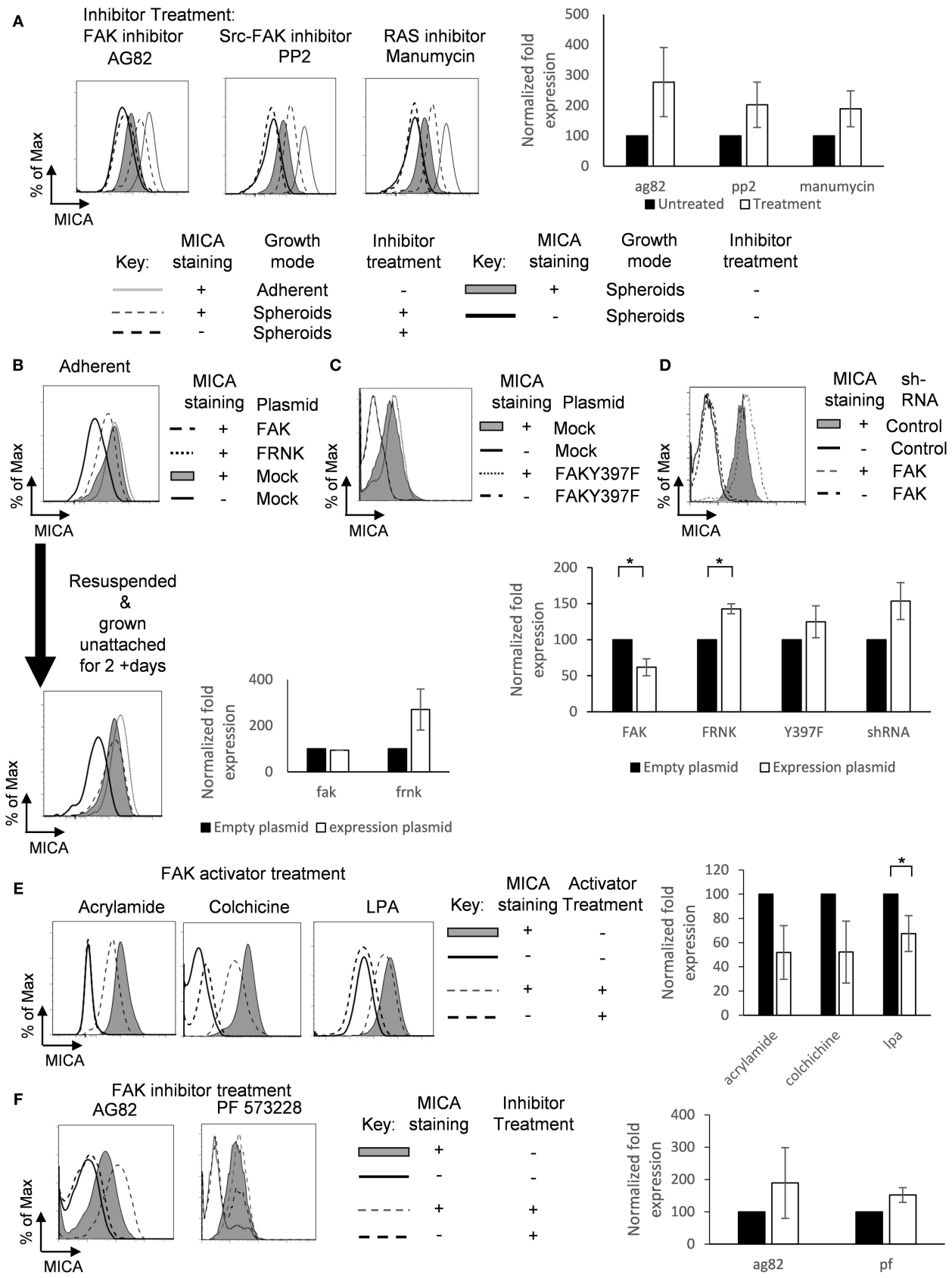


FIGURE 6 | Continued

FIGURE 6 | **Continued**

Focal adhesion kinase (FAK)/Src downregulates MICA surface expression. **(A)** U2OS cells were cultured as either untreated adherent cells (gray line) or as spheroids and treated with (dashed gray line; isotype control staining—dashed black line) or without (shaded gray histogram; isotype control staining—black line) the FAK inhibitors AG82 and PP2 or manumycin. $n = 3$. **(B)** U2OS cells were transfected with FAK (dashed black line), FRNK (dotted gray line), or with empty vector (full shaded gray histogram; isotype control—black line) for 48 h and then analyzed for MICA expression. Some of these cells were then forced to grow in suspension as spheroids. After 2 days, cells were analyzed for MICA expression. $n = 3$. **(C)** Adherent U2OS cells were either transfected with FAKY397F (dotted gray line) or with empty vector (shaded gray histogram; isotype control staining—black line) and stained for MICA expression. **(D)** U2OS cells were also transfected with either scramble control shRNA (shaded gray histogram; isotype control—black line) or shRNA targeting FAK (dashed gray line; isotype control—dashed black line). **(E)** Adherent U2OS cells were left untreated (shaded gray histogram; isotype control—black line) or treated with the FAK activators acrylamide, colchicine, or lysophosphatidic acid and stained for MICA surface expression. **(F)** Adherent U2OS cells were left untreated (shaded grey histogram; isotype control—black line) or with the FAK inhibitors tyrphostin AG82 or PF 573228 and stained for MICA expression (dashed grey line; isotype control staining—dashed black line). $n = 3$ independent experiments.

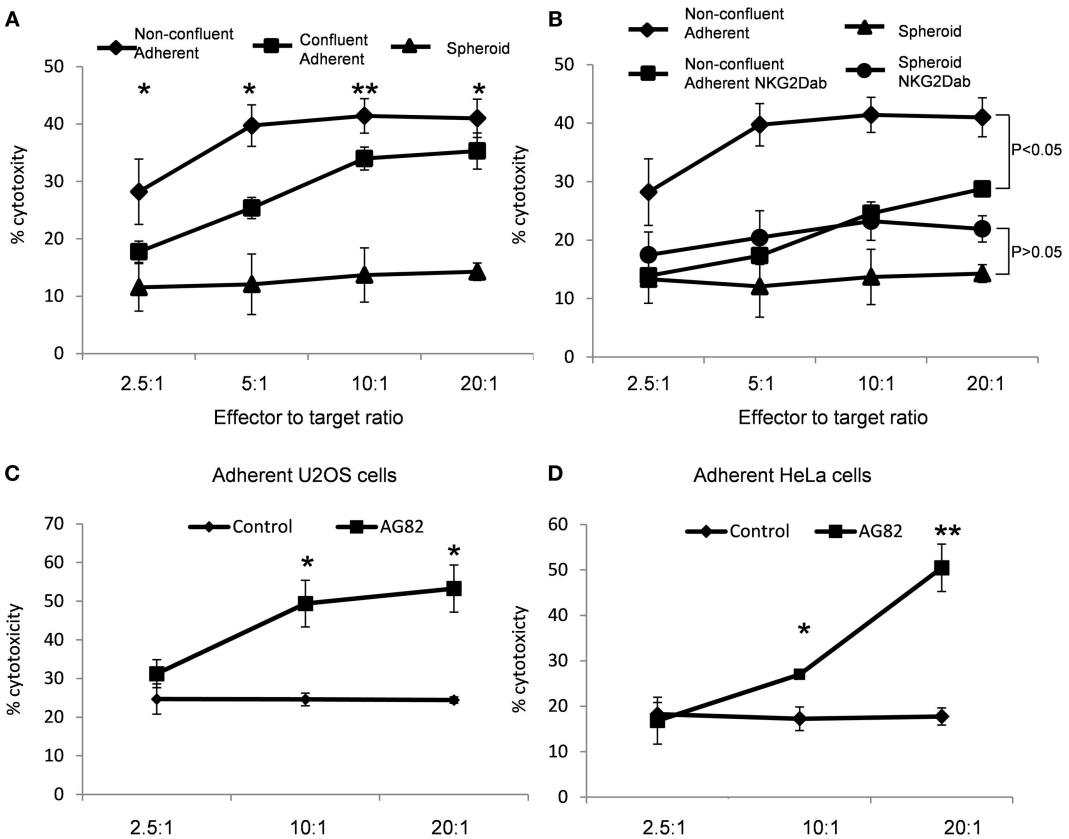


FIGURE 7 | **MICA expression correlates with increased natural killer (NK) cell-mediated cytotoxicity**. **(A)** U2OS cells were either plated to grow as non-confluent adherent cells, as confluent adherent cells or were cultured in suspension. After 5 days, cells were exposed to NK cells in a chromium-release assay. **(B)** Non-confluent cells and spheroids were also subjected to NK cells pretreated with a blocking NKG2D antibody. **(C)** Adherent U2OS and **(D)** HeLa cells were either mock-treated or treated with the focal adhesion kinase inhibitor AG82 prior to exposure to NK cells in a chromium-release assay. Points plotted represent mean of four replicates. Error bars show SEMs. To test for significance, a Student's *t*-test was performed with the degree of cytotoxicity values of each ratio against the other conditions. $^{*}p < 0.05$, $^{**}p < 0.01$.

FAK/Src signaling axis was involved in regulating MICA expression. MICA expression in spheroids was increased by chemical inhibition of FAK/Src or Ras and by overexpression of FRNK or FAKY397F dominant negative FAK mutants. In adherent cells, chemical activation and overexpression of FAK downregulated MICA expression, while the opposite effect was observed following with chemical inhibition of FAK and with shRNA targeted against FAK. Consistent with our observations, TGF-β has been shown to activate FAK and to downregulate MICA expression (22, 31). These data indicate that activation of the FAK/Src pathway has a negative regulatory influence on MICA expression.

Spheroids have high levels of cell–cell contact because they are in a three-dimensional arrangement with other cells. We found that increased cell–cell contact reduced MICA expression on cancer cell lines and that signaling through the FAK/Src axis plays a key role in this. These findings provide a mechanistic foundation for understanding earlier observations in untransformed adherent primary cells (23). Our results show that loss

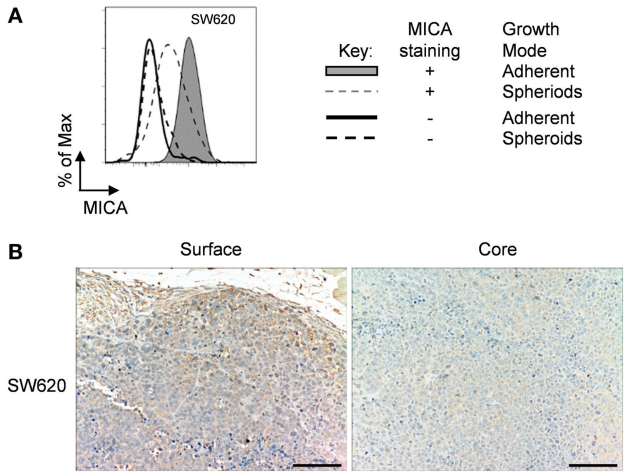


FIGURE 8 | Immunohistochemistry of SW620 xenograft. (A) Adherent SW620 cells were cultured over agarose-coated plates for 5 days to force formation of non-adherent spheroids (dashed gray line) or were cultured under non-confluent adherent conditions (shaded gray histogram) and stained for MICA surface expression (isotype control—black line for adherent cells or dashed black line for non-adherent spheroid suspension cells). **(B)** Immunohistochemistry images of an SW620 xenograft stained for MICA expression showing the surface and the core of the tumor. Bar represents 50 μm.

of cell–cell contact following 1 mM EDTA treatment upregulated MICA expression, but once EDTA levels were high enough to inhibit surface adhesion (5 mM) MICA levels fell to even lower levels than on untreated cells. This may be due to the observation that at lower EDTA concentrations, cells lose cell–cell contact by blocking cadherin adhesive binding activity (32) and have more surfaces to adhere. At higher EDTA levels, integrin signaling is completely blocked (33), although if FAK is not activated, cells would die through anoikis (25). Thus, the effect of loss of surface adhesion is dominant over that of cell–cell contact.

The reduced MICA expression observed on spheroids compared to non-adherent cells is functionally significant, and spheroids are less susceptible to NK cell cytotoxicity. Tumor spheroids are known to be less susceptible to cytotoxic cells, and this has been attributed to reduced antigen presentation associated with downregulation of HSP-70 (3). Transfection of HSP-70 partially restored susceptibility to cytotoxic attack. This effect may in part be due to an upregulation of NKG2D ligands, as HSP-70 transfection has been reported to induce MICA overexpression (34). Here, we could observe that most NKG2D ligands were downregulated following forced suspension. MICA is the NKG2D ligand most highly expressed on many adherent cell lines, and this was significantly downregulated. Although MHC class I expression was also downregulated, this did not make the cells more sensitive; therefore, the NKG2D ligand downregulation appears to be dominant. The MHC class I downregulation may also have an important effect on T cell recognition of metastatic cells.

As MICA and MICB are not expressed in mice (35), many regulatory studies have focused on *in vitro* experiments. To investigate the *in vivo* relevance of our *in vitro* results, we analyzed MICA expression in a tumor xenograft. Here, we could observe that MICA expression was higher on cells at the surface of the tumor than in the core. The immunohistochemistry images showed that cells on the surface of the tumor were more loosely packed, analogous to the non-confluent adherent cells we studied *in vitro*. The core, in contrast, was compact showing more contact and less space between the cells, comparable to over-confluent cells. Therefore, the MICA staining of an *in vivo* tumor correlated with the *in vitro* findings in this study.

The physiological condition where "loss of adhesion" could take place is during tumor cell extravasation. The relationship between MICA expression and metastasis had been observed with uveal melanoma where metastatic melanoma cells have lower MICA expression than primary tumors (36). The failure to induce cell-surface MICA expression upon loss of surface adhesion, thereby facilitating immune evasion, could be a significant step in the development of metastatic cancer. Therefore, in a future study it would be interesting to analyze MICA expression on tumor circulating cells from a range of MICA positive tumors and test if these cells lose MICA expression *in vivo* as a mechanism of immune evasion.

In summary, this study shows that spheroids and confluent adherent cells downregulate MICA expression in a FAK/Src-dependent manner. We have identified a mechanism whereby adhesion and cell contact can modulate the extent to which a cell can be recognized by cytotoxic cells expressing NKG2D. Targeting this pathway may be of therapeutic value in reducing the probability of tumor immune evasion and metastasis.

MATERIALS AND METHODS

Cell Culture

The cell lines 786-O, A549, BS125, primary human fibroblasts, HEK293T, HeLa, Hep3b, HEPG2, HT-1080, HT29, HUH7, MCF7, MO59K, RCC4, SF767, SKOV3, SUSA, SW480, SW620, T47D, U2OS, U343MG, U373MG, U87MG, and ZR751 were cultured in D10: DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, and 4mM L-glutamine. Normal human astrocytes were grown in Astrocyte Medium (ScienceCell) containing astrocyte growth supplement and FCS. BCL-B7, C1R, DAUDI, HL-60, HUT-78, Jurkat, K-562, NK92, NKL, RAJI, THP-1, U937, and YT were cultured in RPMI-1640 with 10% FCS, 50 U/ml of penicillin, and 50 μg/ml of streptomycin (Sigma). NK92 and NKL cell lines were supplemented with IL-2 (500 U/ml) and 1 mM sodium pyruvate. Heparinized blood obtained from healthy donors was collected and centrifuged at 400 × *g* for 20 min over Ficoll-Paque (Sigma, St. Louis, MO, USA). PBMC were collected at the interface, washed twice with DMEM, and analyzed immediately, without culture. To promote growth as spheroids, 24-well plates were coated with 1% agarose in PBS. The wells were then washed twice in full media prior to the addition of these cells.

Inhibition and Activation Treatments

The following inhibitors or activators were used for signaling studies: 1–5 mM EDTA, 10 μM PP2, 1 μM colchicine (Sigma),

6 mM acrylamide (National Diagnostics, Atlanta, GA, USA), 1 μM AG82 FAK/Src inhibitor, 3 μM manumycin (Calbiochem, San Diego, CA, USA), 500nM PF 573228 (R&D Systems, MN, USA), and 5 μM LPA (Enzo Life Sciences).

Transfection

Cells were transfected with FAK, FAKY397F, FRNK (gifts from C. Chen, University of Pennsylvania), shFAK (GATCCCC GAAGTTGGGTTGT CTAGAATTCAAGAGATTCTA GACAA CCCAACTTCTTTTTA), and shScramble in pSIREN (Clontech) using the lipofectamine transfection system per the manufacturer's manual (Invitrogen, Carlsbad, CA, USA).

CFSE Labeling

A total of 1×10^6 cells were pelleted, and 10 μM CFSE staining solution was added directly to the cells, which were gently resuspended and incubated at 37°C for 20 min. Complete medium was then added to stop the reaction. Finally, cells were washed twice with PBS.

Flow Cytometry

For the analysis of NKG2D ligand expression, cells were washed twice and incubated with 5mM EDTA in PBS for 5 min to detach the cells. Spheroids were also treated with 5mM EDTA and manually disrupted to achieve a single cell suspension. Cells were then resuspended in PBS supplemented with 0.03% azide with 5% BSA for blocking. Staining was performed in the same buffer using monoclonal antibodies against MICA (2C10, Santa Cruz, Santa Cruz, CA, USA), MICB (mAb 1599, R&D System, Minneapolis), ULBP1 (mAB 170818, R&D System, Minneapolis), ULBP2 (MAB1298, R&D System, Minneapolis), ULBP3 (Clone 166510, R&D System, Minneapolis), HLA-A, B, C (W6/32, Thermo Fisher, UK), or IgG1, isotype control monoclonal antibodies (eBiosciences, San Diego, CA, USA). Cells were then washed twice and labeled with FITC-labeled polyclonal rabbit anti-mouse IgG (STAR9B, Serotec, Raleigh, NC, USA) or goat anti-mouse IgG Cy5 conjugated (AP124S, Chemicon). Propidium iodide (Sigma) was added at a concentration of 50 μg/ml to identify viable cells. Flow cytometry was performed using a BD®FACSCanto machine and FACSDiva Software (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star).

Chromium-Based Killing Assay

A total of 1×10^6 target cells were dissociated from plates, washed twice in RPMI, resuspended in 100 μl of a 7.4 MBq $Na_2^{51}CrO_4$ solution (GE Healthcare Life Sciences, Piscataway, NJ, USA), diluted in growth media, and then incubated for 1 h in a humidified 5% CO_2 atmosphere at 37°C. After washing three times with RPMI, the labeled cells were resuspended in their growth media and seeded in 96-well plates at 5×10^4 cells per well in quadruplicate. Effector cells were added at the specified effector to target cell ratios (E:T) and were incubated for 4 h at 37°C, 5% CO_2. The supernatant was removed, added to Optiphase Supermix (PerkinElmer), and radioactivity measured with a beta counter (MicroBeta TriLux, PerkinElmer, Waltham, MA, USA). The degree of cytotoxicity was determined according to the formula: % cytotoxicity = [(sample release) − (spontaneous release)]/[(maximum release) − (spontaneous release)]. Spontaneous release was determined by incubating target cells in medium alone. Maximum release of target cells was measured following treatment with 10% detergent Triton X-100 (Sigma). Error bars represent the SD error of the acquired values. To test for significance, a Student's *t*-test was performed with the degree of cytotoxicity values of each ratio against the other conditions. $^*p < 0.05$, $^{**}p < 0.01$.

Semiquantitative PCR

Total RNA was extracted using TRIzol Plus kit (Invitrogen). One microgram of RNA was reverse transcribed into cDNA using OligodT (Invitrogen), and BioScript reverse transcriptase (Bioline). Twenty nanograms of cDNA were subject to semiquantitative PCR reaction using BioTaq polymerase (Bioline) for 28 cycles for MICA and 22 cycles for GAPDH. The PCR primers are MICA forward TCTTCCTGCTTCTGGCTGGCAT; MICA reverse GGGTCATCCTGAGGTCCTTTCCG; GAPDH forward TCCATGACAACTTTGGTATCGTGG; and GAPDH reverse CACCACCCTGTTGCTGTAGCC. PCR reactions were separated by 1% agarose gel and quantified by densitometry using ImageJ software (NIH).

Light Microscopy

Cells were plated in a 12-well plate and treated for 48 h with increasing concentrations of EDTA. Cells were then imaged with a Nikon Eclipse TE2000U fluorescence inverted microscope using a 20× objective and Hamamatsu B&W C4742-95 Orca hi-sensitivity CCD camera using IPLab imaging software.

Western Blotting

Cells were homogenized in lysis buffer (50 mM Tris–HCl pH 7.5, 120 mM NaCl, 1% NP-40, 40 mM β-glycerophosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 25 mM NaF, and 2 μM microcystin-LR). After centrifugation at $15{,}000 \times g$ for 15 min at 4°C, supernatants were collected and soluble protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Fifty micrograms of protein extracts were separated on 8–12% SDS-PAGE and transferred onto PVDF membrane (Millipore, Bedford, MA, USA) by electroblotting. Membranes were blocked with 5% BSA in TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated overnight with (1:100 v:v dilution) primary antibody against MICA (2C10), total FAK (C-20), Tyr397 p-FAK, and Tyr 861FAK and beta-actin (Santa Cruz, CA, USA). Subsequently, membranes were incubated with appropriate HRP-linked secondary antibody (1:2,000 v:v dilution) in PBST (anti-mouse HRP, GE Healthcare, and anti-goat HRP, Santa Cruz) for 1 h at room temperature. Blots were analyzed by enhanced chemiluminescence reagents. Quantification of western blots was done through densitometry using ImageJ (NIH) software.

Immunohistochemistry

IHC was performed using a rabbit polyclonal antibody against MICA (ab62540, Abcam, UK) at 1:30 dilution. For detection,

deparaffinized and rehydrated slides were pretreated in 10 mM citrate buffer, pH 6.0, at 98°C for 60 min for renaturation. The detection ABC method was used per the manufacturer's instructions. MICA specific signals were recorded from slides of xenografts of SW620 cell lines injected in the flank of Hsd nude mice using an automated instrument reagent system (Discovery XT, Ventana Medical System, Inc.) per the user manual. Images of sections were captured (Nikon, YTHM) and analyzed using Image Access Enterprise 7 and ImageJ software. SW620 xenograft slide was a kind gift from Dr. Lei Zhang in Dr. Brian A. Hemmings lab with help from Sandrine Bichet from the Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland. Tissue samples were processed as described previously (37) with the recommendations of the Ethical Committee of the University Hospital of Basel. The protocol was approved by the Ethical Committee of the University Hospital of Basel.

Statistical Analysis

As shown on each figure, *n* indicates the number of experiments using cells from independent experiments. Where three or more experiments were conducted a two-tailed Student's *t*-test with two samples unequal variance type was conducted. Means were considered significantly different when $^{*}p < 0.05$, $^{**}p < 0.01$. Error bars represent the SD of the mean.

AUTHOR CONTRIBUTIONS

GM and CO'C designed the research; GM, DL, and MM performed the experiments; GM and DL performed the statistical analysis; GM, DL, MM, AW, and CO'C analyzed data and wrote the manuscript.

FUNDING

This work was supported by the Medical Research Council (G116/165) and the National Institute for Health Research Oxford Comprehensive Biomedical Research Centre (BRC) Program. GM was supported in part by funding received from the Panamanian National Bureau of Science, Technology and Innovation (SENACYT). AW is recipient of Wellcome Trust Fellowship 092441/Z/10/Z.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00687/full#supplementary-material.

FIGURE S1 | **Schematic diagram and light microscopic images of non-adherent cells, adherent cells, and spheroids**. Growth mode, schematic diagrams, and light microscope images of cells cultured normally in **(A)** adherence, **(B)** suspension, **(C)** over-confluent with naturally detaching cells, or as **(D)** spheroids showing grade of contact, adherence, and MICA expression. Bar represents 50 μm.

FIGURE S2 | **HLA-A, B, C, and NKG2D ligand expression after forced suspension in HeLa and U2OS cells**. **(A)** Adherent HeLa and **(B)** U2OS cell lines were cultured over agarose-coated plates for 5 days to force formation of non-adherent spheroids (dashed gray line) or were cultured under non-confluent adherent conditions (shaded gray histogram) and stained for MICA, MICB, ULBP1, ULBP2, ULBP3, and MHC class I surface expression (isotype control—black line for adherent cells or dashed black line for non-adherent spheroid suspension cells). $n = 3$.

FIGURE S3 | **MICA expression inhibition is not caused by hypoxia, apoptosis, or metalloproteases**. **(A)** U2OS cells were cultured adherently and under hypoxic conditions (1% oxygen) for 48 h (dotted gray line), under hypoxia for 24 h followed by 24 h oxygen–reoxygenation (dashed black line) or cultured in normoxia (shaded gray histogram) and stained for MICA surface expression. Isotype control—black line. $n = 3$. **(B)** U2OS, HeLa, and 786-O adherent cells were either left untreated (full shaded gray histogram; isotype control—thick black line) or incubated under anoxic (0.1% oxygen) conditions for 48 h (dashed thin black line; isotype control—dashed thick black line). $n = 3$. **(C)** U2OS cells were either cultured adherently (gray line; isotype control—black line) or as spheroids and treated with either pifithrin or z-vad (dashed black line) or mock-treated (shaded gray histogram) for 48 h, before staining for MICA expression. The downregulation of MICA on spheroids compared to adherent cells is not abrogated by these inhibitors. $n = 2$. **(D)** U2OS cells were either cultured adherently (gray line), as spheroids (shaded gray histogram; isotype control—black line) or as spheroids and treated with the metalloprotease inhibitor GM6001 (dashed gray line; isotype control—dashed black line) for 48 h while grown in suspension. $n = 2$.

REFERENCES

1. Wolf K, Friedl P. Molecular mechanisms of cancer cell invasion and plasticity. *Br J Dermatol* (2006) 154(Suppl 1):11–5. doi:10.1111/j.1365-2133.2006.07231.x
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144:646–74. doi:10.1016/j.cell.2011.02.013
3. Dangles-Marie V, Richon S, El-Behi M, Echchakir H, Dorothee G, Thiery J, et al. A three-dimensional tumor cell defect in activating autologous CTLs is associated with inefficient antigen presentation correlated with heat shock protein-70 down-regulation. *Cancer Res* (2003) 63:3682–7.
4. Gottfried E, Kunz-Schughart LA, Andreesen R, Kreutz M. Brave little world: spheroids as an in vitro model to study tumor-immune-cell interactions. *Cell Cycle* (2006) 5:691–5. doi:10.4161/cc.5.7.2624
5. Feder-Mengus C, Ghosh S, Weber WP, Wyler S, Zajac P, Terracciano L, et al. Multiple mechanisms underlie defective recognition of melanoma cells cultured in three-dimensional architectures by antigen-specific cytotoxic T lymphocytes. *Br J Cancer* (2007) 96:1072–82. doi:10.1038/sj.bjc.6603664
6. Hynes RO, Zhao Q. The evolution of cell adhesion. *J Cell Biol* (2000) 150:F89–96. doi:10.1083/jcb.150.2.F89
7. Deschesnes RG, Patenaude A, Rousseau JL, Fortin JS, Ricard C, Cote MF, et al. Microtubule-destabilizing agents induce focal adhesion structure disorganization and anoikis in cancer cells. *J Pharmacol Exp Ther* (2007) 320:853–64. doi:10.1124/jpet.106.110957
8. Huang D, Cheung AT, Parsons JT, Bryer-Ash M. Focal adhesion kinase (FAK) regulates insulin-stimulated glycogen synthesis in hepatocytes. *J Biol Chem* (2002) 277:18151–60. doi:10.1074/jbc.M104252200
9. Cox BD, Natarajan M, Stettner MR, Gladson CL. New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. *J Cell Biochem* (2006) 99:35–52. doi:10.1002/jcb.20956
10. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* (2002) 110:673–87. doi:10.1016/S0092-8674(02)00971-6
11. De Sanctis JB, Blanca I, Bianco NE. Secretion of cytokines by natural killer cells primed with interleukin-2 and stimulated with different lipoproteins. *Immunology* (1997) 90:526–33. doi:10.1046/j.1365-2567.1997.00174.x
12. Lanier LL. On guard – activating NK cell receptors. *Nat Immunol* (2001) 2:23–7. doi:10.1038/83130

13. Moretta L, Moretta A. Killer immunoglobulin-like receptors. *Curr Opin Immunol* (2004) 16:626–33. doi:10.1016/j.coi.2004.07.010
14. Bahram S, Inoko H, Shiina T, Radosavljevic M. MIC and other NKG2D ligands: from none to too many. *Curr Opin Immunol* (2005) 17:505–9. doi:10.1016/j.coi.2005.07.016
15. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* (2001) 14:123–33. doi:10.1016/S1074-7613(01)00095-4
16. Yamamoto K, Fujiyama Y, Andoh A, Bamba T, Okabe H. Oxidative stress increases MICA and MICB gene expression in the human colon carcinoma cell line (CaCo-2). *Biochim Biophys Acta* (2001) 1526:10–2. doi:10.1016/S0304-4165(01)00099-X
17. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A* (1996) 93:12445–50. doi:10.1073/pnas.93.22.12445
18. Lin D, Lavender H, Soilleux EJ, O'Callaghan CA. NF-kappaB regulates MICA gene transcription in endothelial cell through a genetically inhibitable control site. *J Biol Chem* (2012) 287:4299–310. doi:10.1074/jbc.M111.282152
19. Waldhauer I, Steinle A. Proteolytic release of soluble UL16-binding protein 2 from tumor cells. *Cancer Res* (2006) 66:2520–6. doi:10.1158/0008-5472.CAN-05-2520
20. Baychelier F, Vieillard V. The modulation of the cell-cycle: a sentinel to alert the NK cells of dangers. *Front Immunol* (2013) 4:325. doi:10.3389/fimmu.2013.00325
21. Grimes DR, Kelly C, Bloch K, Partridge M. A method for estimating the oxygen consumption rate in multicellular tumour spheroids. *J R Soc Interface* (2014) 11:20131124. doi:10.1098/rsif.2013.1124
22. Eisele G, Wischhusen J, Mittelbronn M, Meyermann R, Waldhauer I, Steinle A, et al. TGF-beta and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. *Brain* (2006) 129:2416–25. doi:10.1093/brain/awl205
23. Zou Y, Mirbaha F, Stastny P. Contact inhibition causes strong downregulation of expression of MICA in human fibroblasts and decreased NK cell killing. *Hum Immunol* (2006) 67:183–7. doi:10.1016/j.humimm.2006.02.018
24. Takeichi M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* (1988) 102:639–55.
25. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* (1996) 134:793–9. doi:10.1083/jcb.134.3.793
26. Nakagawa-Yagi Y, Choi DK, Ogane N, Shimada S, Seya M, Momoi T, et al. Discovery of a novel compound: insight into mechanisms for acrylamide-induced axonopathy and colchicine-induced apoptotic neuronal cell death. *Brain Res* (2001) 909:8–19. doi:10.1016/S0006-8993(01)02608-7
27. Kumagai N, Morii N, Fujisawa K, Yoshimasa T, Nakao K, Narumiya S. Lysophosphatidic acid induces tyrosine phosphorylation and activation of MAP-kinase and focal adhesion kinase in cultured Swiss 3T3 cells. *FEBS Lett* (1993) 329:273–6. doi:10.1016/0014-5793(93)80236-N
28. Leibovitz A, Stinson JC, McCombs WB III, McCoy CE, Mazur KC, Mabry ND. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* (1976) 36:4562–9.
29. Boissel N, Rea D, Tieng V, Dulphy N, Brun M, Cayuela JM, et al. BCR/ABL oncogene directly controls MHC class I chain-related molecule A expression in chronic myelogenous leukemia. *J Immunol* (2006) 176:5108–16. doi:10.4049/jimmunol.176.8.5108
30. Windham TC, Parikh NU, Siwak DR, Summy JM, McConkey DJ, Kraker AJ, et al. Src activation regulates anoikis in human colon tumor cell lines. *Oncogene* (2002) 21:7797–807. doi:10.1038/sj.onc.1205989
31. Cai T, Lei QY, Wang LY, Zha XL. TGF-beta 1 modulated the expression of alpha 5 beta 1 integrin and integrin-mediated signaling in human hepatocarcinoma cells. *Biochem Biophys Res Commun* (2000) 274:519–25. doi:10.1006/bbrc.2000.3177
32. Le TL, Yap AS, Stow JL. Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. *J Cell Biol* (1999) 146:219–32. doi:10.1083/jcb.146.999.219
33. Michael KE, Dumbauld DW, Burns KL, Hanks SK, Garcia AJ. Focal adhesion kinase modulates cell adhesion strengthening via integrin activation. *Mol Biol Cell* (2009) 20:2508–19. doi:10.1091/mbc.E08-01-0076
34. Qiao Y, Liu B, Li Z. Activation of NK cells by extracellular heat shock protein 70 through induction of NKG2D ligands on dendritic cells. *Cancer Immun* (2008) 8:12.
35. Carapito R, Bahram S. Genetics, genomics, and evolutionary biology of NKG2D ligands. *Immunol Rev* (2015) 267:88–116. doi:10.1111/imr.12328
36. Vetter CS, Lieb W, Brocker EB, Becker JC. Loss of nonclassical MHC molecules MIC-A/B expression during progression of uveal melanoma. *Br J Cancer* (2004) 91:1495–9. doi:10.1038/sj.bjc.6602123
37. Maier D, Comparone D, Taylor E, Zhang Z, Gratzl O, Van Meir EG, et al. New deletion in low-grade oligodendroglioma at the glioblastoma suppressor locus on chromosome 10q25-26. *Oncogene* (1997) 15:997–1000. doi:10.1038/sj.onc.1201209

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 16 March 2017
doi: 10.3389/fimmu.2017.00293

Role of Distinct Natural Killer Cell Subsets in Anticancer Response

Helena Stabile[1†], Cinzia Fionda[1†], Angela Gismondi[1,2] and Angela Santoni[1,3*]*

[1] Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy, [2] Italian Institute of Technology, Sapienza University of Rome, Rome, Italy, [3] Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy

Natural killer (NK) cells, the prototypic member of innate lymphoid cells, are important effectors of anticancer immune response. These cells can survey and control tumor initiation due to their capability to recognize and kill malignant cells and to regulate the adaptive immune response *via* cytokines and chemokines release. However, several studies have shown that tumor-infiltrating NK cells associated with advanced disease can have profound functional defects and display protumor activity. This evidence indicates that NK cell behavior undergoes crucial alterations during cancer progression. Moreover, a further level of complexity is due to the extensive heterogeneity and plasticity of these lymphocytes, implying that different NK cell subsets, endowed with specific phenotypic and functional features, may be involved and play distinct roles in the tumor context. Accordingly, many studies reported the enrichment of selective NK cell subsets within tumor tissue, whereas the underlying mechanisms are not fully elucidated. A malignant microenvironment can significantly impact NK cell activity, by recruiting specific subpopulations and/or influencing their developmental programming or the acquisition of a mature phenotype; in particular, neoplastic, stroma and immune cells, or tumor-derived factors take part in these processes. In this review, we will summarize and discuss the recently acquired knowledge on the possible contribution of distinct NK cell subsets in the control and/or progression of solid and hematological malignancies. Moreover, we will address emerging evidence regarding the role of different components of tumor microenvironment on shaping NK cell response.

Keywords: natural killer cell subset, tumor microenvironment, natural killer cells, hematological malignancies, solid tumors

OPEN ACCESS

Edited by:
Sandra Laurence Lopez-Verges, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama

Reviewed by:
Christine Susanne Falk, Hannover Medical School, Germany
Kerry S. Campbell, Fox Chase Cancer Center, USA

***Correspondence:**
Angela Gismondi
angela.gismondi@uniroma1.it;
Angela Santoni
angela.santoni@uniroma1.it

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *12 December 2016*
Accepted: *28 February 2017*
Published: *16 March 2017*

Citation:
Stabile H, Fionda C, Gismondi A and Santoni A (2017) Role of Distinct Natural Killer Cell Subsets in Anticancer Response. Front. Immunol. 8:293. doi: 10.3389/fimmu.2017.00293

INTRODUCTION

Natural killer (NK) cells are innate lymphoid cells (ILCs) (1) with a crucial role in immunosurveillance. They display cytotoxic activities against transformed or viral infected cells but are also an important source of chemokines and cytokines highly impacting on adaptive immune responses (2, 3).

Natural killer cell activity is dependent on activating and inhibitory signals transmitted by a large repertoire of surface receptors. Inhibitory receptors prevent NK cells from killing healthy cells and include KIRs, CD94/NKG2A, and ILT2/CD85. The activating receptors recognize self-proteins mainly expressed on stressed target cells and include NCRs (NKp46, NKp30, NKp44), NKG2D, and DNAM1, among others (4).

Natural killer cells develop in the bone marrow (BM) from lineage restricted progenitors, although maturation can also occur in the periphery (5–7). Fully mature NK cells circulate in the peripheral blood (PB), where they represent 5–20% of total lymphocytes, but they are also found in several lymphoid and non-lymphoid organs (8, 9).

Phenotypically, NK cells are defined by the expression of CD56 and the lack of CD3–TCR complex. Moreover, based on CD16 and CD56 expression levels, they are classically distinguished in two subsets: $CD56^{high}CD16^{\pm}$ and $CD56^{low}CD16^{high}$. The $CD56^{low}CD16^{high}$ NK cell subset expresses high levels of KIRs, the maturation marker CD57, and mediates natural and antibody-dependent cellular cytotoxicity, exhibiting high levels of perforin and enhanced killing; $CD56^{high}CD16^{\pm}$ NK cells are characterized by NKG2A, low levels of perforin, and are primarily specialized for cytokine production. It is still debated whether these subsets are functionally distinct NK cells or different stages of maturation. A linear differentiation relationship between $CD56^{high}$ $CD16^{\pm}$ NK cells and $CD56^{low}CD16^{high}$ NK cells has been proposed (10, 11), but it is not supported by observations on human NK cell deficiencies (12, 13); moreover, the possibility that tissue-resident NK cells develop locally is also considered (14).

Besides $CD56^{high}CD16^{\pm}$ and $CD56^{low}CD16^{high}$, additional NK cell subpopulations have been identified under normal and pathological conditions, based on their receptor repertoire (15–17). Thus, human NK cells emerge as a highly heterogeneous and plastic population including subtypes with different and specific functions.

Natural killer cell subsets also differ in tissue distribution that is related to distinct homing properties and/or *in situ* maturation. Tissue-resident NK cells express a different pattern of chemokine and adhesion receptors and also differ from their blood-circulating counterpart (18, 19). PB $CD56^{high}CD16^{\pm}$ NK cells express CD62L, CCR7, CXCR4, and CXCR3 that allow their preferential recruitment to secondary lymphoid organs, tumor, and inflamed tissues (8, 20, 21). Conversely, resident $CD56^{high}$ NK cells lack CD62L but express other adhesion molecules, including the α integrin subunit CD49a and CD103 (22). The $CD56^{low}CD16^{high}$ NK cell subset expresses low level of CD62L and lacks CCR7, but it is characterized by CXCR4, CX_3CR1, CXCR2, and CXCR3 chemokine receptors responsible for their migration into the inflamed sites.

Natural killer cells play a major role in tumor immunosurveillance. They can control tumor initiation but are often inefficacious in advanced disease. More recently, strong NK cell infiltration in established cancers also suggested a role in disease progression (23, 24). Tumor-infiltrating NK cells (TINKs) share phenotypic and functional properties with decidual NK cells (dNKs), well known for their regulatory, pro-angiogenic, and low cytotoxic activities (23, 25, 26).

In tumor microenvironment, several cellular and soluble factors affect NK cell phenotype and function and promote tumor cell evasion from NK cell-mediated recognition and killing (27).

Because the capability of distinct NK cell subsets to exert specific functions, it is extremely important to understand which subpopulations mediate the antitumor response and which environmental factors modulate their activity. Here, we review the role of distinct NK cell subsets in human solid and hematological cancers and the impact of tumor microenvironment on their phenotypic and functional features.

NK CELL SUBSETS IN SOLID TUMORS

Neoplastic transformation was shown to significantly alter NK cell subset localization (**Table 1**), though the exact role of the TINKs subsets remains poorly characterized (28, 29).

The study of TINKs in solid tumors is rather complex as phenotypic alterations can occur following isolation and a comparison with the healthy tissue counterpart is difficult to perform.

Like tissue-resident NK cells that are generally $CD56^{high}CD16^{low}$ and more specialized for cytokine production, a prevalence of $CD56^{high}$ NK cells can infiltrate solid malignancies, although they can exhibit features and/or functions other than those of their circulating and/or healthy counterpart tissue. Thus, a significantly higher frequency of $CD56^{high}perforin^{low}$ NK cells was observed in breast and lung cancers, with respect to normal tissues. $CD56^{high}$ NK cells were poorly cytotoxic, but cytokine producers, and were mainly localized within the stromal compartment. $CD56^{high}perforin^{low}$ accumulation was not attributed to major tumor microenvironment-driven NK cell developmental alterations, but rather to a peculiar chemokine milieu. Indeed, downregulation of CXCL2 that specifically attracts $CD56^{low}$ NK cells and upregulation of CXCL9 and CXCL10 that specifically support $CD56^{high}$ NK cells homing were observed (20, 45).

In breast cancer patients, five different circulating NK subsets were also identified (46): $CD56^{low}CD16^{+}$, $CD56^{low}CD16^{-}$, $CD56^{high}CD16^{-}$, $CD56^{high}CD16^{+}$, and $CD56^{-}CD16^{+}$. A higher percentage of $CD56^{low}CD16^{-}$ and $CD56^{high}CD16^{-}$ subsets were observed both in PB and in advanced invasive mammary tumors. Furthermore, by phenotypic and functional analysis, both subpopulations emerged as more immature ($CD117^{high}CD27^{high}CD57^{low}$) and less functional (low levels of activating receptors, perforin, and granzyme B and degranulation capability). Collectively, these observations suggest that breast tumor microenvironment blocks or reverses NK cell maturation, favoring the emergence of non-cytotoxic NK cells.

Changes in the expression patterns of activating and inhibitory receptors have been also described in tumor-associated $CD56^{high}$ NK cells and have been implicated in their functional deficits. $CD56^{high}$ NK cells, displaying an immature and activated phenotype associated with low or null degranulation potential, were found in prostate tumor and area selected out of the tumor site (30). However, in prostate cancer, lower expression of some activating receptors (NKp46, NKp30, NKG2D, DNAM-1, CD16) and higher expression of the inhibitory receptor ILT2 were observed, with more pronounced effects in NK cells infiltrating metastatic than localized tumors; these latter data indicate that tumor microenvironment can impair NK cytotoxic functions by altering the balance between NK activating and inhibitory receptors. The analysis of NK cell subsets in the lymph nodes of cancer patients revealed comparable numbers of $CD56^{high}$ NK cells in the regional metastatic lymph nodes from stage III melanoma patients (M-LN) and mediastinal lymph nodes from healthy donors (HD). However, 40–60% of $CD56^{high}$ NK cells in M-LN also

TABLE 1 | Phenotype of NK cell subsets in tumors.

Natural killer (NK) cell subset		Tumor	Phenotype	Function		Reference
				Cytokine production	Cytotoxicity	
Solid tumors	$CD56^{high}perforin^{low}$	Lung and breast cancer	$NKG2A^+CD27^+KIR^+$ CD62L downregulation	ND	ND	(20)
	$CD56^{high}CD16^{low}$	Breast, melanoma, and colon cancer	$CD9^+$, $CXCR3^+$	VEGF	ND	(25)
	$CD56^{high}CD16^-$	Non-small cell lung cancer	$KIR^+CD69^+HLA\text{-}DR^+$ NKp44 upregulation	High production of VEGF, PLGF, IL-8	No cytotoxicity	(26)
	$CD56^{high}$	Prostate	NKp46, NKG2D, NKp30, DNAM1, CD16 downregulation ILT2 upregulation	ND	No cytotoxicity	(30)
	$CD56^{hight}CD16^+$	Metastatic lymph nodes adjacent to metastatic melanoma	$NKp46^+$, $NKG2D^+$, $NKp30^+$, CD158 (a, b and e)$^+$	ND	Low cytotoxicity	(31)
	$CD56^{low}$	Metastatic lymph nodes from melanoma patients	$KIR^+CD57^+CD69^+CCR7^+$	ND	High cytotoxicity	(32, 33)
	$CD56^{low}$	Non-small cell lung cancer	$NKp46^+$, NKp80, CD16, NKG2D, and DNAM-1 downregulation	No IFN-γ production	No cytotoxicity	(34)
	CD56hight	Intestinal stromal cancer	$CD16^-KIR^-NKp30c^+$	Reduced production of TNF-α IFN-γ production	Reduced cytotoxicity	(35)
Hematologic tumors	$CD56NCR^{dull}$	AML	$CD16^+KIR^+$	ND	No cytotoxicity	(36)
	$CD56NCR^{high}$	AML	ND	ND	No cytotoxicity	(36)
	$CD56NKp46^{low}$	AML/B-ALL	NKG2A upregulation	No IFN-γ production	No cytotoxicity	(37)
	$CD56^{low}CD16^{low}$	B-ALL/T-ALL	ND	No IFN-γ production	No cytotoxicity	(38)
	$CD56^+$	Myelodysplastic syndromes (MDS)	NKG2D (PB/BM) DNAM1 (BM) downregulation	ND	No cytotoxicity	(39)
	$CD56^{low}$	Multiple myeloma (MM)	DNAM1, CD16, 2B4 downregulation	ND	No cytotoxicity	(40, 41)
	CD56	MM	CD161 downregulation and CD158a upregulation	ND	No cytotoxicity	(40)
	$CD56^{low}$	AML	KIR^+CD57^+	ND	ND	(42)
	$CD56^{low}$	AML	$CD16/CD57^{high}$	ND	ND	(43)
	$CD56^{low}$	MDS	KIR^-NKG2A^-	ND	ND	(44)

up/downregulation, reduction are determined with respect to NK cells from healthy individuals; ND, not determined.

expressed CD16. $CD56^{high}CD16^+$ NK cells displayed an activated phenotype, and their *ex vivo* degranulating capacity inversely correlated with the percentage of malignant cells, suggesting a local tumor-induced suppression of NK cell activation. The prevalence of $CD56^{high}CD16^+$ NK cells in M-LNs was attributed to the maturation and activation of tumor resident $CD56^{high}CD16^-$ NK cells and/or to the migration of PB $CD56^+CD62L^+$ NK cells to M-LNs, where CD16 expression could be upregulated (31).

A relevant property of $CD56^{high}CD16^-$ NK cells within different solid tumors, such as breast, melanoma, colon cancer (25), non-small lung cancer (26) is their pro-angiogenic phenotype possibly responsible for their tumor-promoting role. Indeed, unlike circulating $CD56^{high}CD16^-$ but similar to dNK cells, $CD56^{high}CD16^-$ TINK cells express high levels of CD9, CXCR3, produce VEGF, and have a lower cytotoxic potential, suggesting that similar maturation/polarization mechanisms occur in the decidua and tumor microenvironment of PB NK cells (47–49).

Although substantial evidence indicates $CD56^{high}CD16^{low}$ NK cells as the major TINK, there are also reports on tumor infiltration by $CD56^{low}$ NK cells (32, 33, 46). Enrichment in the tumor infiltrated lymph nodes (TILN) and concomitant reduction of $CD56^{low}$ NK cells in PB were observed in melanoma patients. These $CD56^{low}$ ($CD57^+CD69^+CCR7^+KIR^+$) NK cells were highly cytotoxic against autologous melanoma cells, and, in accordance with their homing into TILN, they expressed CCR7. The reduced proportion of $CD56^{low}$ NK cells in the PB supports the possibility

of a selective recruitment of this subset in TILN. However, *in situ* maturation of CD56^{high} NK into more cytotoxic CD56^{low} NK cells was also suggested because the different chemokine milieu dominated by CXCL8 and CCL2, which may recruit both CD56^{high} and CD56^{dim} CXCR2$^+$/CCR2$^+$ PB NK cells into the TILN (32).

The emerging concept of tissue-specific functions of NK cells together with the selective enrichment of specific subsets in neoplastic tissues indicate that the outcome of antitumor NK cell effector functions is not always predictable and largely depends on the particular tumor microenvironment.

NK CELL SUBSETS IN HEMATOLOGICAL MALIGNANCIES

A large body of evidence indicates that NK cells play a preferential role in the control of the onset and progression of hematological tumors. Moreover, unlike solid cancers where monitoring of PB NK cells could not provide correct information on their tumor-infiltrating counterpart, evaluation of circulating NK cell status can be highly relevant in the context of hematological malignancies.

Abnormal NK cell cytolytic function was observed in acute and chronic leukemia (AML-ALL and CLL-CML), myelodysplastic syndromes (MDS), and multiple myeloma (MM). Yet, most of the studies are focused on PB, but not BM, and poorly address NK cell phenotypic and functional heterogeneity.

The main receptors involved in NK cell recognition and killing of leukemic blasts are NCRs, NKG2D, and DNAM1. According to NCR surface density, unlike NK cells from HD that are mainly NCR^{high} (50), a NCR^{low}CD16$^+$KIR$^+$ NK cell subset that failed to recognize and kill autologous and allogeneic blasts was described in AML patients (36). A smaller cohort of AML patients was also characterized by the presence of the NCR^{high} NK cell subset that showed impaired cytotoxic activity, probably due to NCR ligand down-modulation on leukemic cells. In addition, significant reduction of NKp46 together with increased NKG2A expression was associated with functionally impaired PB NK cells from AML patients with respect to HD (37). Similar to AML, the frequency of PB NCR$^+$ and in particular NKp46$^+$ NK cells from B-ALL patients was lower. Moreover, they also displayed increased NKG2A expression. These phenotypic abnormalities were associated with impaired NK cell killer ability and IFN-γ production in response to autologous blasts (51). As regards to other activating NK receptors, a lower frequency of NKG2D$^+$ and DNAM-1$^+$ NK cells was observed in the context of MDS, AML, and MM (39, 52); moreover, NK cells from MM patients also displayed reduced levels of CD244, CD16, and CD161 (40, 41, 53, 54).

A different scenario was observed with CLL and CML CD56^{low} NK cells which exhibited the same profile of activating and inhibitory receptors of HD but reduced NK cytotoxic ability (55).

Recently, we reported an increased frequency of a newly identified NK cell subset characterized by low levels of CD56 and CD16 (CD56^{low}CD16^{low}) and NKG2A$^+$ in both BM and PB of pediatric B-ALL and T-ALL. In HD, this subset was endowed with both higher cytotoxic activity and IFN-γ producing ability, but it resulted functionally impaired in leukemic patients (38). Similarly, a higher frequency of non-cytotoxic CD56^{low}CD16^{low} NK cells was found in advanced breast cancer (46), suggesting both a preferential homing and functional alterations of this subset in tumor-microenvironment.

Overall, these findings suggest that several mechanisms, including downregulation of activating receptors and/or upregulation of inhibitory receptors on NK cells or modulation of their ligands on cancer cells are responsible for tumor escape from NK cell recognition in hematological malignancies.

In the context of hematological cancers where tumor cells are present in the BM that represents the main site of NK cell differentiation, an important question to address is whether tumor growth also affects NK cell development. Most of the studies, however, addressed this issue examining PB and not BM NK cells. In this regard, Chretien et al. (42) compared the presence of five different stages of NK cell development (CD56^{high}, CD56^{low}/KIR$^-$/CD57$^-$, CD56^{low}/KIR$^+$/CD57$^-$, CD56^{low}/KIR$^-$/CD57$^+$, and CD56^{low}/KIR$^+$/CD57$^+$) in the PB of AML patients and found that one-third of the patients exhibited a significant increase in the proportion of the more mature CD56^{low}/KIR$^+$/CD57$^+$ NK cells at the expenses of more immature CD56^{high} NK cell subset. In addition, a recent study on NK cells from the BM of AML patients showed a reduced frequency of the more mature CD56^{low}CD16/57^{high} NK cell subset that did not correlate with a good prognosis (43). Collectively, these findings, although suggestive of a possible influence of AML cells on NK cell development, are still incomplete, as BM and PB NK cell subsets from the same patient have not been examined, and the possibility that the observed phenotype is due to a preferential migration of more mature CD56^{low}/KIR$^+$/CD57$^+$ NK cells from BM to PB is still open (56).

Moreover, it is increasingly understood the impact of hematological tumors on BM stromal cells, which are crucial for an optimal NK cell differentiation. In this regard, evidences on altered chemokine and cytokine production by BM stromal cells were provided (44), suggesting that effects on NK cell differentiation can be due to the lack of a proper stromal support for NK cell progenitors and/or an altered NK cell subset trafficking.

NK CELL SUBSETS AND TUMOR MICROENVIRONMENT

Several studies indicate that tumor-induced impairment of NK cell functions correlates with alterations of NK cell subset distribution. On the other hand, different immunosuppressive mechanisms can be also responsible for functional NK cell impairment in solid and hematologic malignancies.

Tumor-related soluble factors may be responsible for phenotypic and functional alterations of NK cells, moreover different tumor-resident immune cells, such as M2-polarized macrophages, MDSC, DC, and Treg, may affect NK cell activity, by releasing soluble factors (e.g., IL-10, IDO, PGE_2) or by direct contact-dependent mechanisms (57–59) (**Figure 1**). Although higher amount of TGF-β1, PGE2, IL-10, and IDO were detected in supernatants of solid and hematological tumors, the impact of these soluble factors on NK cell subset distribution was suggested only based on *in vitro* observations (30, 51). Differently, Mamessier et al. performed *in vivo* correlation studies demonstrating that in

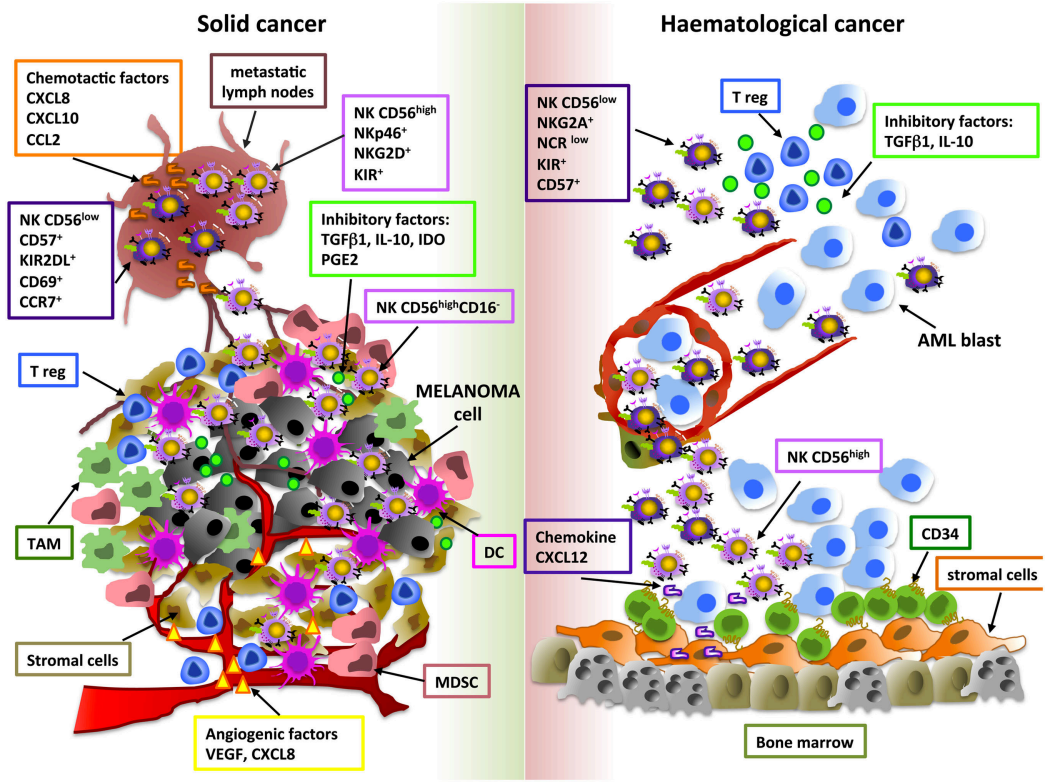


FIGURE 1 | Shaping natural killer (NK) cell subsets in tumor microenvironment. The main cellular and soluble factors affecting NK cell subset distribution in tumor microenvironment are shown. Melanoma and AML are reported as examples of solid and hematological tumor, respectively. For melanoma, tumor and metastatic lymph node (M-LN) infiltrating NK cell subsets are represented. For AML, bone marrow and peripheral blood NK cell subpopulations are indicated.

breast cancer patients decreased expression of activating NK cell receptors (NKp30, NKp46, NKG2D, DNAM-1) or cytotoxic molecules (GZMB) and increased levels of the inhibitory receptor NKG2A on NK cells were associated with high amount of TGF-β1 and PGE2 in tumor supernatants. In particular, TGF-β1 and PGE2 were shown to negatively correlate with molecules related to NK cell cytoxicity and positively correlate with NKG2A receptor expression (60), thus suggesting that these molecules play a role in these regulatory mechanisms.

Among these factors, particular attention has been given to TGF-β1, which has been shown to exert several effects on NK cells, including inhibition of proliferation and *in vitro* NK cell development and differentiation. In this regard, this cytokine was found to reduce the number of NK cells developing from human $CD34^+$ progenitor cells and to promote the conversion of PB $CD56^{high}CD16^+$ NK cells into a dNK-like $CD56^{high}CD16^-$ phenotype (61, 62). Thus, also at tumor site, TGF-β1 may take into account of the pro-angiogenic dNK-like phenotype of tumor-infiltrating NK cells. A number of studies suggest that tumor-derived TGF-β1 also impacts NK development in the context of hematological malignancies. In particular, TGF-β1 overexpression in the BM tumor-microenvironment (MDS, CML, and MM) may be responsible for the suppressive effect of cancer cells on BM stromal cells, thus compromising their supportive role on NK cell maturation (44, 63, 64). Finally, this cytokine may also interfere with intra-tumoral NK cell infiltration *via* modulation of their chemokine receptors (65). In this regard, a peculiar chemokine milieu has been proposed to be important for the recruitment of specific NK cell subpopulations in a number of solid tumors; moreover, altered chemokine expression patterns may also affect NK cell trafficking in hematological malignancies (32, 44, 45). However, higher concentration of chemokines does not always correlate with the presence of these lymphocytes in tumor microenvironment, thus suggesting that other and more complex mechanisms can affect their recruitment (66).

TUMOR ESCAPE FROM NK CELL-MEDIATED RECOGNITION AND KILLING

Elusion of NK cell recognition is a major mechanism of tumor immune evasion. NK cell-activating ligands are expressed on malignant cells, but they can be also released in a soluble form through metalloproteinase-mediated cleavage, exosome secretion, or alternative splicing. Indeed, soluble forms of these ligands are present in the serum or peritoneal fluids of various cancer patients, and their levels positively correlate with tumor stage, metastasis, and poor prognosis (67–69). Reduction of activating ligand expression on cancer cells leads to a less efficient recognition and killing by cytotoxic lymphocytes. Concomitantly, soluble ligands can engage their receptors and cause their internalization

in NK cells; accordingly, a negative correlation between soluble ligands and NKG2D expression on NK cells was largely documented in both solid and hematological tumors (70, 71). However, conflicting results have described either inhibition or promotion of NK cell activation following NKG2D endocytosis (72). An additional escape strategy used by cancer cells is based on the dominance of NK cell inhibitory signals. In several cancer cells, expression of MHC class I molecules binding to inhibitory KIR receptors (KIR2DL2/3, KIR3DL1, and KIR2DL1) results in switch off NK cell effector functions (37, 73–75). Moreover, high levels of non-classical antigens HLA-G (ligand of ILT-2 and KIRDL-4) and HLA-E (ligand of NKG2A/CD94) were found in tumor and serum of cancer patients and were considered independent markers of poor prognosis in various malignancies (76–79). Finally, tumor cell overexpression of other ligands triggering inhibitory signals on NK cells, such as PDL-1/2, contributes to inhibit their susceptibility to NK cell-mediated killing (80, 81).

CONCLUSION

Accumulating evidence indicates that, far from the simple and first distinction in two subsets, NK cells are a very highly heterogeneous population, and different marker combinations can be used to identify distinct subpopulations endowed with specific functional properties. Based on these observations, the role of different NK cell subsets in pathological contexts, including cancer, is increasingly elucidated. Moreover, the emerging evidence about different ILC populations further raise the necessity of a more detailed molecular phenotypic and functional characterization of innate lymphoid subsets in the cancer context. The identification of the role played by the different NK cells both in solid and hematological malignancies would be valuable for the design of novel NK cell targeted therapeutic interventions.

AUTHOR CONTRIBUTIONS

HS, CF, AG, and AS contributed equally to writing and critically revised the paper.

FUNDING

This work was supported by grants from the Italian Association for Cancer Research (AIRC: project #16014 and AIRC 5xmille: project #9962), Istituto Pasteur-Fondazione Cenci Bolognetti and Ministero dell'Istruzione, dell'Università e della Ricerca (Ricerche Universitarie), and the Italian Institute of Technology (A2 project).

REFERENCES

1. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* (2015) 517:293–301. doi:10.1038/nature14189
2. Caligiuri MA. Human natural killer cells. *Blood* (2008) 112:461–9. doi:10.1182/blood-2007-09-077438
3. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science* (2011) 331:44–9. doi:10.1126/science.1198687
4. Lanier LL. NK cell recognition. *Annu Rev Immunol* (2005) 23:225–74. doi:10.1146/annurev.immunol.23.021704.115526
5. Freud AG, Caligiuri MA. Human natural killer cell development. *Immunol Rev* (2006) 214:56–72. doi:10.1111/j.1600-065X.2006.00451.x
6. Freud AG, Yu J, Caligiuri MA. Human natural killer cell development in secondary lymphoid tissues. *Semin Immunol* (2014) 26:132–7. doi:10.1016/j.smim.2014.02.008
7. Di Santo JP, Vosshenrich CA. Bone marrow versus thymic pathways of natural killer cell development. *Immunol Rev* (2006) 214:35–46. doi:10.1111/j.1600-065X.2006.00461.x
8. Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* (2003) 101:3052–7. doi:10.1182/blood-2002-09-2876
9. Santoni A, Carlino C, Gismondi A. Uterine NK cell development, migration and function. *Reprod Biomed Online* (2008) 16:202–10. doi:10.1016/S1472-6483(10)60575-5
10. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, et al. CD56bright. *J Immunol* (2007) 178:4947–55. doi:10.4049/jimmunol.178.8.4947
11. Michel T, Poli A, Cuapio A, Briquemont B, Iserentant G, Ollert M, et al. Human CD56bright NK cells: an update. *J Immunol* (2016) 196:2923–31. doi:10.4049/jimmunol.1502570
12. Gineau L, Cognet C, Kara N, Lach FP, Dunne J, Veturi U, et al. Partial MCM4 deficiency in patients with growth retardation, adrenal insufficiency, and natural killer cell deficiency. *J Clin Invest* (2012) 122:821–32. doi:10.1172/JCI61014
13. Mace EM, Hsu AP, Monaco-Shawver L, Makedonas G, Rosen JB, Dropulic L, et al. Mutations in GATA2 cause human NK cell deficiency with specific loss of the CD56(bright) subset. *Blood* (2013) 121:2669–77. doi:10.1182/blood-2012-09-453969
14. Sojka DK, Tian Z, Yokoyama WM. Tissue-resident natural killer cells and their potential diversity. *Semin Immunol* (2014) 26:127–31. doi:10.1016/j.smim.2014.01.010
15. Lugthart G, Melsen JE, Vervat C, van Ostaijen-Ten Dam MM, Corver WE, Roelen DL, et al. Human lymphoid tissues harbor a distinct CD69+CXCR6+ NK cell population. *J Immunol* (2016) 197:78–84. doi:10.4049/jimmunol.1502603
16. Stegmann KA, Robertson F, Hansi N, Gill U, Pallant C, Christophides T, et al. CXCR6 marks a novel subset of T-bet(lo)Eomes(hi) natural killer cells residing in human liver. *Sci Rep* (2016) 6:26157. doi:10.1038/srep26157
17. Harmon C, Robinson MW, Fahey R, Whelan S, Houlihan DD, Geoghegan J, et al. Tissue-resident Eomes(hi) T-bet(lo) CD56(bright) NK cells with reduced proinflammatory potential are enriched in the adult human liver. *Eur J Immunol* (2016) 46:2111–20. doi:10.1002/eji.201646559
18. Melsen JE, Lugthart G, Lankester AC, Schilham MW. Human circulating and tissue-resident CD56(bright) natural killer cell populations. *Front Immunol* (2016) 7:262. doi:10.3389/fimmu.2016.00262
19. Bernardini G, Sciume G, Santoni A. Differential chemotactic receptor requirements for NK cell subset trafficking into bone marrow. *Front Immunol* (2013) 4:12. doi:10.3389/fimmu.2013.00012
20. Carrega P, Bonaccorsi I, Di CE, Morandi B, Paul P, Rizzello V, et al. CD56(bright) perforin(low) noncytotoxic human NK cells are abundant in both healthy and neoplastic solid tissues and recirculate to secondary lymphoid organs via afferent lymph. *J Immunol* (2014) 192:3805–15. doi:10.4049/jimmunol.1301889
21. Hudspeth K, Donadon M, Cimino M, Pontarini E, Tentorio P, Preti M, et al. Human liver-resident CD56(bright)/CD16(neg) NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways. *J Autoimmun* (2016) 66:40–50. doi:10.1016/j.jaut.2015.08.011
22. Cichocki F, Sitnicka E, Bryceson YT. NK cell development and function – plasticity and redundancy unleashed. *Semin Immunol* (2014) 26:114–26. doi:10.1016/j.smim.2014.02.003
23. Bruno A, Ferlazzo G, Albini A, Noonan DM. A think tank of TINK/TANKs: tumor-infiltrating/tumor-associated natural killer cells in tumor progression and angiogenesis. *J Natl Cancer Inst* (2014) 106:dju200. doi:10.1093/jnci/dju200
24. Cantoni C, Huergo-Zapico L, Parodi M, Pedrazzi M, Mingari MC, Moretta A, et al. NK cells, tumor cell transition, and tumor progression in solid

malignancies: new hints for NK-based immunotherapy? *J Immunol Res* (2016) 2016:4684268. doi:10.1155/2016/4684268
25. Levi I, Amsalem H, Nissan A, Darash-Yahana M, Peretz T, Mandelboim O, et al. Characterization of tumor infiltrating natural killer cell subset. *Oncotarget* (2015) 6:13835–43. doi:10.18632/oncotarget.3453
26. Bruno A, Focaccetti C, Pagani A, Imperatori AS, Spagnoletti M, Rotolo N, et al. The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer. *Neoplasia* (2013) 15:133–42. doi:10.1593/neo.121758
27. Hasmim M, Messai Y, Ziani L, Thiery J, Bouhris JH, Noman MZ, et al. Critical role of tumor microenvironment in shaping NK cell functions: implication of hypoxic stress. *Front Immunol* (2015) 6:482. doi:10.3389/fimmu.2015.00482
28. Carrega P, Ferlazzo G. Natural killer cell distribution and trafficking in human tissues. *Front Immunol* (2012) 3:347. doi:10.3389/fimmu.2012.00347
29. Tallerico R, Garofalo C, Carbone E. A new biological feature of natural killer cells: the recognition of solid tumor-derived cancer stem cells. *Front Immunol* (2016) 7:179. doi:10.3389/fimmu.2016.00179
30. Pasero C, Gravis G, Granjeaud S, Guerin M, Thomassin-Piana J, Rocchi P, et al. Highly effective NK cells are associated with good prognosis in patients with metastatic prostate cancer. *Oncotarget* (2015) 6:14360–73. doi:10.18632/oncotarget.3965
31. Messaoudene M, Fregni G, Fourmentraux-Neves E, Chanal J, Maubec E, Mazouz-Dorval S, et al. Mature cytotoxic CD56(bright)/CD16(+) natural killer cells can infiltrate lymph nodes adjacent to metastatic melanoma. *Cancer Res* (2014) 74:81–92. doi:10.1158/0008-5472.CAN-13-1303
32. Ali TH, Pisanti S, Ciaglia E, Mortarini R, Anichini A, Garofalo C, et al. Enrichment of CD56(dim)KIR + CD57 + highly cytotoxic NK cells in tumour-infiltrated lymph nodes of melanoma patients. *Nat Commun* (2014) 5:5639. doi:10.1038/ncomms6639
33. Holtan SG, Creedon DJ, Thompson MA, Nevala WK, Markovic SN. Expansion of CD16-negative natural killer cells in the peripheral blood of patients with metastatic melanoma. *Clin Dev Immunol* (2011) 2011:316314. doi:10.1155/2011/316314
34. Platonova S, Cherfils-Vicini J, Damotte D, Crozet L, Vieillard V, Validire P, et al. Profound coordinate alterations of intratumoral NK cell phenotype and function in lung carcinoma. *Cancer Res* (2011) 15:5412–22. doi:10.1158/0008-5472.CAN-10-4179
35. Delahaye NF, Rusakiewicz S, Martins I, Ménard C, Roux S, Lyonnet L, et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat Med* (2011) 17:700–7. doi:10.1038/nm.2366
36. Costello RT, Sivori S, Marcenaro E, Lafage-Pochitaloff M, Mozziconacci MJ, Reviron D, et al. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. *Blood* (2002) 99:3661–7. doi:10.1182/blood.V99.10.3661
37. Stringaris K, Sekine T, Khoder A, Alsuliman A, Razzaghi B, Sargeant R, et al. Leukemia-induced phenotypic and functional defects in natural killer cells predict failure to achieve remission in acute myeloid leukemia. *Haematologica* (2014) 99:836–47. doi:10.3324/haematol.2013.087536
38. Stabile H, Nisti P, Morrone S, Pagliara D, Bertaina A, Locatelli F, et al. Multifunctional human CD56 low CD16 low natural killer cells are the prominent subset in bone marrow of both healthy pediatric donors and leukemic patients. *Haematologica* (2015) 100:489–98. doi:10.3324/haematol.2014.116053
39. Epling-Burnette PK, Bai F, Painter JS, Rollison DE, Salih HR, Krusch M, et al. Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. *Blood* (2007) 109:4816–24. doi:10.1182/blood-2006-07-035519
40. Konjevic G, Vuletic A, Mirjacic MK, Colovic N, Colovic M, Jurisic V. Decreased CD161 activating and increased CD158a inhibitory receptor expression on NK cells underlies impaired NK cell cytotoxicity in patients with multiple myeloma. *J Clin Pathol* (2016) 69:1009–16. doi:10.1136/jclinpath-2016-203614
41. Costello RT, Boehrer A, Sanchez C, Mercier D, Baier C, Le TT, et al. Differential expression of natural killer cell activating receptors in blood versus bone marrow in patients with monoclonal gammopathy. *Immunology* (2013) 139:338–41. doi:10.1111/imm.12082
42. Chretien AS, Granjeaud S, Gondois-Rey F, Harbi S, Orlanducci F, Blaise D, et al. Increased NK cell maturation in patients with acute myeloid leukemia. *Front Immunol* (2015) 6:564. doi:10.3389/fimmu.2015.00564
43. Aggarwal N, Swerdlow SH, TenEyck SP, Boyiadzis M, Felgar RE. Natural killer cell (NK) subsets and NK-like T-cell populations in acute myeloid leukemias and myelodysplastic syndromes. *Cytometry B Clin Cytom* (2016) 90:349–57. doi:10.1002/cyto.b.21349
44. Geyh S, Oz S, Cadeddu RP, Frobel J, Bruckner B, Kundgen A, et al. Insufficient stromal support in MDS results from molecular and functional deficits of mesenchymal stromal cells. *Leukemia* (2013) 27:1841–51. doi:10.1038/leu.2013.193
45. Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, et al. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells. *Cancer* (2008) 112:863–75. doi:10.1002/cncr.23239
46. Mamessier E, Pradel LC, Thibult ML, Drevet C, Zouine A, Jacquemier J, et al. Peripheral blood NK cells from breast cancer patients are tumor-induced composite subsets. *J Immunol* (2013) 190:2424–36. doi:10.4049/jimmunol.1200140
47. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* (2006) 12:1065–74. doi:10.1038/nm1452
48. Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, et al. Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood* (2008) 111:3108–15. doi:10.1182/blood-2007-08-105965
49. Carlino C, Trotta E, Stabile H, Morrone S, Bulla R, Soriani A, et al. Chemerin regulates NK cell accumulation and endothelial cell morphogenesis in the decidua during early pregnancy. *J Clin Endocrinol Metab* (2012) 97:3603–12. doi:10.1210/jc.2012-1102
50. Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, et al. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur J Immunol* (1999) 29:1656–66. doi:10.1002/(SICI)1521-4141(199905)29:05<1656::AID-IMMU1656>3.0.CO;2-1
51. Rouce RH, Shaim H, Sekine T, Weber G, Ballard B, Ku S, et al. The TGF-beta/SMAD pathway is an important mechanism for NK cell immune evasion in childhood B-acute lymphoblastic leukemia. *Leukemia* (2016) 30:800–11. doi:10.1038/leu.2015.327
52. Sanchez-Correa B, Gayoso I, Bergua JM, Casado JG, Morgado S, Solana R, et al. Decreased expression of DNAM-1 on NK cells from acute myeloid leukemia patients. *Immunol Cell Biol* (2012) 90:109–15. doi:10.1038/icb.2011.15
53. El-Sherbiny YM, Meade JL, Holmes TD, McGonagle D, Mackie SL, Morgan AW, et al. The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res* (2007) 67:8444–9. doi:10.1158/0008-5472.CAN-06-4230
54. Fauriat C, Mallet F, Olive D, Costello RT. Impaired activating receptor expression pattern in natural killer cells from patients with multiple myeloma. *Leukemia* (2006) 20:732–3. doi:10.1038/sj.leu.2404096
55. Chen CI, Koschmieder S, Kerstiens L, Schemionek M, Altvater B, Pscherer S, et al. NK cells are dysfunctional in human chronic myelogenous leukemia before and on imatinib treatment and in BCR-ABL-positive mice. *Leukemia* (2012) 26:465–74. doi:10.1038/leu.2011.239
56. Hejazi M, Manser AR, Frobel J, Kundgen A, Zhao X, Schonberg K, et al. Impaired cytotoxicity associated with defective natural killer cell differentiation in myelodysplastic syndromes. *Haematologica* (2015) 100:643–52. doi:10.3324/haematol.2014.118679
57. Szczepanski MJ, Szajnik M, Welsh A, Whiteside TL, Boyiadzis M. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-beta1. *Haematologica* (2011) 96:1302–9. doi:10.3324/haematol.2010.039743
58. Smyth MJ, Teng MW, Swann J, Kyparissoudis K, Godfrey DI, Hayakawa Y. CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol* (2006) 176:1582–7. doi:10.4049/jimmunol.176.3.1582
59. Shenghui Z, Yixiang H, Jianbo W, Kang Y, Laixi B, Yan Z, et al. Elevated frequencies of CD4(+) CD25(+) CD127lo regulatory T cells is associated to poor prognosis in patients with acute myeloid leukemia. *Int J Cancer* (2011) 129:1373–81. doi:10.1002/ijc.25791

60. Mamessier E, Sylvain A, Thibult ML, Houvenaeghel G, Jacquemier J, Castellano R, et al. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *J Clin Invest* (2011) 121:3609–22. doi:10.1172/JCI45816
61. Allan DS, Rybalov B, Awong G, Zuniga-Pflucker JC, Kopcow HD, Carlyle JR, et al. TGF-beta affects development and differentiation of human natural killer cell subsets. *Eur J Immunol* (2010) 40:2289–95. doi:10.1002/eji.200939910
62. Keskin DB, Allan DS, Rybalov B, Andzelm MM, Stern JN, Kopcow HD, et al. TGFbeta promotes conversion of CD16+ peripheral blood NK cells into. *Proc Natl Acad Sci U S A* (2007) 104:3378–83. doi:10.1073/pnas.0611098104
63. Diaz-Blanco E, Bruns I, Neumann F, Fischer JC, Graef T, Rosskopf M, et al. Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients with CML in chronic phase. *Leukemia* (2007) 21:494–504. doi:10.1038/sj.leu.2404549
64. Bruns I, Cadeddu RP, Brueckmann I, Frobel J, Geyh S, Bust S, et al. Multiple myeloma-related deregulation of bone marrow-derived CD34(+) hematopoietic stem and progenitor cells. *Blood* (2012) 120:2620–30. doi:10.1182/blood-2011-04-347484
65. Castriconi R, Dondero A, Bellora F, Moretta L, Castellano A, Locatelli F, et al. Neuroblastoma-derived TGF-beta1 modulates the chemokine receptor repertoire of human resting NK cells. *J Immunol* (2013) 190:5321–8. doi:10.4049/jimmunol.1202693
66. Halama N, Michel S, Kloor M, Zoernig I, Benner A, Spille A, et al. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer Res* (2011) 71:5670–7. doi:10.1158/0008-5472.CAN-11-0268
67. Baragano RA, Suarez-Alvarez B, Lopez-Larrea C. Secretory pathways generating immunosuppressive NKG2D ligands: new targets for therapeutic intervention. *Oncoimmunology* (2014) 3:e28497. doi:10.4161/onci.28497
68. Zingoni A, Cecere F, Vulpis E, Fionda C, Molfetta R, Soriani A, et al. Genotoxic stress induces senescence-associated ADAM10-dependent release of NKG2D MIC ligands in multiple myeloma cells. *J Immunol* (2015) 195:736–48. doi:10.4049/jimmunol.1402643
69. Fionda C, Soriani A, Zingoni A, Santoni A, Cippitelli M. NKG2D and DNAM-1 ligands: molecular targets for NK cell-mediated immunotherapeutic intervention in multiple myeloma. *Biomed Res Int* (2015) 2015:178698. doi:10.1155/2015/178698
70. Hilpert J, Grosse-Hovest L, Grunebach F, Buechele C, Nuebling T, Raum T, et al. Comprehensive analysis of NKG2D ligand expression and release in leukemia: implications for NKG2D-mediated NK cell responses. *J Immunol* (2012) 189:1360–71. doi:10.4049/jimmunol.1200796
71. Paschen A, Baingo J, Schadendorf D. Expression of stress ligands of the immunoreceptor NKG2D in melanoma: regulation and clinical significance. *Eur J Cell Biol* (2014) 93:49–54. doi:10.1016/j.ejcb.2014.01.009
72. Molfetta R, Quatrini L, Zitti B, Capuano C, Galandrini R, Santoni A, et al. Regulation of NKG2D expression and signaling by endocytosis. *Trends Immunol* (2016) 37:790–802. doi:10.1016/j.it.2016.08.015
73. Varbanova V, Naumova E, Mihaylova A. Killer-cell immunoglobulin-like receptor genes and ligands and their role in hematologic malignancies. *Cancer Immunol Immunother* (2016) 65:427–40. doi:10.1007/s00262-016-1806-9
74. Sanchez-Correa B, Morgado S, Gayoso I, Bergua JM, Casado JG, Arcos MJ, et al. Human NK cells in acute myeloid leukaemia patients: analysis of NK cell-activating receptors and their ligands. *Cancer Immunol Immunother* (2011) 60:1195–205. doi:10.1007/s00262-011-1050-2
75. Verheyden S, Demanet C. Susceptibility to myeloid and lymphoid leukemia is mediated by distinct inhibitory KIR-HLA ligand interactions. *Leukemia* (2006) 20:1437–8. doi:10.1038/sj.leu.2404279
76. Schutt P, Schutt B, Switala M, Bauer S, Stamatis G, Opalka B, et al. Prognostic relevance of soluble human leukocyte antigen-G and total human leukocyte antigen class I molecules in lung cancer patients. *Hum Immunol* (2010) 71:489–95. doi:10.1016/j.humimm.2010.02.015
77. Yie SM, Hu Z. Human leukocyte antigen-G (HLA-G) as a marker for diagnosis, prognosis and tumor immune escape in human malignancies. *Histol Histopathol* (2011) 26:409–20. doi:10.14670/HH-26.409
78. Maki G, Hayes GM, Naji A, Tyler T, Carosella ED, Rouas-Freiss N, et al. NK resistance of tumor cells from multiple myeloma and chronic lymphocytic leukemia patients: implication of HLA-G. *Leukemia* (2008) 22:998–1006. doi:10.1038/leu.2008.15
79. McWilliams EM, Mele JM, Cheney C, Timmerman EA, Fiazuddin F, Strattan EJ, et al. Therapeutic CD94/NKG2A blockade improves natural killer cell dysfunction in chronic lymphocytic leukemia. *Oncoimmunology* (2016) 5:e1226720. doi:10.1080/2162402X.2016.1226720
80. Kearl TJ, Jing W, Gershan JA, Johnson BD. Programmed death receptor-1/programmed death receptor ligand-1 blockade after transient lymphodepletion to treat myeloma. *J Immunol* (2013) 190:5620–8. doi:10.4049/jimmunol.1202005
81. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* (2010) 116:1757–66. doi:10.1002/cncr.24899

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 18 January 2017
doi: 10.3389/fimmu.2016.00694

STATs in NK-Cells: The Good, the Bad, and the Ugly

Dagmar Gotthardt† *and Veronika Sexl**

Department for Biomedical Sciences, Institute of Pharmacology and Toxicology, University of Veterinary Medicine, Vienna, Austria

Natural killer (NK)-cells are major players in the fight against viral infections and transformed cells, but there is increasing evidence attributing a disease-promoting role to NK-cells. Cytokines present in the tumor microenvironment shape NK-cell maturation, function, and effector responses. Many cytokines signal *via* the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway that is also frequently altered and constitutively active in a broad range of tumor cells. As a consequence, there are currently major efforts to develop therapeutic strategies to target this pathway. Therefore, it is of utmost importance to understand the role and contributions of JAK–STAT molecules in NK-cell biology—only this knowledge will allow us to predict effects of JAK–STAT inhibition for NK-cell functions and to successfully apply precision medicine. We will review the current knowledge on the role of JAK–STAT signaling for NK-cell functions and discuss conditions involved in the switch from NK-cell tumor surveillance to disease promotion.

Keywords: JAK–STAT, tumor surveillance, cytotoxicity, immunologic, mouse models, NK cells, VEGF-A, tumor promotion

OPEN ACCESS

Edited by:
Vincent Vieillard, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Evelyn Ullrich, Goethe University Frankfurt, Germany
Laura Eugenia Velazquez, French Institute of Health and Medical Research, France

***Correspondence:**
Veronika Sexl
veronika.sexl@vetmeduni.ac.at

†Present address:
Dagmar Gotthardt, Department of Microbiology and Immunology, University of California San Francisco, San Francisco, CA, USA

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *14 November 2016*
Accepted: *28 December 2016*
Published: *18 January 2017*

Citation:
Gotthardt D and Sexl V (2017) STATs in NK-Cells: The Good, the Bad, and the Ugly. Front. Immunol. 7:694. doi: 10.3389/fimmu.2016.00694

INTRODUCTION

Natural killer (NK) cells are major players of the innate immune system and immediate effector cells against viral infections, pathogens, and malignant cells. In humans, NK-cells compromise 5–15% of circulating blood lymphocytes and are further sub-divided based on the expression of the cell adhesion molecule CD56 and the low affinity Fc-receptor CD16 into $CD3^{-}CD56^{bright}CD16^{-}$ and $CD3^{-}CD56^{dim}CD16^{+}$ NK-cells. $CD56^{bright}$ NK-cells are mainly found in lymph nodes, produce cytokines upon activation, and possess only minor cytotoxic potential. Upon maturation to $CD56^{dim}$ cells—the majority of circulating NK-cells in healthy humans representing approximately 90% of NK-cells—gain significant cytotoxic potential (1, 2). There is ample evidence for the ability of NK-cells to recognize and lyse a broad variety of tumor cells (3–5). The ability of NK-cell-mediated immune surveillance extends to the prevention of metastatic spread (6–8), which is currently one of the dominating clinical problems in cancer therapy.

Initially described to function without prior sensitization, accumulating evidence demonstrates that NK-cell effector function is a complex and tightly regulated process (9–12). This ensures rapid effector reactions while preventing autoimmunity (13). During development, NK-cells undergo a licensing process that shapes their responsive steady state. NK-cells lacking inhibitory receptors or unable to recognize cognate MHC molecules maintain a hyporesponsive state (13, 14). Even if fully developed and equipped to act against target cells, NK-cells require a costimulatory signal to pull

the final trigger. Cytokines provided by the microenvironment or the ligation of activating receptors serve as promoting signals (15, 16). NK-cell activation is thus also controlled by the availability of cytokines including type I interferons (IFNs) from TLR^+ cells. IFNAR signaling in dendritic cells leads to the subsequent production of IL-15 that is trans-presented to activate NK-cells (17). Besides IL-15, also IL-2 produced by $CD4^+$ T cells stimulates NK-cell activation while regulatory T cells (T_{regs}) inhibit NK-cell responses in a TGF-β-dependent manner. Moreover, T_{regs} expressing the high affinity IL-2 receptor alpha chain CD25 limit the availability of IL-2 for NK-cells (18, 19).

Another layer of complexity is added by the escape mechanisms of tumor cells. Tumor cells evade NK-cell recognition by several mechanisms including changes in expression of MHC class I or secretion of cytokines and mediators impeding NK-cell responses (20). Immunosuppressive cytokines such as TGF-β or adenosine in the tumor microenvironment block NK-cell maturation and their cytotoxic potential or act indirectly by recruiting suppressor cells (21, 22). Cytokines have thus both abilities; they may activate or block NK-cells.

Most cytokines influencing NK-cell functions signal *via* Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, a conserved pathway transmitting extracellular signals from the cell surface to the nucleus (23). The JAK–STAT pathway is frequently altered and constitutively active in a broad range of tumors. There are major efforts to develop therapeutic strategies to target components of this pathway (24–26). It is thus critical to comprehend the role of JAK–STAT molecules in NK-cell biology. This knowledge will enable to predict effects of JAK–STAT inhibition for NK-cells, a prerequisite for precision medicine.

JAK–STAT

Cytokine binding to a respective receptor on the cell surface leads to the activation of receptor-associated tyrosine kinases, the JAKs. Once activated, JAKs trans-phosphorylate each other, thereby creating docking sites for signal transducer and activator of transcription (STAT) molecules. Subsequent to binding, STATs become activated by JAK-mediated tyrosine phosphorylation and form homo- or heterodimers, translocate to the nucleus where they regulate transcription (27, 28). Four distinct JAK kinases (JAK1, 2, 3, and TYK2) as well as seven different STAT proteins exist (STAT1, 2, 3, 4, 5A, 5B, and 6). One cytokine may activate more than one member of the JAK and/or STAT family (29). **Table 1** summarizes our current knowledge on JAK–STAT signaling in NK-cells.

JAKs: THE DRIVER OF THE STATs

One cytokine may activate more than one JAK and each JAK targets more than one STAT protein. This multilayered and complex activation pattern creates sometimes elaborate phenotypes upon deletion or inhibition of single components (46). The distinct roles of JAK kinases for NK-cell biology are on the edge of being unraveled, currently only limited information is available.

Treatment with the JAK1/JAK2 inhibitor ruxolitinib reduces NK-cell numbers, impairs their proliferation, maturation, and cytolytic capacity. Application of ruxolitinib in a murine breast cancer model enhanced metastatic spread by interfering with NK-cell functions (7, 47). The fact that ruxolitinib efficiently inhibits JAK1 and JAK2 but also with low affinity JAK3, makes it difficult to assign specific roles to distinct members of the JAK family. NK-cells fail to develop in *Jak3*$^{-/-}$ mice—a phenotype that is mirrored in patients harboring *Jak3* mutations. These patients suffer from a SCID phenotype lacking T and NK-cells (48–50). The contribution of JAK1 and JAK2 on NK-cell development and function needs to be further explored. While JAK3 is predominantly expressed in the hematopoietic compartment, JAK1 and JAK2 are ubiquitously expressed and *Jak1* and *Jak2* knockouts are perinatal/embryonic lethal (51, 52). JAK1 has been reported to be crucial for lymphopoiesis, and both JAK1 and JAK3 are important upstream kinases mediating IL-15-dependent signaling and subsequent STAT5 activation (52–54). It is attractive to speculate that loss of JAK1 would as well induce the loss of peripheral NK-cells.

Experiments using *Jak2*$^{-}$ conditional knockout mice uncovered a critical role for JAK2 in NK-cell maturation (7). Breast cancer metastasis related to impaired NK-cell function was enhanced in mice treated with the JAK2-specific inhibitor BSK805. Simultaneous treatment with IL-15 prevented the enhanced metastasis provoked by JAK2 inhibition. This indicates that BSK805-mediated JAK2 inhibition does not affect IL-15-mediated responses in NK-cells presumably acting *via* JAK1 and JAK3 (7). Only the generation and analysis of NK cell-specific conditional knockout mice will allow us to characterize the individual effects of JAKs on NK-cell development and effector function.

In contrast to other JAKs, *Tyk2*$^{-/-}$ NK-cells are present at normal numbers but show impaired IL-12/IL-18-mediated signaling with reduced STAT4 activation. Consequently, *Tyk2*$^{-/-}$ NK-cells possess a severely impaired cytolytic activity, do not efficiently clear certain infections, and display an impaired tumor immune surveillance (55–58). In line, patients with autosomal recessive *Tyk2* mutations suffer from recurrent bacterial and viral infections and display impaired NK-cell responses (59).

THE GOOD: STAT1: IT TURNS THE KILLING ON

STAT1 and STAT2 are well studied transcription factors and important for signals in response to IFNs (60). Our knowledge on STAT2-regulated NK-cell functions is limited; it is known that STAT2 controls viral load during LCMV infections (61). In contrast, STAT1 effects have been characterized in more detail. STAT1 is a crucial regulator of IFN-γ production and NK-cell cytotoxicity (60–62). *Stat1*-deficient mice are highly susceptible to bacterial and viral infections. *Stat1*$^{-/-}$ mice show reduced expression of MHC class I molecules, which is thought to lead to hyporesponsive, unlicensed NK-cells (63, 64). It is currently unclear whether the impaired cytotoxicity is solely the consequence of the impaired licensing or whether STAT1 fulfills other major

TABLE 1 | Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling in natural killer (NK)-cells (27, 30–45).

Cytokine	Receptor-associated JAKs	Activated STATs	Function	Effect induced by
IL-2	JAK1, JAK3	STAT1, STAT3, STAT5	Proliferation	STAT5
	JAK2	STAT4	Activation	STAT1/4/5; STAT3?
IL-7	JAK1, JAK3	STAT5	Survival of $CD56^{bright}$ NK-cells, upregulation of FasL	STAT5
			Development of distinct NK-cell subsets	
IL-12	JAK2, TYK2	STAT1, STAT3, STAT4	Activation	STAT1/4
			Induction of *Vegf-A* expression	STAT3?
IL-15	JAK1, JAK3	STAT5	Survival, maturation, proliferation	STAT5
		STAT3	Activation	STAT5, STAT3?
IL-10	JAK1	STAT3	Activation	STAT3
			Induction of *Vegf-A* expression	STAT3?
IL-21	JAK1, JAK3	STAT1, STAT3	Antiproliferative (mouse NK-cells), proliferation (human NK-cells)	STAT3?
			Maturation, activation	STAT1?
			Induction of *Vegf-A* expression	STAT3?
IL-27	JAK1	STAT1, STAT3, STAT5	Activation	Unknown
			Increased ADCC	STAT5?
			Increased IL-10 production	STAT3?
			Increased viability	STAT5?
			Decreased proliferation	STAT3?
Interferon-α/β	JAK1, TYK2	STAT1, STAT3	Maturation	STAT1; STAT4?
			Activation	STAT1/3/4
			Induction of *Vegf-A* expression	STAT3?

functions. The complexity of STAT1 signaling in innate immunity is further highlighted by the existence of a non-canonical STAT1 pathway. $STAT1^{Y701F}$ mutant proteins that cannot be activated by JAKs in the canonical manner partially rescue impaired cytolytic responses of *Stat1*$^{-/-}$ NK-cells. One potential explanation for this unexpected phenomenon is the finding that STAT1 locates to the immunological synapse when NK-cells conjugate target cells. In line, STAT1 has been shown to bind proteins involved in cell junction formation at the immunological synapse during tumor cell recognition (65). Moreover, *ex vivo* derived NK-cells show a constitutive phosphorylation of the STAT1-S727 residue restraining NK-cell cytotoxicity. This phosphorylation is present without any stimulus and prior to tyrosine phosphorylation, thus deviating from the canonical STAT activation (6, 28). These observations point at a complex and multilayered function of STAT1 in NK-cells and suggest STAT1 as a central node integrating several processes.

Many effects described in *Stat1*-deficient mice are mirrored in patients. STAT1 deficiency in humans is an autosomal recessive immune disorder; null mutations are associated with recurrent bacterial and viral infections indicating impaired NK-cell activities although no detailed information is available so far (66–70).

THE UGLY: STAT3: AVOIDING AUTOIMMUNITY OR THE TARGET FOR NK-CELL THERAPY?

While cytokines such as IL-12, IL-15, IL-21, and type I IFNs induce STAT3 tyrosine phosphorylation in NK-cells, the most potent activation is achieved by treatment with the immunosuppressive and anti-inflammatory cytokine IL-10 (71). Many tumors harbor constitutively active STAT3 that triggers the release of immunosuppressive cytokines such as IL-10 or TGF-β. These tumor-derived cytokines further induce a pronounced STAT3 phosphorylation in infiltrating immune cells. There, induced STAT3 activation is considered to impair tumor immune surveillance and allows the tumor to escape immune control (72, 73). High levels of STAT3 phosphorylation in the tumor stroma often correlate with loss of intact tumor immune surveillance (74). This effect is of therapeutic interest as STAT3 inhibitors are currently developed to treat patients suffering from cancer of various origin (75, 76). There is dual hope in these STAT3-directed therapies; on the one side, they are expected to block STAT3-mediated growth promoting and pro-survival signals in the tumor cells themselves. On the other hand, STAT3 inhibitors directly act on the infiltrating immune cells and might boost their cytotoxic behavior.

There is first evidence that this concept holds true for NK-cells. Studies in mouse models uncovered that STAT3 activation in NK-cells indeed suppresses cytotoxicity. The deletion of STAT3 in NK-cells enhanced cytotoxicity in melanoma and leukemia models (71, 77) and resulted in a prolonged survival (71). The absence of STAT3 was paralleled by an increased expression of perforin and granzyme B and the activating receptor DNAM-1. There is conflicting evidence if and how STAT3 also regulates the expression of the activating NKG2D receptor in NK-cells. In human, NK-cells stimulation with IL-10 and IL-21 induces NKG2D expression in a STAT3-dependent manner. Similar results were obtained in a mouse study showing enhanced NKG2D-mediated antitumor responses upon IL-21 treatment (78, 79). Against the expectations, *Stat3*$^{-/-}$ NK-cells isolated from *Stat3*$^{fl/fl}$*Ncr1*-Cre^{Tg} mice, where deletion of STAT3 is restricted to $NKp46^{+}$ cells, show no changes in NKG2D expression (71). In contrast, NK-cells analyzed from *Stat3*$^{fl/fl}$*VavCre* mice showed reduced NKG2D expression (79). The controversy is further heated by a study showing that IL-21 stimulation inhibits NKG2D expression of IL-2-cultured primary human NK-cells (80). Several scenarios

may explain these conflicting results; one may envision that STAT3 is involved in epigenetic processes that control NKG2D expression and that occur prior to NKp46 expression. In such a scenario, the deletion of STAT3 in a NKp46^{+} population would be too late in NK-cell development to interfere with NKG2D expression. Alternatively, the regulation of NKG2D expression in NK-cells might require cell extrinsic-cues that depend on STAT3 and are lost in *Stat3$^{fl/fl}$VavCre* mice upon deletion in the entire hematopoietic system (79).

Of note, STAT3 inhibition in tumors has been shown to enhance immunogenicity even in tumors that do not depend on STAT3 for survival and growth. One of the mechanisms how immunogenicity is increased is the enhanced expression of NKG2D ligands on tumor cells (81, 82).

Another consequence of STAT3 deletion in NK-cells is an increased expression level of STAT5 (71). As described below, STAT5 is a potent stimulator of NK-cell survival and cytotoxicity. It remains to be determined how any STAT3-directed therapy will interfere with the delicate balance of STAT3-mediated suppression and STAT5-mediated activation of NK-cell cytotoxicity. This is of particular relevance when employing cytokines that act *via* both STAT proteins, e.g., IL-15. It is attractive to speculate that IL-15-induced STAT3 activation may serve to counteract the IL-15-STAT5-mediated NK-cell cytotoxicity to prevent autoimmunity. A detailed understanding of the mechanisms governing the repression of NK-cell overshoots is of utmost therapeutic importance. Cancer therapies aim at increasing the potential of killers while avoiding self-destruction.

THE GOOD: STAT4: YOU BETTER HAVE MORE

STAT4 is a prerequisite for IL-12-mediated cytotoxicity and IFN-γ production in murine and human NK-cells (83, 84). Additionally, STAT4 has been described to induce T-bet and IL-10 in NK-cells and to be involved in the generation of memory NK-cells after MCMV infection (83, 85, 86). Direct binding of STAT4 to the perforin promoter has been reported in human NK-cells (87). Besides its potent activation by IL-12 stimulation, high basal levels of STAT4 protein expression have been detected in murine and human NK-cells (61). In contrast to other immune cells, IL-2 treatment activates STAT4 in NK-cells and enhances responses to IL-12 by upregulating of the IL12R (38, 84). It is attractive to speculate that the constitutively high expression levels of STAT4 represent a "ready-to-go" repertoire that enables NK-cells to immediately react on cytokine exposure. This hypothesis is supported by the fact that NK-cells represent the first line of defense against pathogens—their rapid and efficient activation being a prerequisite. In line, tolerogenic NK-cells have been reported in the context of liver transplantation, where immunosuppression subsequently decreased STAT4 levels and resulting in hyporesponsive NK-cells (88).

Although IL-12 possesses the potential to also activate STAT1 and STAT3, STAT4 appears to be crucial in mediating IL-12-induced signaling and IFN-γ production. The role of IL-12-induced STAT1 and STAT3 activation for IFN-γ production is currently unclear. It may represent an evolutionary backup to induce a second wave of IFN-γ response. On the other hand, STAT1 and STAT3 may act as feedback loop and prevent successive production. In fact, binding of several STAT molecules to the IFN-γ promoter has been reported (71, 89).

THE GOOD: STAT5: TEACHES NK-CELLS HOW TO DRIVE

STAT5 transmits signals downstream of IL-2 and IL-15, and its expression is indispensable for the survival of peripheral NK-cells (90). STAT5 exists of two homologs, STAT5A and STAT5B, that share more than 90% sequence identity and arose by gene duplication (91). There is evidence that the loss of STAT5B, but not STAT5A reduces NK-cell numbers and impairs cytolytic responses (92). This is mirrored in patients harboring *Stat5b* deficiencies and suffering from NK-cell lymphopenia, recurrent bacterial and viral infections, several clinical pathologies, and high morbidity (67, 93). While the deletion of STAT5B only reduces NK-cell numbers to 50%, the targeted deletion of STAT5A and STAT5B in NK-cells induces apoptosis and leads to a complete loss of peripheral NK-cells (90). These data indicate that both STAT5 isoforms are involved in NK-cell maturation and survival (90). Survival of STAT5-deficient NK-cells can be rescued by the enhanced expression of the anti-apoptotic gene *Bcl-2* and allows studying the role of STAT5 for other NK-cell functions. STAT5 is not only regulating NK-cell survival, proliferation, and cytotoxicity but also drives cell maturation (94) by driving the expression of transcription factors involved in NK-cell maturation and survival (94). Besides allowing NK-cell maturation and cytotoxicity, STAT5 suppresses the tumor-promoting potential of NK-cells (94). Similar to myeloid cells, NK-cells have the potential to support tumor growth by secreting VEGF-A (94, 95). VEGF-A expression and thus tumor promotion is suppressed by STAT5 with STAT5B being the relevant isoform (94). There is accumulating evidence for the existence of VEGF-A secreting tumor infiltrating NK-cells in patients suffering from small lung cell cancer, breast, and colon tumors (96, 97). These tumor-promoting NK-cells are immature (CD56^{bright}), and their presence has been correlated to poor disease prognosis in several studies (98–100). Therefore, it is attractive to speculate that IL-2- and IL-15-mediated STAT5 activation in cancer patients does not only activate NK-cell cytotoxicity but also reverts pro-angiogenic effects. Decidual NK-cells have been the first NK-cells reported to produce VEGF to promote trophoblast invasion and remodeling of spiral arteries (101–103). Uterine NK-cells are poorly cytotoxic with a particular cytokine profile (101). It remains to be elucidated whether STAT5 is also involved in VEGF-A production in the decidua. A suppressive cytokine milieu such as TGF-β in the uterus or hypoxic conditions might dampen STAT5 signaling and represent a prerequisite for VEGF-A transcription. Evolution brought two types of NK-cells into light: besides being effective killers NK-cells have acquired to adapt to immunosuppressive cytokines and to switch to a tolerogenic but pro-angiogenic behavior.

THE BAD: STAT6: STILL SOME MISSING BRICKS

Activation of STAT6 has been reported to drive IL-5 and IL-13 production in cultured NK-cells and to limit cytotoxic responses (104). In line, studies with *Stat6*$^{-/-}$ mice showed increased viral resistance and higher cytolytic activity of NK-cells in the absence of STAT6 (105). However, a positive correlation of STAT6 expression and IFN-γ production was reported after costimulating murine NK-cells with IL-4 and IL-2 (106). Further studies need to explore whether a STAT6 blockade would be a potential therapeutic option to enhance responses in human NK-cells.

CONCLUSION

The JAK–STAT pathway is evolutionary highly conserved; thus, the human situation nicely matches the findings in experimental animal models. In that line, many insights that we gained from murine NK-cells can be translated to human NK-cells. **Figure 1** summarizes our current knowledge on the role of STATs in NK-cell functions. In general, STAT1, STAT4, and STAT5 stimulate NK-cell maturation and cytotoxicity, whereas STAT3 and STAT6 negatively impact on NK-cell activity. It is attractive to speculate that the suppressive role of STAT3 and STAT6 is important to prevent NK-cell overshoots and autoimmunity. STAT5 is the only STAT family member that is indispensable for NK-cells since it governs survival and growth in addition to cytotoxicity and maturation. It may thus be seen as NK-cell master regulator.

As shown for macrophages NK-cells not only inhibit but also promote tumor formation, e.g., by producing VEGF-A. So far, STAT5 has been shown to prevent NK-cell-mediated tumor promotion by suppressing VEGF-A. However, it is unclear if and how other family members contribute to the switch from tumor suppression to tumor progression. Another layer of complexity is added by the fact that STATs rarely act alone but are embedded in a network of signaling events depending on the microenvironment and stimuli present. Signal integration is required to determine outcomes; at its lowest level integration of activity arising from various STAT family members is needed as even a single cytokine can activate multiple STATs (listed in **Table 1**). Some cytokines activate STAT family members with opposing functions such as IL-12 or type I IFNs. Further research will have to link NK activity and biological outcomes to cytokine-induced STAT activation and their synergic and/or antagonistic roles. The evolving field of systems biology may be of help to address these issues and/or to even predict the complex biologically and medically relevant questions *in vivo* at high pace to optimize current cancer therapies.

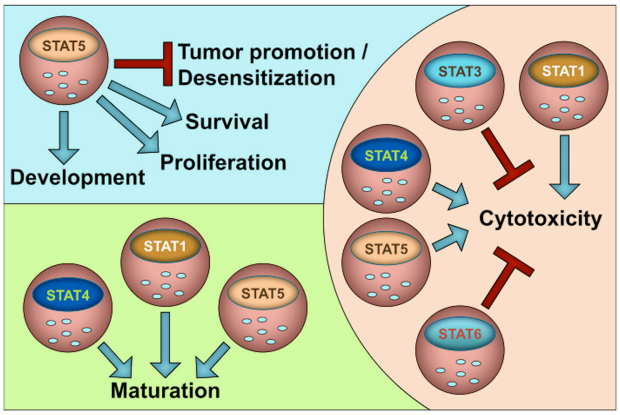


FIGURE 1 | The role of STATs for NK cell homeostasis and function.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

The authors thank the entire JAK–STAT community in Vienna for the many discussions on JAK–STAT signaling in NK-cells. They are grateful to Peter Martinek for his help with the graphical illustration and to Andrea Hölbl-Kovacic for detailed corrections and editing the manuscript.

FUNDING

The authors were supported by the PhD program "Inflammation and Immunity" FWF W1212 and the grant FWF P28571-B21.

REFERENCES

1. Caligiuri MA. Human natural killer cells. *Blood* (2008) 112:461–9. doi:10.1182/blood-2007-09-077438
2. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* (2001) 22:633–40. doi:10.1016/S1471-4906(01)02060-9
3. Waldhauer I, Steinle A. NK-cells and cancer immunosurveillance. *Oncogene* (2008) 27:5932–43. doi:10.1038/onc.2008.267
4. Vermijlen D, Luo D, Froelich CJ, Medema JP, Kummer JA, Willems E, et al. Hepatic natural killer cells exclusively kill splenic/blood natural killer-resistant tumor cells by the perforin/granzyme pathway. *J Leukoc Biol* (2002) 72:668–76.
5. Giezeman-Smits KM, Gorter A, van Vlierberghe RL, Giezeman-Smits KM, v Eendenburg JD, Eggermont AM, et al. The regulatory role of CD45 on rat NK-cells in target cell lysis. *J Immunol Ref J Immunol UCSF Libr* (2016) 163:71–6.
6. Putz EM, Gotthardt D, Hoermann G, Csiszar A, Wirth S, Berger A, et al. CDK8-mediated STAT1-S727 phosphorylation restrains NK-cell cytotoxicity and tumor surveillance. *Cell Rep* (2013) 4(3):437–44. doi:10.1016/j.celrep.2013.07.012
7. Bottos A, Gotthardt D, Gill JW, Gattelli A, Frei A, Tzankov A, et al. Decreased NK-cell tumour immunosurveillance consequent to JAK inhibition enhances metastasis in breast cancer models. *Nat Commun* (2016) 7:12258. doi:10.1038/ncomms12258
8. Langers I, Renoux VM, Thiry M, Delvenne P, Jacobs N. Natural killer cells: role in local tumor growth and metastasis. *Biologics* (2012) 6:73–82. doi:10.2147/BTT.S23976
9. Cichocki F, Miller JS, Anderson SK, Bryceson YT. Epigenetic regulation of NK-cell differentiation and effector functions. *Front Immunol* (2013) 4:55. doi:10.3389/fimmu.2013.00055
10. Orr MT, Lanier LL. Natural killer cell education and tolerance. *Cell* (2010) 142:847–56. doi:10.1016/j.cell.2010.08.031

11. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* (2008) 9:495–502. doi:10.1038/ni1581
12. Watzl C, Urlaub D, Fasbender F, Claus M. Natural killer cell regulation – beyond the receptors. *F1000Prime Rep* (2014) 6:87. doi:10.12703/P6-87
13. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol* (2006) 6:520–31. doi:10.1038/nri1863
14. Yokoyama WM, Kim S. How do natural killer cells find self to achieve tolerance? *Immunity* (2006) 24:249–57. doi:10.1016/j.immuni.2006.03.006
15. Bryceson YT, March ME, Ljunggren H-G, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev* (2006) 214:73–91. doi:10.1111/j.1600-065X.2006.00457.x
16. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SE, Yagita H, et al. Activation of NK-cell cytotoxicity. *Mol Immunol* (2005) 42:501–10. doi:10.1016/j.molimm.2004.07.034
17. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* (2007) 26:503–17. doi:10.1016/j.immuni.2007.03.006
18. Kerdiles Y, Ugolini S, Vivier E. T cell regulation of natural killer cells. *J Exp Med* (2013) 210:1065–8. doi:10.1084/jem.20130960
19. Ralainirina N, Poli A, Michel T, Poos L, Andrès E, Hentges F, et al. Control of NK-cell functions by CD4+CD25+ regulatory T cells. *J Leukoc Biol* (2007) 81:144–53. doi:10.1189/jlb.0606409
20. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK-cell-based immunotherapy for malignant diseases. *Cell Mol Immunol* (2013) 10:230–52. doi:10.1038/cmi.2013.10
21. Naganuma H, Sasaki A, Satoh E, Nagasaka M, Nakano S, Isoe S, et al. Transforming growth factor-beta inhibits interferon-gamma secretion by lymphokine-activated killer cells stimulated with tumor cells. *Neurol Med Chir (Tokyo)* (1996) 36:789–95. doi:10.2176/nmc.36.789
22. Mittal D, Young A, Stannard K, Yong M, Teng MWL, Allard B, et al. Antimetastatic effects of blocking PD-1 and the adenosine A2A receptor. *Cancer Res* (2014) 74:3652–8. doi:10.1158/0008-5472.CAN-14-0957
23. Imada K, Leonard WJ. The Jak-STAT pathway. *Mol Immunol* (2000) 37:1–11. doi:10.1016/S0161-5890(00)00018-3
24. Yu H, Jove R. The STATs of cancer – new molecular targets come of age. *Nat Rev Cancer* (2004) 4:97–105. doi:10.1038/nrc1275
25. Springuel L, Renauld J-C, Knoops L. JAK kinase targeting in hematologic malignancies: a sinuous pathway from identification of genetic alterations towards clinical indications. *Haematologica* (2015) 100:1240–53. doi:10.3324/haematol.2015.132142
26. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer* (2009) 9:798–809. doi:10.1038/nrc2734
27. Murray PJ. The JAK-STAT signaling pathway: input and output integration. *J Immunol* (2007) 178:2623–9. doi:10.4049/jimmunol.178.5.2623
28. Li WX. Canonical and non-canonical JAK-STAT signaling. *Trends Cell Biol* (2008) 18:545–51. doi:10.1016/j.tcb.2008.08.008
29. Kallal LE, Biron CA. Changing partners at the dance: variations in STAT concentrations for shaping cytokine function and immune responses to viral infections. *JAKSTAT* (2013) 2:e23504. doi:10.4161/jkst.23504
30. Liu Z, Yang L, Cui Y, Wang X, Guo C, Huang Z, et al. IL-21 enhances NK-cell activation and cytolytic activity and induces Th17 cell differentiation in inflammatory bowel disease. *Inflamm Bowel Dis* (2009) 15:1133–44. doi:10.1002/ibd.20923
31. Brady J, Hayakawa Y, Smyth MJ, Nutt SL. IL-21 induces the functional maturation of murine NK-cells. *J Immunol* (2004) 172:2048–58. doi:10.4049/jimmunol.172.4.2048
32. Mocellin S, Panelli M, Wang E, Rossi CR, Pilati P, Nitti D, et al. IL-10 stimulatory effects on human NK-cells explored by gene profile analysis. *Genes Immun* (2004) 5:621–30. doi:10.1038/sj.gene.6364135
33. Michaud A, Dardari R, Charrier E, Cordeiro P, Herblot S, Duval M. IL-7 enhances survival of human CD56bright NK-cells. *J Immunother* (2010) 33:382–90. doi:10.1097/CJI.0b013e3181cd872d
34. Wendt K, Wilk E, Buyny S, Schmidt RE, Jacobs R. Interleukin-21 differentially affects human natural killer cell subsets. *Immunology* (2007) 122:486–95. doi:10.1111/j.1365-2567.2007.02675.x
35. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* (1993) 178:1041–8. doi:10.1084/jem.178.3.1041
36. Pillet A-H, Bugault F, Thèze J, Chakrabarti LA, Rose T. A programmed switch from IL-15- to IL-2-dependent activation in human NK-cells. *J Immunol* (2009) 182:6267–77. doi:10.4049/jimmunol.0801933
37. DeBlaker-Hohe DF, Yamauchi A, Yu CR, Horvath-Arcidiacono JA, Bloom ET. IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK-cells. *Cell Immunol* (1995) 165:33–43. doi:10.1006/cimm.1995.1184
38. Wang KS, Ritz J, Frank DA. IL-2 induces STAT4 activation in primary NK-cells and NK-cell lines, but not in T cells. *J Immunol* (1999) 162:299–304.
39. Carson WE, Giri JG, Lindemann MJ, Linett ML, Ahdieh M, Paxton R, et al. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med* (1994) 180:1395–403. doi:10.1084/jem.180.4.1395
40. Matikainen S, Paananen A, Miettinen M, Kurimoto M, Timonen T, Julkunen I, et al. IFN-alpha and IL-18 synergistically enhance IFN-gamma production in human NK-cells: differential regulation of Stat4 activation and IFN-gamma gene expression by IFN-alpha and IL-12. *Eur J Immunol* (2001) 31:2236–45. doi:10.1002/1521-4141(200107)31:7<2236::AID-IMMU2236>3.3.CO;2-7
41. Mizutani T, Neugebauer N, Putz EM, Moritz N, Simma O, Zebedin-Brandl E, et al. Conditional IFNAR1 ablation reveals distinct requirements of type I IFN signaling for NK-cell maturation and tumor surveillance. *Oncoimmunology* (2012) 1:1027–37. doi:10.4161/onci.21284
42. Ziblat A, Domaica CI, Spallanzani RG, Iraolagoitia XLR, Rossi LE, Avila DE, et al. IL-27 stimulates human NK-cell effector functions and primes NK-cells for IL-18 responsiveness. *Eur J Immunol* (2015) 45:192–202. doi:10.1002/eji.201444699
43. Matsui M, Kishida T, Nakano H, Yoshimoto K, Shin-Ya M, Shimada T, et al. Interleukin-27 activates natural killer cells and suppresses NK-resistant head and neck squamous cell carcinoma through inducing antibody-dependent cellular cytotoxicity. *Cancer Res* (2009) 69:2523–30. doi:10.1158/0008-5472.CAN-08-2793
44. Laroni A, Gandhi R, Beynon V, Weiner HL. IL-27 imparts immunoregulatory function to human NK-cell subsets. *PLoS One* (2011) 6:e26173. doi:10.1371/journal.pone.0026173
45. Vosshenrich CAJ, Ranson T, Samson SI, Corcuff E, Colucci F, Rosmaraki EE, et al. Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK-cell precursors and peripheral NK-cells in vivo. *J Immunol* (2005) 174:1213–21. doi:10.4049/jimmunol.174.3.1213
46. Stark GR, Darnell JE. The JAK-STAT pathway at twenty. *Immunity* (2012) 36:503–14. doi:10.1016/j.immuni.2012.03.013
47. Schönberg K, Rudolph J, Vonnahme M, Parampalli S, Cornez I, Hejazi M, et al. JAK inhibition impairs NK-cell function. *Cancer Res* (2015) 75(11):2187–99. doi:10.1158/0008-5472.CAN-14-3198
48. Park SY, Saijo K, Takahashi T, Osawa M, Arase H, Hirayama N, et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* (1995) 3:771–82. doi:10.1016/1074-7613(95)90066-7
49. Nosaka T, van Deursen JM, Tripp RA, Thierfelder WE, Witthuhn BA, McMickle AP, et al. Defective lymphoid development in mice lacking Jak3. *Science* (1995) 270:800–2. doi:10.1126/science.270.5237.800
50. Roberts JL, Lengi A, Brown SM, Chen M, Zhou Y-J, O'Shea JJ, et al. Janus kinase 3 (JAK3) deficiency: clinical, immunologic, and molecular analyses of 10 patients and outcomes of stem cell transplantation. *Blood* (2004) 103:2009–18. doi:10.1182/blood-2003-06-2104
51. Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, Pfeffer K. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* (1998) 93:397–409. doi:10.1016/S0092-8674(00)81168-X
52. Rodig SJ, Meraz MA, White JM, Lampe PA, Riley JK, Arthur CD, et al. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell* (1998) 93:373–83. doi:10.1016/S0092-8674(00)81166-6
53. Delconte RB, Kolesnik TB, Dagley LF, Rautela J, Shi W, Putz EM, et al. CIS is a potent checkpoint in NK-cell-mediated tumor immunity. *Nat Immunol* (2016) 17:816–24. doi:10.1038/ni.3470

54. Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. *Immunol Rev* (2009) 228:273–87. doi:10.1111/j.1600-065X.2008.00754.x
55. Stoiber D, Kovacic B, Schuster C, Schellack C, Karaghiosoff M, Kreibich R, et al. TYK2 is a key regulator of the surveillance of B lymphoid tumors. *J Clin Invest* (2004) 114:1650–8. doi:10.1172/JCI22315
56. Prchal-Murphy M, Witalisz-Siepracka A, Bednarik KT, Putz EM, Gotthardt D, Meissl K, et al. In vivo tumor surveillance by NK-cells requires TYK2 but not TYK2 kinase activity. *Oncoimmunology* (2015) 4:e1047579. doi:10.1080/2162402X.2015.1047579
57. Schleicher U, Mattner J, Blos M, Schindler H, Rollinghoff M, Karaghiosoff M, et al. Control of *Leishmania major* in the absence of Tyk2 kinase. *Eur J Immunol* (2004) 34:519–29. doi:10.1002/eji.200324465
58. Shimoda K, Tsutsui H, Aoki K, Kato K, Matsuda T, Numata A, et al. Partial impairment of interleukin-12 (IL-12) and IL-18 signaling in Tyk2-deficient mice. *Blood* (2002) 99:2094–9. doi:10.1182/blood.V99.6.2094
59. Kreins AY, Ciancanelli MJ, Okada S, Kong X-F, Ramírez-Alejo N, Kilic SS, et al. Human TYK2 deficiency: mycobacterial and viral infections without hyper-IgE syndrome. *J Exp Med* (2015) 212:1641–62. doi:10.1084/jem.20140280
60. Au-Yeung N, Mandhana R, Horvath CM. Transcriptional regulation by STAT1 and STAT2 in the interferon JAK-STAT pathway. *JAKSTAT* (2013) 2:e23931. doi:10.4161/jkst.23931
61. Miyagi T, Gil MP, Wang X, Louten J, Chu WM, Biron CA. High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells. *J Exp Med* (2007) 204:2383–96. doi:10.1084/jem.20070401
62. Liang S, Wei H, Sun R, Tian Z. IFNalpha regulates NK-cell cytotoxicity through STAT1 pathway. *Cytokine* (2003) 23:190–9. doi:10.1016/S1043-4666(03)00226-6
63. Lesinski GB, Anghelina M, Zimmerer J, Bakalakos T, Badgwell B, Parihar R, et al. The antitumor effects of IFN-alpha are abrogated in a STAT1-deficient mouse. *J Clin Invest* (2003) 112:170–80. doi:10.1172/JCI16603
64. Robbins SH, Tessmer MS, Van Kaer L, Brossay L. Direct effects of T-bet and MHC class I expression, but not STAT1, on peripheral NK-cell maturation. *Eur J Immunol* (2005) 35:757–65. doi:10.1002/eji.200425797
65. Putz EM, Majoros A, Gotthardt D, Prchal-Murphy M, Zebedin-Brandl EM, Fux DA, et al. Novel non-canonical role of STAT1 in natural killer cell cytotoxicity. *Oncoimmunology* (2016) 5(9):e1186314. doi:10.1080/2162402X.2016.1186314
66. Vairo D, Tassone L, Tabellini G, Tamassia N, Gasperini S, Bazzoni F, et al. Severe impairment of IFN-γ and IFN-α responses in cells of a patient with a novel STAT1 splicing mutation. *Blood* (2011) 118:1806–17. doi:10.1182/blood-2011-01-330571
67. Jouanguy E, Gineau L, Cottineau J, Béziat V, Vivier E, Casanova J-L. Inborn errors of the development of human natural killer cells. *Curr Opin Allergy Clin Immunol* (2013) 13:589–95. doi:10.1097/ACI.0000000000000011
68. Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S, et al. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat Genet* (2003) 33:388–91. doi:10.1038/ng1097
69. Chapgier A, Kong X-F, Boisson-Dupuis S, Jouanguy E, Averbuch D, Feinberg J, et al. A partial form of recessive STAT1 deficiency in humans. *J Clin Invest* (2009) 119:1502–14. doi:10.1172/JCI37083
70. Boisson-Dupuis S, Kong X-F, Okada S, Cypowyj S, Puel A, Abel L, et al. Inborn errors of human STAT1: allelic heterogeneity governs the diversity of immunological and infectious phenotypes. *Curr Opin Immunol* (2012) 24:364–78. doi:10.1016/j.coi.2012.04.011
71. Gotthardt D, Putz EM, Straka E, Kudweis P, Biaggio M, Poli V, et al. Loss of STAT3 in murine NK-cells enhances NK-cell-dependent tumor surveillance. *Blood* (2014) 124:2370–9. doi:10.1182/blood-2014-03-564450
72. Kortylewski M, Yu H. Role of Stat3 in suppressing anti-tumor immunity. *Curr Opin Immunol* (2008) 20:228–33. doi:10.1016/j.coi.2008.03.010
73. Cacalano NA. Regulation of natural killer cell function by STAT3. *Front Immunol* (2016) 7:128. doi:10.3389/fimmu.2016.00128
74. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* (2007) 7:41–51. doi:10.1038/nri1995
75. Yue P, Turkson J. Targeting STAT3 in cancer: how successful are we? *Expert Opin Investig Drugs* (2009) 18:45–56. doi:10.1517/13543780802565791
76. Siveen KS, Sikka S, Surana R, Dai X, Zhang J, Kumar AP, et al. Targeting the STAT3 signaling pathway in cancer: role of synthetic and natural inhibitors. *Biochim Biophys Acta* (2014) 1845:136–54. doi:10.1016/j.bbcan.2013.12.005
77. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med* (2005) 11:1314–21. doi:10.1038/nm1325
78. Takaki R, Hayakawa Y, Nelson A, Sivakumar PV, Hughes S, Smyth MJ, et al. IL-21 enhances tumor rejection through a NKG2D-dependent mechanism. *J Immunol* (2005) 175:2167–73. doi:10.4049/jimmunol.175.4.2167
79. Zhu S, Phatarpekar PV, Denman CJ, Senyukov VV, Somanchi SS, Nguyen-Jackson HT, et al. Transcription of the activating receptor NKG2D in natural killer cells is regulated by STAT3 tyrosine phosphorylation. *Blood* (2014) 124:403–11. doi:10.1182/blood-2013-05-499707
80. Burgess SJ, Marusina AI, Pathmanathan I, Borrego F, Coligan JE. IL-21 down-regulates NKG2D/DAP10 expression on human NK and CD8+ T cells. *J Immunol* (2006) 176:1490–7. doi:10.4049/jimmunol.176.3.1490
81. Bedel R, Thiery-Vuillemin A, Grandclement C, Balland J, Remy-Martin J-P, Kantelip B, et al. Novel role for STAT3 in transcriptional regulation of NK immune cell targeting receptor MICA on cancer cells. *Cancer Res* (2011) 71:1615–26. doi:10.1158/0008-5472.CAN-09-4540
82. Fionda C, Malgarini G, Soriani A, Zingoni A, Cecere F, Iannitto ML, et al. Inhibition of glycogen synthase kinase-3 increases NKG2D ligand MICA expression and sensitivity to NK-cell-mediated cytotoxicity in multiple myeloma cells: role of STAT3. *J Immunol* (2013) 190:6662–72. doi:10.4049/jimmunol.1201426
83. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* (2004) 20:477–94. doi:10.1016/S1074-7613(04)00076-7
84. Wang KS, Frank DA, Ritz J. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood* (2000) 95:3183–90.
85. Sun JC, Madera S, Bezman NA, Beilke JN, Kaplan MH, Lanier LL. Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J Exp Med* (2012) 209:947–54. doi:10.1084/jem.20111760
86. Grant LR, Yao Z-J, Hedrich CM, Wang F, Moorthy A, Wilson K, et al. Stat4-dependent, T-bet-independent regulation of IL-10 in NK-cells. *Genes Immun* (2008) 9:316–27. doi:10.1038/gene.2008.20
87. Yamamoto K, Shibata F, Miyasaka N, Miura O. The human perforin gene is a direct target of STAT4 activated by IL-12 in NK-cells. *Biochem Biophys Res Commun* (2002) 297:1245–52. doi:10.1016/S0006-291X(02)02378-1
88. Jamil KM, Hydes TJ, Cheent KS, Cassidy SA, Traherne JA, Jayaraman J, et al. STAT4-associated natural killer cell tolerance following liver transplantation. *Gut* (2016). doi:10.1136/gutjnl-2015-309395
89. Xu X, Sun YL, Hoey T. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science* (1996) 273:794–7. doi:10.1126/science.273.5276.794
90. Eckelhart E, Warsch W, Zebedin E, Simma O, Stoiber D, Kolbe T, et al. A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. *Blood* (2011) 117:1565–73. doi:10.1182/blood-2010-06-291633
91. Berger A, Sexl V, Valent P, Moriggl R. Inhibition of STAT5: a therapeutic option in BCR-ABL1-driven leukemia. *Oncotarget* (2014) 5:9564–76. doi:10.18632/oncotarget.2465
92. Imada K, Bloom ET, Nakajima H, Horvath-Arcidiacono JA, Udy GB, Davey HW, et al. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *J Exp Med* (1998) 188:2067–74. doi:10.1084/jem.188.11.2067
93. Bezrodnik L, Di Giovanni D, Caldirola MS, Azcoiti ME, Torgerson T, Gaillard MI. Long-term follow-up of STAT5B deficiency in three Argentinian patients: clinical and immunological features. *J Clin Immunol* (2015) 35:264–72. doi:10.1007/s10875-015-0145-5
94. Gotthardt D, Putz EM, Grundschober E, Prchal-Murphy M, Straka E, Kudweis P, et al. STAT5 is a key regulator in NK-cells and acts as a molecular switch from tumor surveillance to tumor promotion. *Cancer Discov* (2016) 6:414–29. doi:10.1158/2159-8290.CD-15-0732

95. Coffelt SB, Hughes R, Lewis CE. Tumor-associated macrophages: effectors of angiogenesis and tumor progression. *Biochim Biophys Acta* (2009) 1796:11–8. doi:10.1016/j.bbcan.2009.02.004
96. Levi I, Amsalem H, Nissan A, Darash-Yahana M, Peretz T, Mandelboim O, et al. Characterization of tumor infiltrating natural killer cell subset. *Oncotarget* (2015) 6:13835–43. doi:10.18632/oncotarget.3453
97. Bruno A, Focaccetti C, Pagani A, Imperatori AS, Spagnoletti M, Rotolo N, et al. The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer. *Neoplasia* (2013) 15:133–42. doi:10.1593/neo.121758
98. Mamessier E, Sylvain A, Thibult ML, Houvenaeghel G, Jacquemier J, Castellano R, et al. Human breast cancer cells enhance self tolerance by promoting evasion from NK-cell antitumor immunity. *J Clin Invest* (2011) 121:3609–22. doi:10.1172/JCI45816
99. Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, et al. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56brightCD16- cells and display an impaired capability to kill tumor cells. *Cancer* (2008) 112:863–75. doi:10.1002/cncr.23239
100. Hejazi M, Manser AR, Fröbel J, Kündgen A, Zhao X, Schönberg K, et al. Impaired cytotoxicity associated with defective natural killer cell differentiation in myelodysplastic syndromes. *Haematologica* (2015) 100:643–52. doi:10.3324/haematol.2014.118679
101. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK-cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* (2006) 12:1065–74. doi:10.1038/nm1452
102. Chakraborty D, Rumi MAK, Konno T, Soares MJ. Natural killer cells direct hemochorial placentation by regulating hypoxia-inducible factor dependent trophoblast lineage decisions. *Proc Natl Acad Sci U S A* (2011) 108:16295–300. doi:10.1073/pnas.1109478108
103. Fraser R, Whitley GS, Johnstone AP, Host AJ, Sebire NJ, Thilaganathan B, et al. Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *J Pathol* (2012) 228:322–32. doi:10.1002/path.4057
104. Katsumoto T, Kimura M, Yamashita M, Hosokawa H, Hashimoto K, Hasegawa A, et al. STAT6-dependent differentiation and production of IL-5 and IL-13 in murine NK2 cells. *J Immunol* (2004) 173:4967–75. doi:10.4049/jimmunol.173.8.4967
105. Mahalingam S, Karupiah G, Takeda K, Akira S, Matthaei KI, Foster PS. Enhanced resistance in STAT6-deficient mice to infection with ectromelia virus. *Proc Natl Acad Sci U S A* (2001) 98:6812–7. doi:10.1073/pnas.111151098
106. Bream JH, Curiel RE, Yu C-R, Egwuagu CE, Grusby MJ, Aune TM, et al. IL-4 synergistically enhances both IL-2- and IL-12-induced IFN-gamma expression in murine NK-cells. *Blood* (2003) 102:207–14. doi:10.1182/blood-2002-08-2602

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 20 March 2017
doi: 10.3389/fimmu.2017.00301

Immunoregulatory Role of NK Cells in Tissue Inflammation and Regeneration

*Annie Tosello-Trampont[1], Fionna A. Surette[1], Sarah E. Ewald[1,2] and Young S. Hahn[1,2]**

[1] Beirne B. Carter Center for Immunology Research, Charlottesville, VA, USA, [2] Department of Microbiology, University of Virginia, Charlottesville, VA, USA

NK cells represent an important first line of defense against viral infection and cancer and are also involved in tissue homeostasis. Studies of NK cell activation in the last decade have revealed that they are able to respond to the inflammatory stimuli evoked by tissue damage and contribute to both progression and resolution of diseases. Exacerbation of the inflammatory response through interactions between immune effector cells facilitates the progression of non-alcoholic fatty liver disease (NAFLD) into steatosis, cirrhosis, and hepatocellular carcinoma (HCC). When hepatic damage is incurred, macrophage activation is crucial for initiating cross talk with neighboring cells present in the liver, including hepatocytes and NK cells, and the importance of this interaction in shaping the immune response in liver disease is increasingly recognized. Inflicted structural damage can be in part regenerated *via* the process of self-limiting fibrosis, though persistent hepatic damage will lead to chronic fibrosis and loss of tissue organization and function. The cytotoxic activity of NK cells plays an important role in inducing hepatic stellate cell apoptosis and thus curtailing the progression of fibrosis. Alternatively, in some diseases, such as HCC, NK cells may become dysregulated, promoting an immunosuppressive state where tumors are able to escape immune surveillance. This review describes the current understanding of the contributions of NK cells to tissue inflammation and metabolic liver diseases and the ongoing effort to develop therapeutics that target the immunoregulatory function of NK cells.

Keywords: liver, NK cells, fibrosis, immunoregulation, regeneration

OPEN ACCESS

Edited by:
Bree Foley,
Telethon Kids Institute, Australia

Reviewed by:
Hui Peng,
University of Science and Technology of China, China
Silke Paust,
Baylor College of Medicine, USA

****Correspondence:***
Young S. Hahn
ysh5e@virginia.edu

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *16 November 2016*
Accepted: *03 March 2017*
Published: *20 March 2017*

Citation:
Tosello-Trampont A, Surette FA, Ewald SE and Hahn YS (2017) Immunoregulatory Role of NK Cells in Tissue Inflammation and Regeneration. Front. Immunol. 8:301. doi: 10.3389/fimmu.2017.00301

INTRODUCTION

The liver plays a critical role in functioning as both a metabolic and immunological site. It receives a dual blood supply; one-quarter is oxygen-rich blood delivered by the hepatic artery, while the remaining three-quarters is blood draining from the gastrointestinal tract and spleen *via* the portal vein, enriched in dietary- and environmental-antigen (1). Liver sinusoidal endothelial cells (LSECs) form the walls of hepatic sinusoids and present numerous fenestrations, allowing blood to contact the underlying hepatocytes. Slow blood flow in hepatic sinusoids allows a better interaction between circulating lymphocytes, liver sinusoidal endothelium, and hepatocytes to facilitate the clearance of gut-derived antigens by liver-resident cells (2). To compensate for the high exposure to circulating antigens, the liver must maintain a tolerant microenvironment in which there is constant low-level suppression of immune responses. Liver immune cells are educated to permit immunological tolerance to self-antigens, environmental, and dietary antigens, during homeostasis, but can initiate both

innate and adaptive immune responses in the context of infection (3). In humans and mice, the liver is largely composed of hepatocytes (80% of the liver mass), while the remaining 20% is made up of non-parenchymal cells including lymphocytes, myeloid cells, Kupffer cells (liver-resident macrophages, KCs), HSCs, and LSECs (4, 5). NK cells are enriched in the liver, representing 25–30% of human liver lymphocytes compared to 10–20% of total peripheral blood mononuclear cell (PBMC) lymphocytes (6). However, during chronic hepatitis B and C, NK cell numbers are increased through recruitment by KC-secreted chemokines (7, 8), and the survival of NK cells is enhanced by cytokine production from Kupffer cells, LSECs, and T cells (9). The high immunological load present during infection, a large proportion of which are NK cells, results in a unique immune environment.

NK cells are widely distributed in both lymphoid (bone marrow and liver) and non-lymphoid organs (peripheral blood, lung, and uterus) and bridge the gap between innate and adaptive immune responses. They conduct immunosurveillance by probing cells *via* their inhibitory receptors [NKG2A and the Ly-49 family in mice, and killer-immunoglobulin-like receptor (KIR) and NKG2A in humans] to determine whether the correct self major histocompatibility complex (MHC) is expressed and to ensure tolerance against healthy cells. In humans and mice, NK cells can detect infected, transformed, or stressed cells with their activating receptors (NKG2D and NKp46), resulting in their activation. NK cell activation can be triggered many ways, including cross-linking of activating receptors (NKG2D and NKp46) with simultaneous disengagement of inhibitory receptors (NKG2A) or by various cytokines such as type I IFNs, IL-2, IL-12, IL-15, and IL-18. Additionally, NK cells can be directly activated through CD16A signaling that triggers antibody-dependent cell-mediated cytotoxicity (ADCC) or receive signals through toll-like receptors (TLRs) expressed on their surface, which recognize pathogen-associated molecular patterns (PAMPs) expressed by injured cells (10). Upon activation, NK cells can become cytotoxic and release lytic granules (perforin, granzymes) or induce death signals through expression of death receptors (TRAIL/TRAIL-R, FasL/Fas) (11, 12). While NK cells are able to mediate their functions in an antigen-independent, innate manner, recent investigations have suggested that liver-resident NK cells are capable of acquiring antigen-specific memory. In studies that utilized murine models, it was shown that a persistent and transferable NK cell memory response is generated to haptens and viruses and that the retention of this memory population requires CXCR6 expression (13). This antigen-specific NK memory has further been studied in non-human primates, where it has been maintained up to 5 years (14). However, the underlying mechanisms for the generation of NK memory responses still remain to be elucidated.

The interplay between NK cells and their surrounding tissues and immune cells shapes NK cell maturation and function. In the liver, cross talk between NK cells and macrophages during various phases of liver injury-induced inflammation allows NK cells to regulate both inflammatory and anti-inflammatory macrophages (**Figure 1**). Hepatic macrophages play a central role in the pathogenesis of chronic liver disease (15). They can exert dual responses depending on their origin, the phase of liver immune response, the developmental stage of a disease, or the acute/chronic profile of a disease. Macrophages can differentiate into a wide range of pro-inflammatory/classical M1 to immunoregulatory/alternative M2 macrophage profile under inflammatory conditions during liver injury (16). M1 macrophages are activated by pro-inflammatory cytokines (IFN-γ, IL-12, or TNF-α) and/or microbial products (LPS) and can phagocytose bacteria and virus, as well as release pro-inflammatory cytokines and chemokines. M2 macrophages are induced by IL-4, IL-10, and IL-13 produced by various cell types (16). There are several subtypes of M2 macrophages whose functions vary between wound healing (fibrogenic activity and tissue repair), restorative function (matrix resolution and tissue repair), or pro-inflammatory ("turnoff immune response") activity.

Moreover, NK cell function can be shaped by inflammasomes, which are triggered in response to cell damage. Inflammasome components are expressed in hepatocytes, KCs, and macrophages and contribute to the induction of liver inflammation *via* pyroptotic cell death and release of alarmins as well as the cleavage and secretion of IL-1β and IL-18. The inflammasome sensor NLRP3 responds to extracellular ATP released from dying cells and to reactive oxygen species triggered by phagosome damage from engulfment of particulates (17, 18). Mice deficient in NLRP3 are protected in diet-induced and some infection models of liver injury (19–24). Viral double-stranded DNA can trigger the inflammasome by binding AIM2 expressed on hepatocytes (25). In the case of hepatitis C virus (HCV) infection, both hepatocytes and KC have been shown to release IL-1β and IL-18. Notably, KCs appear to be responsible for activating NK cells *via* this mechanism (25–27). While cell death and release of alarmins exacerbate liver inflammation, IL-18 exerts a protective function to limit liver injury. This may in part be due to the NK cell-mediated activation of other immune mediators, including the induction of PD-L1 and FasL, as well as the essential role of cytokine in eliciting NK memory (28–31). These findings support the pathophysiological role of inflammasomes in hepatic inflammation and liver injury.

CHARACTERISTICS OF LIVER NK CELLS

Liver NK cells are a unique cell population in terms of frequency, phenotype, and function (32). Due to its anatomical structure, blood supply, immune resident cell repertoire, and the constant exposure to dietary- and gut-derived antigens, the tolerogenic liver microenvironment may be conducive for the enrichment and characteristics of liver NK cells (33). While the majority of NK cells originate in bone marrow from progenitor NK cells (NKPs), some NK cells evolve in other lymphoid tissues such as the thymus and lymph nodes (34, 35). Compared to bone marrow-derived NK cells, NK cells developing from the thymus demonstrate a higher expression of GATA-3 and CD127 and require IL-7 signals for their development (34). The earliest committed NKPs in mouse bone marrow are identified as $DX5^{-}CD161^{-}CD122^{+}$ and are dependent on the Id2 transcription factor (36). NKPs then acquire the expression of phenotypic markers NK1.1, NKp46, CD94/NKG2 receptors, Ly49 receptors, and DX5 to become mature NK cells. The expression of CD11b and CD27 are used to define the maturation status of mouse NK cells. NK cell maturation is categorized into four stages based on the acquisition of CD11b

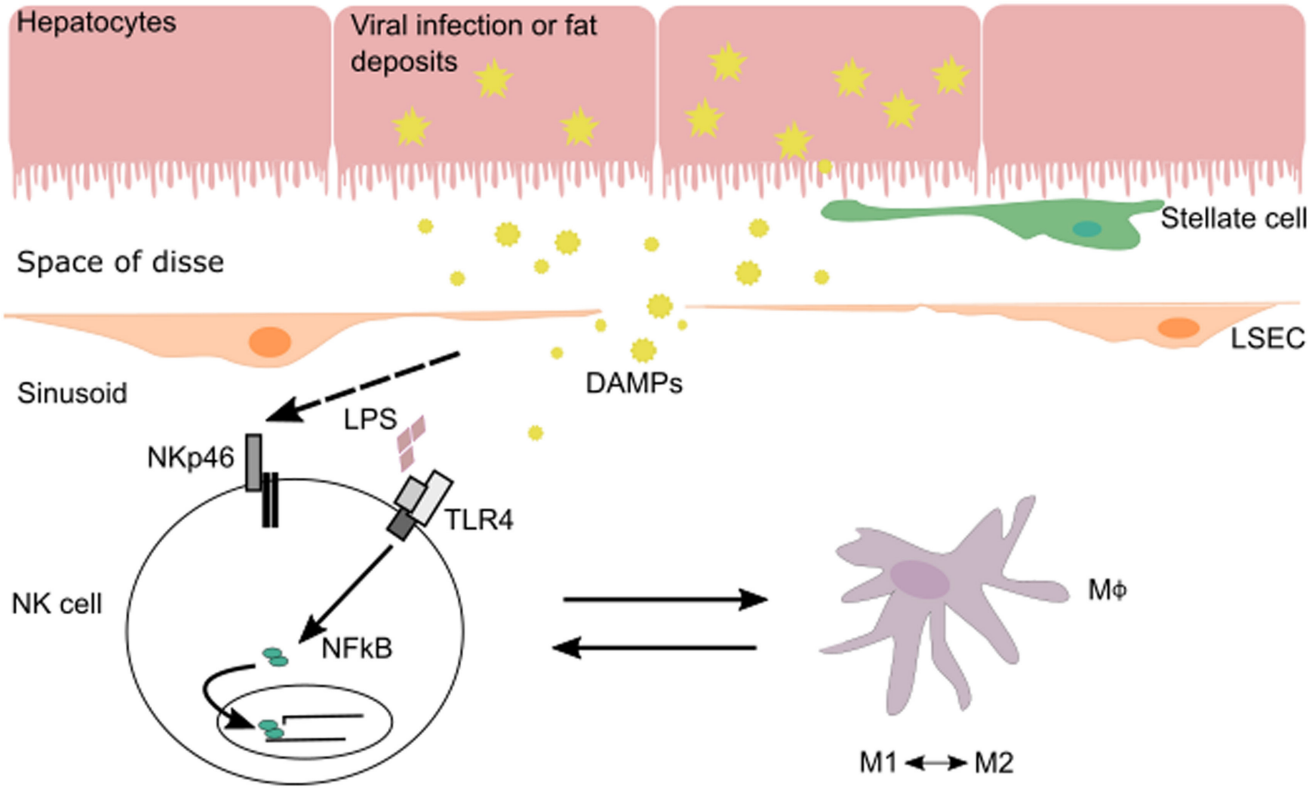


FIGURE 1 | In the context of viral infection or fatty liver disease, NK cells can be stimulated *via* their activating receptors or by toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns and DAMPs expressed by injured cells. During liver injury-induced inflammation, NK cells are able to regulate inflammatory (M1) and anti-inflammatory (M2) macrophages depending on the phase of the immune response and stage of disease.

expression and the loss of CD27. The most immature NK cells are defined as $CD11b^{-}CD27^{-}$, and these progress to $CD11b^{-}CD27^{+}$, then $CD11b^{+}CD27^{+}$, and finally become the most mature NK cells by expressing $CD11b^{+}CD27^{-}$. Mature $CD11b^{+}$ $CD27^{-}$NK cells are predominant in the spleen, and immature $CD11b^{-}CD27^{+}$ NK cells are more prevalent in the liver (37).

The liver is populated by conventional NK (cNK) cells and resident NK cells that are distinguished by the mutually exclusive expression of the integrins DX5 and CD49a, respectively. Mouse NK cells are usually identified by the cell surface phenotype of $CD3^{-}DX5^{+}$. The expression of NK activating receptor, NKp46, is also used as a phenotypic marker in mice and humans, with cNK cells defined by the phenotype of $CD49a^{-}NKp46^{+}DX5^{+}$ $CD3^{-}$, and liver-resident NK cells identified by $CD49a^{+}NKp46^{+}$ $DX5^{-}CD3^{-}$, with low expression of CD11b and Ly49, and high expression of TRAIL (38). During homeostasis, cNK cells are highly migratory, circulating throughout the body and primarily found in bone marrow, blood, and spleen. Activated cNK cells produce a large amount of cytokines but display low cytolytic activity. Recently, liver-resident NK cells have been identified as members of innate lymphoid cells (ILCs) and constitute the group 1 ILCs, along with ILC1s (39–42). Both NK cells and ILC1s are characterized by their expression of the transcription factor T-bet, but NK cells are distinctive in their expression of EOMES. While not in the scope of this review, there are numerous others that cover ILC biology, nomenclature, and the vast differences in transcriptional regulation, receptor expression, and localization that may be helpful (43–45). Liver-resident NK cells have more cytotoxicity, but less IFN-γ production following IL-12/IL-18 stimulation than NK cells in the blood and spleen under homeostatic conditions (31). In contrast, a recent report demonstrates that ILC1s are able to produce higher levels of cytokines (IFN-γ) than cNK cells following PMA/ionomycin stimulation (46). Within the liver, $CD49a^{+}DX5^{-}$ NK cells are found at a significantly higher frequency compared to other sites (bone marrow, spleen, blood) and selectively reside in hepatic sinusoids. Liver $CD49a^{+}DX5^{-}$ NK cells express high levels of TRAIL and have cytotoxic activity against tumor cells. $TRAIL^{+}$ NK cells predominate in fetal and neonatal mice, and in adulthood are present in the liver, but not the spleen. Tissue-resident $CD49a^{+}DX5^{-}$ NK cells are also found in the uterus and skin (46).

Human NK cells are $CD3^{-}CD56^{+}$ lymphocytes and can be divided into two subsets based on the level of expression of CD56 and CD16. $CD16^{+}CD56^{dim}$ NK cells represent 90% of blood and spleen NK cells and demonstrate higher cytotoxic activity than $CD16^{-}CD56^{bright}$ NK cells by producing high levels of granzymes and perforin. $CD16^{+}CD56^{dim}$ NK cells have high expression of KIR and express intermediate-affinity IL-2 receptor resulting in low expansion capacity under IL-2 stimulation. Representing only 10% of blood NK cells, $CD16^{-}CD56^{bright}$

NK cells are predominant in secondary lymphoid organs such as lymph nodes. In the liver, both populations are present with the same frequency. $CD16^{-}CD56^{bright}$ NK cells exhibit less cytolytic activity than $CD16^{+}CD56^{dim}$ NK cells but demonstrate the same cytotoxicity after prolonged activation. $CD16^{-}CD56^{bright}$ NK cells express high- and intermediate-affinity IL2-receptor facilitating their *in vivo* and *in vitro* expansion under low doses of IL-2. Additionally, they are more responsive to stimulation by pro-inflammatory cytokines. Upon activation, they produce cytokines (IFN-γ, IL-10, GM-CSF, and TNF-α) and chemokines. A recent study suggested that $CD16^{-}CD56^{bright}$ NK cells might represent liver-resident NK cells with high expression of CD69, an activation marker, and expression of CXCR6 and CCR5 to retain them in the liver (47). The development and the differentiation of human liver-resident NK cells are still undefined. As an antiviral effect, NK cells secrete granzyme B and perforin to lyse virally infected cells and induce their apoptosis and secrete cytokines such as TNF-α and IFN-γ to further the immune response (48). In particular, NK IFN-γ production has direct cytotoxic effects on virally-infected cells, elicits an antiviral state in the uninfected cells, and induces chemotaxis to recruit adaptive immune cells (6, 49, 50). HCV patients with chronic infection show poor NK cell responses compared to patients with resolved HCV infection. The *in vitro* studies on co-cultures of NK cells with HCV-conditioned $CD33^{+}$ PBMCs demonstrated a reduced level of IFN-γ production but no effect on granzyme B release. This suppression of NK cell-derived IFN-γ production has been shown to be mediated by $CD33^{+}CD11b^{lo}HLA\text{-}DR^{lo}$ myeloid-derived suppressor cells (MDSCs) *via* an arginase-1-dependent inhibition of mTOR activation (**Figure 2**) (51). These results identify the induction of MDSCs in HCV infection as a potent immune evasion strategy that suppresses antiviral NK cell responses.

ROLE OF NK CELLS IN LIVER DISEASE

As a result of tight regulatory controls, loss of liver tissue can be regenerated *via* the process of self-limiting fibrosis (3). Indeed, the liver can be fully recovered structurally and functionally even after a loss of 50% of its hepatocytes. While fibrosis can be beneficial in an acute sense, repeated hepatic damages due to persistent inflammation or infection can lead to chronic fibrosis, resulting in a loss of liver tissue organization and function and eventually, cirrhosis (3). At this stage, if cirrhosis fails to regress to early stage fibrosis, there is a high risk for developing irreversible hepatocellular carcinoma (HCC). The most common causes of fibrosis are chronic viral infections (HBV, HCV), frequent use of hepatotoxic drugs (acetaminophen, ibuprofen), or metabolic syndromes [non-alcoholic fatty liver disease (NAFLD)] that trigger chronic inflammation. Fibrosis results in activation of HSCs and macrophages that produce extracellular matrix (ECM), forming scar tissue. As a result of liver damage, dying hepatocytes are cleared by liver macrophages, which trigger an inflammatory response by secreting cytokines and chemokines to recruit other cells to the wound site and initiate the process of tissue repair. In an inflamed hepatic microenvironment, HSCs become activated and differentiate into myofibroblasts that produce

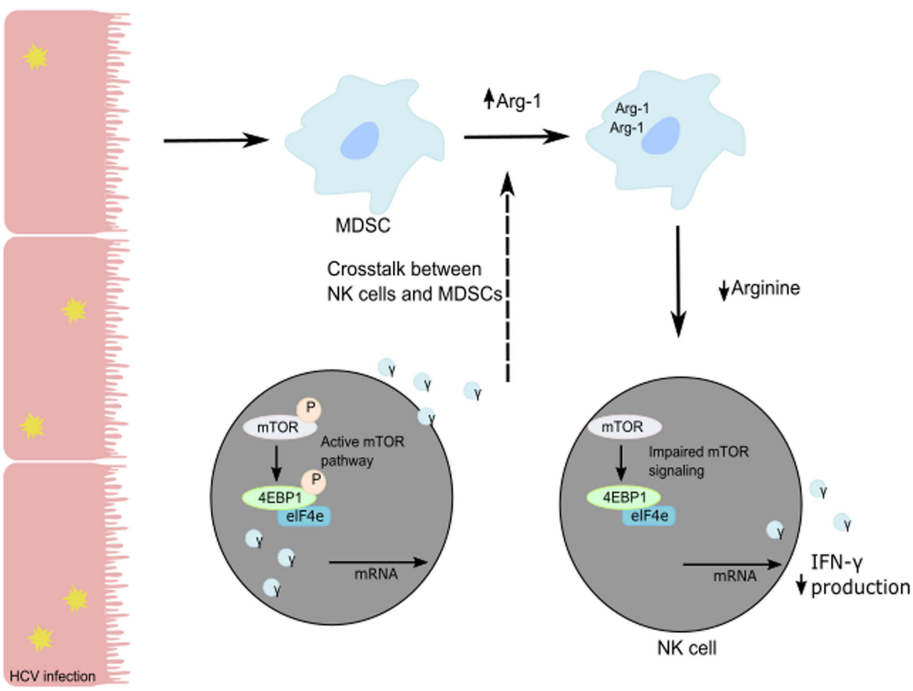


FIGURE 2 | Suppression of NK cell-derived IFN-γ production is mediated by $CD33^{+}CD11b^{lo}HLA\text{-}DR^{lo}$ myeloid-derived suppressor cells (MDSCs) following a reduction in available arginine *via* an Arg-1 mechanism. During HCV infection, HCV core protein induces MDSC development. Following the loss of arginine, the mTOR pathway is unable to efficiently translate IFN-γ mRNA, and the subsequent loss of IFN-γ protein production and secretion from NK cells in the liver results in immune evasion by HCV.

a large amount of fibrous proteins (collagen, elastin, laminin, fibronectin). The progression of fibrosis depends on the balance between production of ECM components and their degradation by metalloproteinase produced by restorative M2 macrophages. In this review, we will focus on the dual role of NK cells in the initiation, progression, and resolution of liver fibrosis and how it is regulated by the cross talk of NK cells with surrounding macrophages and stellate cells.

Role of NK Cells in Fibrosis

NK cells play a paradoxical role in the development of liver fibrosis. The cytotoxic activity of NK cells can curtail the development of fibrosis by killing HSC-derived myofibroblasts through engagement of NKG2D receptor with its ligand, RAE-1 (in mouse) expressed by early activated HSCs (52, 53). In humans, NK cells preferentially kill senescent-activated HSCs expressing MICA following recognition by NKG2D receptor on NK cells (54, 55). In addition, the expression of NKp46 ligand on human and mouse-activated HSCs can also trigger NK cell-mediated cytotoxic activity, ameliorating liver fibrosis (56). The ability of NK cells to kill HSCs seems to be dependent on the activation stage of HSCs and expression of specific molecules; quiescent HSCs and activated HSCs that express Timp-1 are resistant to apoptotic signals (57) (**Figure 3**). HSCs are the most sensitive to NK cytotoxicity during early activation and senescence stages.

In humans and mice, IFN-γ-producing NK cells have been demonstrated to negatively regulate fibrosis. *In vitro* and *in vivo* studies in mouse have shown an anti-fibrotic effect of NK cell-derived IFN-γ, which induces HSC apoptosis and cell cycle arrest. However, clinical trials with IFN-γ proved ineffective; the anti-fibrotic action of IFN-γ might depend on its targeted delivery to HSCs (58, 59). Additionally, IFN-γ-producing $DX5^{+}NKp46^{+}$ cNK cells are increased during non-alcoholic steatohepatitis (NASH), a cause of fibrosis, and skew the polarization of activated KC and liver macrophages toward an M1 profile, rather than toward a fibrosis-inducing M2 macrophage profile (60). Furthermore, NK cells release IL-22 upon activation, which can be anti-fibrogenic (61–63).

While IL-22 plays a beneficial role during acute inflammatory events, prolonged and excessive production of IL-22 may have the opposite effect (62). Continuous exposure to proliferative and antiapoptotic signals may drive cells to change phenotype and become cancerous as demonstrated in IL-22 transgenic mice where IL-22 primes the liver to be more susceptible to tumor development (64). Additionally, in HBV-infected patients and HBV transgenic mice, IL-22 exacerbates chronic inflammation and the development of fibrosis by promoting Th17 cell responses in the liver (65–68). The dual nature of IL-22, and by extension, NK cells, between protection and inflammation may depend on the tissue and inflammatory conditions. Due to technical challenges, it is difficult to evaluate which specific NK cell subset (i.e., conventional or liver-resident NK cells) has anti-fibrogenic effects. Most of the *in vitro* studies use cNK cells from human blood or mouse spleens and *in vivo* depletion experiments using

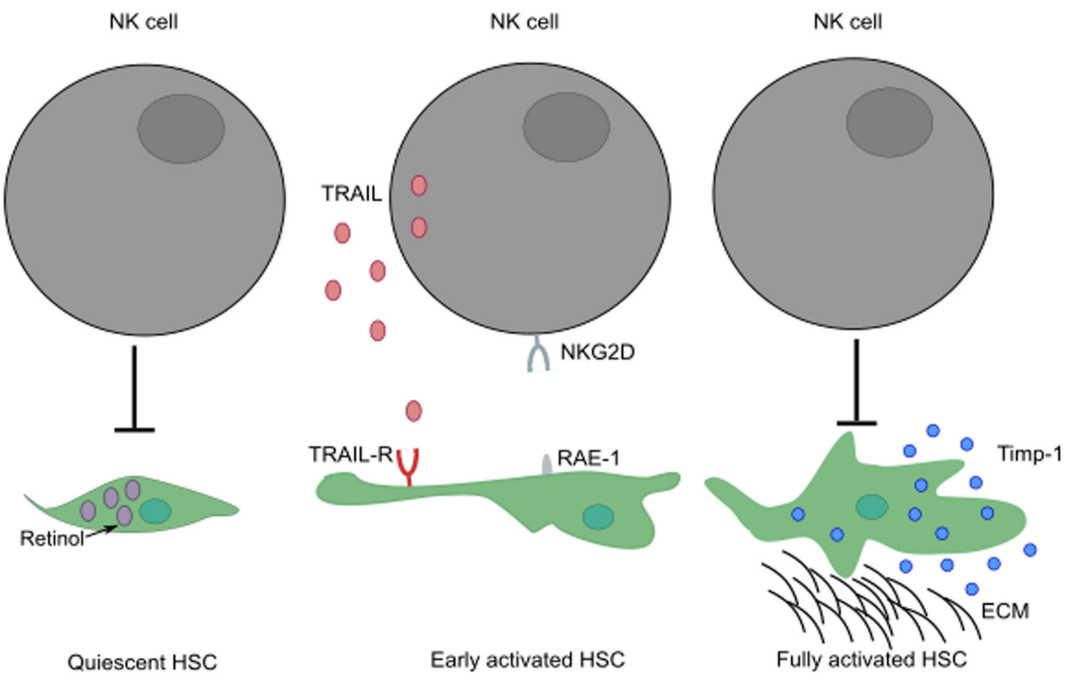


FIGURE 3 | The activation stage of HSCs can influence the ability of NK cells to exert cytotoxic activity. While quiescent HSCs are resistant to apoptotic signals, their activation leads to increased surface expression of TRAIL-R. Following engagement of their activating receptors, NK cells can conduct cytotoxic activity by releasing lytic granules or by transduction of death signals through death receptor/ligand interactions (i.e., TRAIL/TRAIL-R, FasL/Fas). Fully activated HSCs expressing Timp-1 are protected from apoptosis. These HSCs can differentiate into myofibroblasts that produce large amounts of fibrous proteins, which can lead to fibrosis when not restrained. ECM, extracellular matrix.

antibodies do not efficiently remove all liver-resident NK cells. Few liver-resident NK cells can be purified from a mouse liver, significantly prohibiting adoptive transfer.

In contrast to the anti-fibrogenic properties of some NK cells, other studies have demonstrated that NK cells can enhance liver injury by killing stressed hepatocytes *via* engagement of NKG2D, NKp30, and/or TRAIL. Additionally, they can accomplish this *via* IFN-γ-induced apoptosis, leading to the development of fibrosis (69–74). For example, patients with NASH show an increase in hepatocyte Fas expression and apoptosis, which correlates with the severity of the disease (75). This pathogenic role of NK cells has also been identified in human autoimmune liver diseases, such as primary biliary cirrhosis, where NK cells promote the killing of biliary epithelial cells *via* TRAIL and by releasing cytokines that promote adaptive immunity (76, 77). In parallel, IL-10-producing NK cells delay primary biliary cirrhosis by annihilating the adaptive immune response in killing autologous DCs and T cells (78).

Role of NK Cells in Tissue Regeneration

As many studies have now demonstrated the regulatory role of NK cells in fibrosis, compelling evidence also points to a role for NK cells in tissue regeneration (79, 80). Hepatic NK cells can interact with surrounding parenchymal and non-parenchymal cells to influence the release of growth hormones, cytokines, and chemokines within the proliferating hepatic tissues. Hepatic NK cells promoted liver regeneration after partial hepatectomy (81). NK cells may have a beneficial effect on liver tissue regeneration by secreting TNF-α and IL-22 during inflammation, which stimulate the proliferation of hepatocytes to replace dying hepatocytes (64, 82). NK cells might also play a role in liver regeneration by cross talk with hepatic macrophages known to regulate the differentiation of hepatic progenitor cells into hepatocytes (83). All of the regenerative effects of NK cells are dependent on optimal NK cell activation, as overactivation actually prevents liver regeneration (84). Indeed, when strongly activated, NK cells produce excessive amounts of IFN-γ and lose self-tolerance, compromising liver repair (85). To maintain self-tolerance during regenerative hyperplasia, hepatocytes upregulate the poliovirus receptor (PVR/CD155) after partial hepatectomy, which engages the regulatory molecule T cell Ig and ITIM domain (TIGIT) on NK cells (84). NK cell effector functions used to resolve inflammation also shape the recruitment and differentiation of stem cells for tissue regeneration. NK cells enhance the recruitment of mesenchymal stem cells (MSCs) by producing a variety of chemokines, such as, NAP-2 (CXCL7), GRO-b (CXCL2), GRO-g (CXCL3), IL-8, and RANTES (86). MSCs have the capacity to differentiate into specific lineages that promote tissue repair. The cross talk between NK cells and MSCs has been demonstrated in several studies, in particular, during bone repair where NK cells mediate the recruitment of MSCs. Taken together, the dual role of NK cells on fibrosis and tissue regeneration could be explained by their differentiation from actively cytotoxic cells to regulatory cells that produce cytokines supportive of tissue repair. Regulatory NK cells might be induced as a result of NK cell interactions with surrounding cells such as immune cells (KC and liver macrophages), effector cells of connective tissue (fibroblasts, HSCs), or stem cells.

Role of NK Cells in HCC

In mouse models of cancer and human cancer patients, NK cells are often dysfunctional, with reduced cytotoxic activity, impaired production of cytokines, and an inability to efficiently kill abnormal cells (87–90). In patients with HCC, there is also a significant reduction in both the percentage and number of total liver NK cells, as well as peripheral $CD56^{dim}CD16^{pos}$ subsets (91, 92). At all stages of HCC, these $CD56^{dim}CD16^{pos}$ NK cells have a decreased ability to produce cytokine, with IFN-γ production following PMA/ionomycin stimulation decreasing from 50 to 5% between healthy and HCC donors (93). Total NK cells show reduced production of Granzyme A, Granzyme B, and perforin once HCC has progressed past stage I (93). NK cells cultured with cancer-associated fibroblasts from HCC (H-CAFs) downregulate NKG2D and NKp46 and decrease expression of Granzyme B, perforin, TNF-α, and INF-γ (94). Following PGE2 blockade by the inhibitor NS398, these NK cells increased TNF-α and IFN-γ production, an effect that was further amplified with the addition of the indoleamine 2,3-dioxygenase (IDO) inhibitor, 1-MT (94). Further investigation into the interactions that contribute to dysregulated NK cells and by extension, the immunosuppressive state that facilitates HCC progression, is necessary to fully understand the tumor microenvironment and how to target therapies to these interactions.

THERAPEUTICS TARGETED TO NK CELLS

The immunoregulatory function of NK cells in infection and inflammatory diseases makes these cells good therapeutic targets for controlling infection and preventing the development of chronic inflammatory diseases (88). Several preclinical therapeutic approaches have been used to target NK cells for treating HCC. Therapies included induction of the activation of NK cells (with cytokines such as IL-2, IL-12, IL-15, IL-21, IFN-α, and IFN-γ) or performing adoptive transfers of activated NK cells. Type I IFNs has been shown to have antiviral, anti-fibrotic, and antitumor effects, likely due to their ability to stimulate NK cytolytic activity (37). IL-12 and IL-18 are also potent activators of NK cells that produce IFN-γ and TNF-α and have antiviral effects (95). Activation can also be achieved by blocking NK cell inhibitory receptors with monoclonal antibodies (mAb) (anti-NKG2A or anti-KIR), or by activating NK activating receptors to enhance NK cell ADCC against tumor cells.

Therapeutic approaches have been designed to use specific mAb to target co-inhibitory molecules (i.e., immune checkpoint inhibitors) that stop immune responses against tumor cells (96). Blocking these immune checkpoint inhibitors would release immune cells, including NK cells, from inhibitory mechanisms to restore their full immune activity. These immune checkpoint inhibitors are well described in the context of T cell exhaustion (97, 98). They include cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed death 1 (PD-1), lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and mucin-domain-containing-3 (TIM-3), and B and T lymphocyte attenuator (BTLA). The expression of PD-1 and Tim-3 on liver NK cells from patients with HBV-related HCC is increased and also associated with immune cell exhaustion (99, 100). The promising results from clinical trials

using mAb against immune checkpoint inhibitors might also prove to be a likely therapeutic strategy for liver disease, especially in combination with drugs enhancing NK cell activation. Recent studies have shown that the expression of Tim-3 on peripheral $CD56^+$ NK cells correlates with expression of serological markers of liver fibrosis in patients with advanced schistosomiasis (101). Blocking PD-1, CTLA-4, or Tim-3 pathway with mAbs could protect patients from fibrosis, cirrhosis, and HCC by restoring NK cell function. Several mAbs against PD-1 (ipilimumab, pembrolizumab, nivolumab), PD-L1 (atezolizumab, durvalumab), or CTLA-4 (tremelimumab/CP-675,206) are currently being tested in clinical trials against HCC.

NK cell activation can also be achieved through blocking NK inhibitory receptors such as KIR, NKG2A/CD94, or TIGIT. Cancer cells or virus-infected cells upregulate the expression of MHC class I molecules (HLA in humans) as an immune escape mechanism in order to evade killing by NK and T cells. MHC I molecules in mice, and HLA molecules in humans, engage NK inhibitory receptors like Ly49 and NKG2A and KIR, respectively, to prevent NK cell activation. Anti-NKG2A antibodies are already under investigation in several clinical trials alone, or in combination with other anticancer drugs (ibrutninb), mAb-like anti-EGFR (cetuximab), or anti-PD-L1 (duvalumab). New clinical studies are in progress to test the antitumor effect of anti-KIR monoclonal antibody in combination with other drugs.

Taken together, the combination of NK cell-based therapy with conventional therapies might become an efficient approach to cure or lessen the burden of liver cancers. More clinical trials are needed to evaluate the safety and efficiency of these combined approaches. In addition, NK cells play different roles depending on the developmental stage of a specific disease, emphasizing the importance of investigating the spatiotemporal role of NK cells in cancer and fibrosis. It is worth noting that most NK cell studies are done with peripheral and splenic NK cells, not with tissue-resident NK cells. More investigations are required to determine the specific role of each NK cell subset, how these subsets contribute to the development of tissue-specific disease, and the most effective therapeutic strategies for each disease.

SUMMARY AND CONCLUSION

NK cells play crucial roles in regulation of chronic inflammatory diseases such as tissue fibrosis and cancer. Thus, the understanding of NK-mediated immunoregulation would provide insight into designing therapeutics against viral infection and inflammatory diseases. NK cells have a protective role in the development of liver fibrosis in the model of NAFLD development where they regulate the tight balance between liver inflammation and repair through macrophage polarization. Thus, the identification of NK cells as upstream regulators of macrophage function provides a new cellular target to modulate macrophage-mediated inflammation in chronic liver diseases. Further exploration of the interplay between myeloid cells and NK cells may thus help identify key molecular regulators that can resolve chronic inflammation and restore immune homeostasis.

AUTHOR CONTRIBUTIONS

AT-T reviewed the literature and wrote the manuscript. YH, FS, and SE contributed to and edited the manuscript.

ACKNOWLEDGMENTS

We would like to thank Emily Mercadante and members of the Hahn lab for their input on this manuscript.

FUNDING

This work is supported by grants from the National Institutes of Health (R01AI09812601).

REFERENCES

1. Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol* (2013) 14(10):996–1006. doi:10.1038/ni.2691
2. Racanelli V, Rehermann B. The liver as an immunological organ. *Hepatology* (2006) 43(2 Suppl 1):S54–62. doi:10.1002/hep.21060
3. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol* (2014) 14(3):181–94. doi:10.1038/nri3623
4. Kmieć Z. Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol* (2001) 161:1–151. doi:10.1007/978-3-642-56553-3
5. Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gastroenterology* (2001) 120(1):250–60. doi:10.1053/gast.2001.20947
6. Żeromski J, Mozer-Lisewska I, Kaczmarek M, Kowala-Piaskowska A, Sikora J. NK cells prevalence, subsets and function in viral hepatitis C. *Arch Immunol Ther Exp* (2011) 59(6):449–55. doi:10.1007/s00005-011-0145-y
7. Bonorino P, Ramzan M, Camous X, Dufeu-Duchesne T, Thelu MA, Sturm N, et al. Fine characterization of intrahepatic NK cells expressing natural killer receptors in chronic hepatitis B and C. *J Hepatol* (2009) 51(3):458–67. doi:10.1016/j.jhep.2009.05.030
8. Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology* (2009) 137(3):1151–60. doi:10.1053/j.gastro.2009.05.047
9. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. *Immunol Rev* (2007) 220:169–82. doi:10.1111/j.1600-065X.2007.00563.x
10. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat Immunol* (2010) 11(5):373–84. doi:10.1038/ni.1863
11. Hamerman JA, Ogasawara K, Lanier LL. NK cells in innate immunity. *Curr Opin Immunol* (2005) 17(1):29–35. doi:10.1016/j.coi.2004.11.001
12. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* (2001) 7(1):94–100. doi:10.1038/83416
13. Paust S, Gill HS, Wang B, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol* (2010) 11(12):1127–35. doi:10.1038/ni.1953
14. Reeves RK, Li H, Jost S, Blass E, Li H, Schafer JL, et al. Antigen-specific NK cell memory in rhesus macaques. *Nat Immunol* (2015) 16(9):927–32. doi:10.1038/ni.3227
15. Schuppan D, Kim YO. Evolving therapies for liver fibrosis. *J Clin Invest* (2013) 123(5):1887–901. doi:10.1172/JCI66028

16. Labonte AC, Tosello-Trampont AC, Hahn YS. The role of macrophage polarization in infectious and inflammatory diseases. *Mol Cells* (2014) 37(4):275–85. doi:10.14348/molcells.2014.2374
17. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* (2010) 464(7293):1357–61. doi:10.1038/nature08938
18. He Y, Hara H, Nunez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci* (2016) 41(12):1012–21. doi:10.1016/j.tibs.2016.09.002
19. Guo S, Yang C, Diao B. The NLRP3 inflammasome and IL-1β accelerate immunologically mediated pathology in experimental viral fulminant hepatitis. *PLoS Pathog* (2015) 11(9):e1005155. doi:10.1371/journal.ppat.1005216
20. Huang H, Chen H-W, Evankovich J, Yan W, Rosborough BR, Nace GW, et al. Histones activate the NLRP3 inflammasome in Kupffer cells during sterile inflammatory liver injury. *J Immunol* (2013) 191(5):2665–79. doi:10.4049/jimmunol.1202733
21. Kamo N, Ke B, Ghaffari A. SC/caspase-1/IL-1β signaling triggers inflammatory responses by promoting HMGB1 induction in liver ischemia/reperfusion injury. *Hepatology* (2013) 58(1):351–62. doi:10.1002/hep.26320
22. Vandanmagsar B, Youm Y-H, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med* (2011) 17(2):179–88. doi:10.1038/nm.2279
23. Negash AA, Ramos HJ, Crochet N, Lau DTY, Doehle B, Papic N, et al. IL-1β production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease. *PLoS Pathog* (2013) 9(4):e1003330. doi:10.1371/journal.ppat.1003330
24. Maltez V, Tubbs AL, Cook KD, Aachoui Y, Falcone EL, Holland SM, et al. Inflammasomes coordinate pyroptosis and natural killer cell cytotoxicity to clear infection by a ubiquitous environmental bacterium. *Immunity* (2015) 43(5):987–97. doi:10.1016/j.immuni.2015.10.010
25. Zannetti C, Roblot G, Charrier E. Characterization of the inflammasome in human Kupffer cells in response to synthetic agonists and pathogens. *J Immunol* (2016) 197(1):356–67. doi:10.4049/jimmunol.1502301
26. Pan X, Xu H, Zheng C, Li M, Zou X, Cao H, et al. Human hepatocytes express absent in melanoma 2 and respond to hepatitis B virus with interleukin-18 expression. *Virus Genes* (2016) 52(4):445–52. doi:10.1007/s11262-016-1327-9
27. Movita D, Van de Garde MD, Biesta P, Kreefft K, Haagmans B, Zuniga E, et al. Inflammatory monocytes recruited to the liver within 24 hours after virus-induced inflammation resemble Kupffer cells but are functionally distinct. *J Virol* (2015) 89(9):4809–17. doi:10.1128/JVI.03733-14
28. Dupaul-Chicoine J, Arabzadeh A, Dagenais M, Douglas T, Champagne C, Morizot A, et al. The Nlrp3 inflammasome suppresses colorectal cancer metastatic growth in the liver by promoting natural killer cell tumoricidal activity. *Immunity* (2015) 43(4):751–63. doi:10.1016/j.immuni.2015.08.013
29. Terme M, Ullrich E, Aymeric L, Meinhardt K, Coudert JD, Desbois M, et al. Cancer-induced immunosuppression: IL-18-elicited immunoablative NK cells. *Cancer Res* (2012) 72(11):2757–67. doi:10.1158/0008-5472.CAN-11-3379
30. Terme M, Ullrich E, Aymeric L, Meinhardt K, Desbois M, Delahaye N, et al. IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res* (2011) 71(16):5393–9. doi:10.1158/0008-5472.CAN-11-0993
31. Van den Boorn JG, Jakobs C, Hagen C, Renn M, Luiten RM, Melief CJ, et al. Inflammasome-dependent induction of adaptive NK cell memory. *Immunity* (2016) 44(6):1406–21. doi:10.1016/j.immuni.2016.05.008
32. Lassen MG, Lukens JR, Dolina JS, Brown MG, Hahn YS. Intrahepatic IL-10 maintains NKG2A(+)Ly49(-) liver NK cells in a functionally hyporesponsive state. *J Immunol* (2010) 184(5):2693–701. doi:10.4049/jimmunol.0901362
33. Robinson MW, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. *Cell Mol Immunol* (2016) 13(3):267–76. doi:10.1038/cmi.2016.3
34. Di Santo JP. Natural killer cell developmental pathways: a question of balance. *Annu Rev Immunol* (2006) 24:257–86. doi:10.1146/annurev.immunol.24.021605.090700
35. Geiger TL, Sun JC. Development and maturation of natural killer cells. *Curr Opin Immunol* (2016) 39:82–9. doi:10.1016/j.coi.2016.01.007
36. Rosmaraki EE, Douagi I, Roth C, Colucci F, Cumano A, Di Santo JP. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* (2001) 31(6):1900–9. doi:10.1002/1521-4141(200106)31:6<1900::AID-IMMU1900>3.0.CO;2-M
37. Krueger PD, Lassen MG, Qiao HH, Hahn YS. Regulation of NK cell repertoire and function in the liver. *Crit Rev Immunol* (2011) 31(1):43–52. doi:10.1615/CritRevImmunol.v31.i1.40
38. Peng H, Jiang X, Chen Y, Sojka DK, Wei H, Gao X, et al. Liver-resident NK cells confer adaptive immunity in skin-contact inflammation. *J Clin Invest* (2013) 123(4):1444–56. doi:10.1172/JCI66381
39. Klose CS, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) 157(2):340–56. doi:10.1016/j.cell.2014.03.030
40. Peng H, Tian Z. Re-examining the origin and function of liver-resident NK cells. *Trends Immunol* (2015) 36(5):293–9. doi:10.1016/j.it.2015.03.006
41. Serafini N, Vosshenrich CA, Di Santo JP. Transcriptional regulation of innate lymphoid cell fate. *Nat Rev Immunol* (2015) 15(7):415–28. doi:10.1038/nri3855
42. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol* (2016) 17(7):758–64. doi:10.1038/ni.3482
43. Sonnenberg G, Artis D. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. *Nat Med* (2015) 21(7):698–708. doi:10.1038/nm.3892
44. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells – a proposal for uniform nomenclature. *Nat Rev Immunol* (2013) 13(2):145–9. doi:10.1038/nri3365
45. Jiao Y, Huntington ND, Belz GT, Seillet C. Type 1 innate lymphoid cell biology: lessons learnt from natural killer cells. *Front Immunol* (2016) 7:426. doi:10.3389/fimmu.2016.00426
46. Sojka DK, Plougastel-Douglas B, Yang L, Pak-Wittel MA, Artyomov MN, Ivanova Y, et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *Elife* (2014) 3:e01659. doi:10.7554/eLife.01659
47. Hudspeth K, Donadon M, Cimino M, Pontarini E, Tentorio P, Preti M, et al. Human liver-resident CD56(bright)/CD16(neg) NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways. *J Autoimmun* (2016) 66:40–50. doi:10.1016/j.jaut.2015.08.011
48. Ahlenstiel G. The natural killer cell response to HCV infection. *Immune Netw* (2013) 13(5):168–76. doi:10.4110/in.2013.13.5.168
49. Roda JM, Parihar R, Magro C, Nuovo GJ, Tridandapani S, Carson WE. Natural killer cells produce T cell-recruiting chemokines in response to antibody-coated tumor cells. *Cancer Res* (2006) 66(1):517–26. doi:10.1158/0008-5472.CAN-05-2429
50. Sancéau J, Sondermeyer P, Béranger F, Falcoff R, Vaquero C. Intracellular human gamma-interferon triggers an antiviral state in transformed murine L cells. *Proc Natl Acad Sci U S A* (1987) 84(9):2906–10. doi:10.1073/pnas.84.9.2906
51. Goh CC, Roggerson KM, Lee HC, Golden-Mason L, Rosen HR, Hahn YS. Hepatitis C virus-induced myeloid-derived suppressor cells suppress NK cell IFN-γ production by altering cellular metabolism via arginase-1. *J Immunol* (2016) 196(5):2283–92. doi:10.4049/jimmunol.1501881
52. Melhem A, Muhanna N, Bishara A, Alvarez CE, Ilan Y, Bishara T, et al. Anti-fibrotic activity of NK cells in experimental liver injury through killing of activated HSC. *J Hepatol* (2006) 45(1):60–71. doi:10.1016/j.jhep.2005.12.025
53. Radaeva S, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. *Gastroenterology* (2006) 130(2):435–52. doi:10.1053/j.gastro.2005.10.055
54. Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, et al. Senescence of activated stellate cells limits liver fibrosis. *Cell* (2008) 134(4):657–67. doi:10.1016/j.cell.2008.06.049

55. Sagiv A, Burton DG, Moshayev Z, Vadai E, Wensveen F, Ben-Dor S, et al. NKG2D ligands mediate immunosurveillance of senescent cells. *Aging (Albany NY)* (2016) 8(2):328–44. doi:10.18632/aging.100897
56. Gur C, Doron S, Kfir-Erenfeld S, Horwitz E, Abu-Tair L, Safadi R, et al. NKp46-mediated killing of human and mouse hepatic stellate cells attenuates liver fibrosis. *Gut* (2012) 61(6):885–93. doi:10.1136/gutjnl-2011-301400
57. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, et al. Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. *Hepatology* (2002) 36(4):850–60. doi:10.1053/jhep.2002.35625
58. Bansal R, Prakash J, De Ruiter M, Poelstra K. Interferon gamma peptidomimetic targeted to hepatic stellate cells ameliorates acute and chronic liver fibrosis in vivo. *J Control Release* (2014) 179:18–24. doi:10.1016/j.jconrel.2014.01.022
59. Bansal R, Prakash J, Post E, Beljaars L, Schuppan D, Poelstra K. Novel engineered targeted interferon-gamma blocks hepatic fibrogenesis in mice. *Hepatology* (2011) 54(2):586–96. doi:10.1002/hep.24395
60. Tosello-Trampont AC, Krueger P, Narayanan S, Landes SG, Leitinger N, Hahn YS. NKp46(+) natural killer cells attenuate metabolism-induced hepatic fibrosis by regulating macrophage activation in mice. *Hepatology* (2016) 63(3):799–812. doi:10.1002/hep.28389
61. Radaeva S, Sun R, Pan HN, Hong F, Gao B. Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology* (2004) 39(5):1332–42. doi:10.1002/hep.20184
62. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. *Int Immunol* (2011) 23(3):159–63. doi:10.1093/intimm/dxr001
63. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, Flavell RA. Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity* (2007) 27(4):647–59. doi:10.1016/j.immuni.2007.07.023
64. Park O, Wang H, Weng H, Feigenbaum L, Li H, Yin S, et al. In vivo consequences of liver-specific interleukin-22 expression in mice: implications for human liver disease progression. *Hepatology* (2011) 54(1):252–61. doi:10.1002/hep.24339
65. Zhao J, Zhang Z, Luan Y, Zou Z, Sun Y, Li Y, et al. Pathological functions of interleukin-22 in chronic liver inflammation and fibrosis with hepatitis B virus infection by promoting T helper 17 cell recruitment. *Hepatology* (2014) 59(4):1331–42. doi:10.1002/hep.26916
66. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity* (2004) 21(2):241–54. doi:10.1016/j.immuni.2004.07.007
67. Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J Immunol* (2005) 174(6):3695–702. doi:10.4049/jimmunol.174.6.3695
68. Ma HL, Liang S, Li J, Napierata L, Brown T, Benoit S, et al. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. *J Clin Invest* (2008) 118(2):597–607. doi:10.1172/JCI33263
69. Beraza N, Malato Y, Sander LE, Al-Masaoudi M, Freimuth J, Riethmacher D, et al. Hepatocyte-specific NEMO deletion promotes NK/NKT cell- and TRAIL-dependent liver damage. *J Exp Med* (2009) 206(8):1727–37. doi:10.1084/jem.20082152
70. Fernandez-Alvarez S, Gutierrez-de Juan V, Zubiete-Franco I, Barbier-Torres L, Lahoz A, Pares A, et al. TRAIL-producing NK cells contribute to liver injury and related fibrogenesis in the context of GNMT deficiency. *Lab Invest* (2015) 95(2):223–36. doi:10.1038/labinvest.2014.151
71. Ishiyama K, Ohdan H, Ohira M, Mitsuta H, Arihiro K, Asahara T. Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. *Hepatology* (2006) 43(2):362–72. doi:10.1002/hep.21035
72. Mantovani S, Mele D, Oliviero B, Barbarini G, Varchetta S, Mondelli MU. NKp30 isoforms in patients with chronic hepatitis C virus infection. *Immunology* (2015) 146(2):234–42. doi:10.1111/imm.12495
73. Ochi M, Ohdan H, Mitsuta H, Onoe T, Tokita D, Hara H, et al. Liver NK cells expressing TRAIL are toxic against self hepatocytes in mice. *Hepatology* (2004) 39(5):1321–31. doi:10.1002/hep.20204
74. Zou Y, Bao J, Pan X, Lu Y, Liao S, Wang X, et al. NKP30-B7-H6 interaction aggravates hepatocyte damage through up-regulation of interleukin-32 expression in hepatitis B virus-related acute-on-chronic liver failure. *PLoS One* (2015) 10(8):e0134568. doi:10.1371/journal.pone.0134568
75. Feldstein AE, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* (2003) 125(2):437–43. doi:10.1016/S0016-5085(03)00907-7
76. Shimoda S, Harada K, Niiro H, Shirabe K, Taketomi A, Maehara Y, et al. Interaction between toll-like receptors and natural killer cells in the destruction of bile ducts in primary biliary cirrhosis. *Hepatology* (2011) 53(4):1270–81. doi:10.1002/hep.24194
77. Tian Z, Gershwin ME, Zhang C. Regulatory NK cells in autoimmune disease. *J Autoimmun* (2012) 39(3):206–15. doi:10.1016/j.jaut.2012.05.006
78. Schleinitz N, Vely F, Harle JR, Vivier E. Natural killer cells in human autoimmune diseases. *Immunology* (2010) 131(4):451–8. doi:10.1111/j.1365-2567.2010.03360.x
79. Boukouaci W, Lauden L, Siewiera J, Dam N, Hocine HR, Khaznadar Z, et al. Natural killer cell crosstalk with allogeneic human cardiac-derived stem/progenitor cells controls persistence. *Cardiovasc Res* (2014) 104(2):290–302. doi:10.1093/cvr/cvu208
80. Kumar P, Thakar MS, Ouyang W, Malarkannan S. IL-22 from conventional NK cells is epithelial regenerative and inflammation protective during influenza infection. *Mucosal Immunol* (2013) 6(1):69–82. doi:10.1038/mi.2012.49
81. Graubardt N, Fahrner R, Trochsler M, Keogh A, Breu K, Furer C, et al. Promotion of liver regeneration by natural killer cells in a murine model is dependent on extracellular adenosine triphosphate phosphohydrolysis. *Hepatology* (2013) 57(5):1969–79. doi:10.1002/hep.26008
82. Cosgrove BD, Cheng C, Pritchard JR, Stolz DB, Lauffenburger DA, Griffith LG. An inducible autocrine cascade regulates rat hepatocyte proliferation and apoptosis responses to tumor necrosis factor-alpha. *Hepatology* (2008) 48(1):276–88. doi:10.1002/hep.22335
83. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nat Med* (2012) 18(4):572–9. doi:10.1038/nm.2667
84. Bi J, Zheng X, Chen Y, Wei H, Sun R, Tian Z. TIGIT safeguards liver regeneration through regulating natural killer cell-hepatocyte crosstalk. *Hepatology* (2014) 60(4):1389–98. doi:10.1002/hep.27245
85. Sun R, Gao B. Negative regulation of liver regeneration by innate immunity (natural killer cells/interferon-gamma). *Gastroenterology* (2004) 127(5):1525–39. doi:10.1053/j.gastro.2004.08.055
86. Almeida CR, Caires HR, Vasconcelos DP, Barbosa MA. NAP-2 secreted by human NK cells can stimulate mesenchymal stem/stromal cell recruitment. *Stem Cell Reports* (2016) 6(4):466–73. doi:10.1016/j.stemcr.2016.02.012
87. Cerwenka A, Lanier LL. Natural killer cell memory in infection, inflammation and cancer. *Nat Rev Immunol* (2016) 16(2):112–23. doi:10.1038/nri.2015.9
88. Mandal A, Viswanathan C. Natural killer cells: in health and disease. *Hematol Oncol Stem Cell Ther* (2015) 8(2):47–55. doi:10.1016/j.hemonc.2014.11.006
89. Patel P, Schutzer SE, Pyrsopoulos N. Immunobiology of hepatocarcinogenesis: ways to go or almost there? *World J Gastrointest Pathophysiol* (2016) 7(3):242–55. doi:10.4291/wjgp.v7.i3.242
90. Tian Z, Chen Y, Gao B. Natural killer cells in liver disease. *Hepatology* (2013) 57(4):1654–62. doi:10.1002/hep.26115
91. Liu H, Deng W, Li J, Tang Y, Zhang L, Cui Y, et al. Peripheral blood lymphocyte subset levels differ in patients with hepatocellular carcinoma. *Oncotarget* (2016) 7(47):77558–64. doi:10.18632/oncotarget.13041
92. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, et al. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. *Hepatology* (2009) 50(3):799–807. doi:10.1002/hep.23054
93. Cai L, Zhang Z, Zhou L, Wang H, Fu J, Zhang S, et al. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. *Clin Immunol* (2008) 129(3):428–37. doi:10.1016/j.clim.2008.08.012
94. Li T, Yang Y, Hua X, Wang G, Liu W, Jia C, et al. Hepatocellular carcinoma-associated fibroblasts trigger NK cell dysfunction via PGE2 and IDO. *Cancer Lett* (2012) 318(2):154–61. doi:10.1016/j.canlet.2011.12.020

95. Lee SH, Miyagi T, Biron CA. Keeping NK cells in highly regulated antiviral warfare. *Trends Immunol* (2007) 28(6):252–9. doi:10.1016/j.it.2007.04.001
96. Su M, Huang CX, Dai AP. Immune checkpoint inhibitors: therapeutic tools for breast cancer. *Asian Pac J Cancer Prev* (2016) 17(3):905–10. doi:10.7314/APJCP.2016.17.3.905
97. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* (2010) 363(8):711–23. doi:10.1056/NEJMoa1003466
98. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–64. doi:10.1038/nrc3239
99. Li H, Wu K, Tao K, Chen L, Zheng Q, Lu X, et al. Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. *Hepatology* (2012) 56(4):1342–51. doi:10.1002/hep.25777
100. Zeng Z, Shi F, Zhou L, Zhang MN, Chen Y, Chang XJ, et al. Upregulation of circulating PD-L1/PD-1 is associated with poor post-cryoablation prognosis in patients with HBV-related hepatocellular carcinoma. *PLoS One* (2011) 6(9):e23621. doi:10.1371/journal.pone.0023621
101. Wu QW, Zhu X, Fu X, Yang JS, Cao ZG, Pu C. [Expression of Tim-3 on peripheral CD56(+) NK cells and its correlation with liver fibrosis in patients with advanced schistosomiasis]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* (2015) 33(5):346–50.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 25 April 2017
doi: 10.3389/fimmu.2017.00465

Natural Killer Cells in Graft-versus-Host-Disease after Allogeneic Hematopoietic Cell Transplantation

Federico Simonetta[1,2], *Maite Alvarez*[1] *and Robert S. Negrin*[1]*

[1] Division of Blood and Marrow Transplantation, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA, [2] Division of Hematology, Department of Oncology, Geneva University Hospitals, University of Geneva, Geneva, Switzerland

Allogeneic hematopoietic cell transplantation (HCT) is a well-established therapeutic modality effective for a variety of hematological malignancies but, unfortunately, is associated with significant morbidity and mortality related to cancer relapse as well as to transplant-related complications including graft-versus-host-disease (GvHD). Natural killer (NK) cells are the first donor-derived lymphocyte subset to recover after HCT, and their crucial role in protection against cancer relapse and infections is well established. Conversely, the role played by NK cells in GvHD is still controversial. Early studies suggested a participation of NK cells in GvHD induction or exacerbation. Subsequently, experimental evidence obtained in mice as well observational studies performed in humans led to a model in which NK cells play a regulatory role in GvHD by repressing alloreactive T cell responses. This widely accepted model has been recently challenged by clinical evidence indicating that NK cells can in some cases promote GvHD. In this review, we summarize available knowledge about the role of NK cells in GVHD pathogenesis. We review studies uncovering cellular mechanisms through which NK cells interact with other immune cell subsets during GvHD leading to a model in which NK cells naturally suppress GvHD through their cytotoxic ability to inhibit T cell activation unless exogenous hyperactivation lead them to produce proinflammatory cytokines that can conversely sustain T cell-mediated GvHD induction.

Keywords: natural killer cells, graft-versus-host-disease, bone marrow transplantation, HSCT, tolerance

OPEN ACCESS

Edited by:
Sandra Laurence Lopez-Verges, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama

Reviewed by:
Subramaniam Malarkannan, Medical College of Wisconsin, USA
Evelyn Ullrich, Goethe University Frankfurt, Germany

***Correspondence:**
Robert S. Negrin
negrs@stanford.edu

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *05 February 2017*
Accepted: *05 April 2017*
Published: *25 April 2017*

Citation:
Simonetta F, Alvarez M and Negrin RS (2017) Natural Killer Cells in Graft-versus-Host-Disease after Allogeneic Hematopoietic Cell Transplantation. Front. Immunol. 8:465. doi: 10.3389/fimmu.2017.00465

INTRODUCTION

Natural killer (NK) cells are the first donor-derived lymphocyte subsets to recover after hematopoietic cell transplantation (HCT), preceding by several months the reconstitution of adaptive T and B lymphocytes. NK cells have been the focus of significant attention in the HCT field over the last four decades. Studies of the role of NK cells in bone marrow engraftment demonstrated that host NK cells persisting after conditioning can contribute to graft rejection (1) while donor NK cells can promote hematopoietic engraftment (2). At the same time, several preclinical and clinical studies focusing on NK cell alloreactivity in anticancer responses identified donor NK cells as crucial players in preventing cancer relapse after HCT for hematologic malignancies (3, 4). Less well established, however, is the role of NK cells in graft-versus-host-disease (GvHD), a major complication of HCT. While the classical model of GvHD pathogenesis includes, together with donor-derived T cells, donor-derived NK cells in the immune-pathological activation leading to GvHD (5), evidence from preclinical

models as well as from studies in human HCT recipients led to a more complex picture where NK cells could either promote or prevent GvHD.

In this review, we summarize the available knowledge about the role of NK cells in GvHD pathogenesis. After reviewing preclinical and clinical studies uncovering cellular mechanisms through which NK cells interact with other immune cell subsets during GvHD, we propose a new model in which distinct effector mechanisms determine the pathogenic or regulatory role of NK cells in promotion or control of GvHD, respectively. Finally, we discuss the impact that GvHD can in turn have on NK cell biology and the potential consequences in the context of HCT.

EARLY STUDIES

The first study suggesting a relationship between NK cells and GvHD development was reported by Lopez and coworkers from the Sloan Kettering Cancer Center showing a significant association between GvHD development and pre-transplant levels of NK cell activity, as measured by cytotoxic assays performed using herpes simplex virus type 1-infected fibroblast as target cells, in peripheral blood of a small and heterogeneous cohort of 13 patients undergoing different protocols of HCT (6). Importantly, most of the patients included in the series underwent HCT after myeloablative conditioning, and no information was provided about NK cell activity after transplantation. Shortly thereafter, Livnat et al. (7) and Dokhelar et al. (8) addressed the same issue assessing NK cell activity against the K562 leukemic cell line both before and after HCT and obtained contradictory results finding either no relationship (7) or a positive association (8) between early posttransplant NK cell activity and GvHD development. Despite the contradictory conclusions obtained and the limitations of the studies including the heterogeneity of the patients cohorts as well as of the analytical methods employed, these early studies opened the way to numerous studies addressing the role of NK cells in GvHD.

A first approach has been to investigate the presence of NK cells in GvHD target organs. In the mouse parent-into-F1 (P > F1) model of GvHD, increased NK cell activity measured against YAC lymphoma target cells was detected in spleen (9–11), lymph nodes (9, 10), thymus (9, 12), and intestinal intraepithelial lymphocytes (10) from mice with active GvHD. Similarly, in murine minor mismatch HCT models, large granular lymphocytes displaying an immunophenotype characteristic of NK cells infiltrated the skin (13), liver, and intestine (14) from animals with acute GVHD. Importantly, the use of congenic markers demonstrated that these cells were of donor origin (14). Accordingly, the study of biopsies obtained from skin (15–17), liver (18, 19), and intestinal (20) of patients with acute GvHD showed the presence of NK cells among the lymphoid population infiltrating these GvHD target tissues. The study of biopsies obtained from female patients transplanted with male donor grafts confirmed in humans the donor origin of the NK cells infiltrating tissues during GvHD (16). The target tissues infiltration by NK cells during GvHD, both in mice and humans, supported a model in which NK cells may induce, or at least contribute to, GvHD development. Attempts were, therefore, made to obtain experimental evidence supporting this hypothesis, first by using NK cell depleting antibodies directed against the cell surface glycolipid asialo GM1 or to the cell surface NKR-P1 family receptor NK1.1. However, results from reports employing this approach were inconsistent, few studies suggested a reduction of GvHD upon treatment of recipients (21–23) while most studies employing antibody depletion on donor cells showed only minimal if any impact on GvHD development (23–27). This discrepancy suggested that depleting antibodies exerted their effect through the depletion of an effector cell population appearing after HCT rather then by depleting NK cells contained in the graft. Further, the epitopes recognized by anti-asialo GM1 and anti-NK1.1 antibodies are expressed by several immune cell subsets other than NK cells, including activated T cells involved in GvHD development (28–30), making it impossible to distinguish between an NK and a T cell directed effect. Ghayur et al. used a complimentary approach employing beige mice carrying a homozygous *bg* mutation that leads to severe deficiency in NK cell function. Adoptive transfer of *bg/bg* splenocytes failed to induce GvHD in a P > F1 model, while transfer of heterozygous +/*bg* induced hepatic GvHD, suggesting that donor NK cells were responsible for GvHD induction (31). However, even in this model, a functional deficit in adaptive T cells from beige mice complicates the interpretation of the results (32, 33).

NK CELL CYTOTOXIC FUNCTIONS AND GvHD PREVENTION

While murine models based on antibody depletion or genetic alteration of NK cells failed to provide consistent evidence for a role of NK cells in GvHD pathogenesis, the adoptive transfer of NK cells offered unexpected insights. In an attempt to promote bone marrow engraftment in a major mismatch murine model, Murphy and coworkers adoptively transferred NK cells purified from C.B-17 severe combined immunodeficiency (SCID) (H-2^d) mice into lethally irradiated C57BL/6J (H-2^b) mice together with non-T-cell depleted bone marrow cells from BALB/cJ (H-2^d) mice with or without splenocytes (2). In mice not receiving splenocytes, transferred NK cells did not induce GvHD, thus questioning the NK GvHD-inducing potential suggested by antibody depletion studies. More interestingly, in mice receiving splenocytes, activated NK cells prevented the development of GvHD that invariably lead to death of mice injected with BM cells and splenocytes alone. This unexpected result revealed not only that NK cells can be adoptively transferred safely in this major mismatch model without inducing GvHD but also that they can prevent T cell-mediated GvHD development. The results of this first study were confirmed during the years by several other reports (3, 34–39) and numerous studies in humans suggested that higher numbers of NK cells (40–47) and the presence of NK cell alloreactivity (3, 4, 48–50) reduce GvHD development.

In particular, NK cell alloreactivity has been found to be crucial for NK cell-mediated protection from GvHD. Ruggeri et al. showed in a major mismatch HCT murine model that alloreactive Ly49 ligand-mismatched NK cell infusion prevented T cell-induced GvHD, while administration of even large numbers of non-alloreactive Ly49 ligand-matched NK cells

provided no protection (3). These results were subsequently confirmed by Lundqvist et al. who further extended this observation showing that, although inefficient in preventing GvHD, Ly49 ligand-matched NK cells displayed an antitumor activity similar to Ly49 ligand-mismatched NK cells (35). The need of Ly49 ligand-mismatch for GvHD control by NK cells prompted some investigators to silence Ly49C to induce alloreactivity with promising results (51). Alloreactive NK cells were shown to indirectly inhibit T cell proliferation and GvHD induction by depleting antigen-presenting cells (APCs) (3, 38) through their cytolytic activity, the c-Kit$^-$CD27$^-$CD11b$^+$ NK cells being the most potent in this effect (38). In particular, the expression of the activating receptor KIR2DS1, which binds to HLA-C2, seems to contribute to the APCs' killing and it was even able to override the inhibition mediated by the expression of the inhibitory receptor NKG2A, which binds to HLA-E in humans or Qa-1b in mouse (50). Similarly, proportions of donor-derived NK cells expressing the activating receptor CD94/NKG2C, which recognize as well HLA-E/Qa-1b, were lower in HLA-matched and HLA-mismatched HCT recipients with acute or chronic GvHD compared with patients without GvHD (52). Accordingly, patients with acute or chronic GvHD displayed a lower ratio of CD94/NKG2C to CD94/NKG2A on NK cells suggesting a competition for the same ligands between NKG2C and NKG2A that would result in NK cell activation or suppression, respectively (52). Finally, Ghadially et al. suggested that NK cell-mediated killing of APC during GvHD is mediated by the stimulation of NKp46 receptor by still unknown ligand(s) expressed by dendritic cells (DCs) as the absence of NKp46 on donor NK cells leads to increased stimulation of donor T cells by DCs (53), resulting in increased tissue damage (54).

In addition to this indirect, APC-killing mechanism, others and we have shown that NK cells can suppress GvHD by directly lysing activated T cells. *In vitro* evidence obtained in murine (55) and human (56, 57) cells showed that T cells during activation upregulate stress molecules acting as ligands for the NK activating receptor NKG2D, thus becoming targets of NK cell-mediated killing. In a major mismatch HCT model, we showed that allogeneic T cells upregulate the NKG2D ligand Rae1γ and perhaps other molecules during GvHD and thus become susceptible to NK cell-mediated killing through a NKG2D-dependent cell lysis (37). Noval Rivas and coworkers obtained very similar results in a minor mismatch model of chronic GvHD induced by adoptive transfer of monoclonal anti-male CD4 T cells into lymphopenic male mice (58). Interestingly, we observed in our system an increased ratio of splenic donor regulatory T cells (Treg) to total donor conventional CD4$^+$ and CD8$^+$ T cells (Tcon) in the presence of NK cells, suggesting a differential susceptibility of Treg and Tcon to NK cell-mediated cell lysis leading to an immune-regulatory environment that eventually contributes to GvHD suppression (37). Direct T cell killing by NK cells can, therefore, be considered as a complimentary mechanism of GvHD suppression, in addition to the aforementioned modulation by APC-killing, which can be particularly important at GvHD tissues sites. Accordingly, we have shown that, after transplantation, NK cells traffic to GvHD target organs following a spatial and temporal distribution very similar to T cells (59) offering them the opportunity to target activated T cell at the effector site. However, in contrast to T cells, NK cells have a more limited persistence, which may in part explain their reduced capacity for GVHD induction. Interestingly, GvHD prevention by T cell killing at tissue sites can be exerted as well by residual tissue resident recipient NK cells eventually persisting after conditioning depending on conditioning intensity as it has been recently shown in a minor mismatch murine model (60). T cell killing by NK cells appears to be dependent on both perforin production (37, 60) and FAS-mediated induction of apoptosis (37, 58, 61). Collectively, these models demonstrated that NK cells can suppress GvHD development through their cytotoxic function either directly, by depleting activated alloreactive T cells, or indirectly, by depleting APC and preventing T cell stimulation (**Figure 1**, left panel).

NK CELL CYTOKINE PRODUCTION AND GvHD INDUCTION

In addition to their cytolytic potential, NK cell can modulate immune responses through cytokine production. Whether this mechanism can participate in GvHD prevention by NK cells is unclear. One of the early studies showed that administration of anti-TGFβ monoclonal antibody significantly limited the NK cell suppressive effect on GvHD (34). However, no evidence was provided that NK cells were indeed the source of TGFβ and administration of exogenous TGFβ failed to prevent GvHD development, indicating that TGFβ contribution to GvHD suppression is only partial and through a mechanism still to be completely uncovered.

Although it is unclear if NK cells production of immune-suppressive cytokines can prevent GvHD, it is established that pro-inflammatory cytokine production by NK cells can contribute to GvHD development. In a xenogeneic model, Xun et al. showed that *in vitro* interleukin-2 (IL-2)-activated human NK cells producing interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were able to induce acute GvHD upon transfer into SCID mice (62, 63). Interestingly, NK cells were found in GvHD target tissues in juxtaposition to damaged cells and produced *in situ* IFN-γ and TNF-α (62). Although the limitations of the xenogeneic model should be taken into account, the results from the aforementioned experiments suggest that, when pre-activated to produce the pro-inflammatory cytokines IFN-γ and TNF-α, NK cells can indeed promote rather than prevent GvHD development. In accordance, while NK depletion by NK1.1 antibodies had no effect on GvHD when employed on steady-state donor splenocytes (25), it significantly prevented GvHD when employed on splenocytes obtained from donor mice previously treated with the toll-like receptor 3 stimulator polyinosinic:polycytidylic acid (poly I:C) (64, 65) by reducing IFN-γ production (65). Further, higher proportions of IFN-γ-producing NK cells after HCT have been shown to be associated in humans with an increased incidence of acute GvHD (66). Collectively, these studies provide evidence for a promoting role of NK cells in GvHD, opposite from the suppressive role exerted by cytolysis, through the production of pro-inflammatory cytokines that may act directly to induce cell damage or indirectly

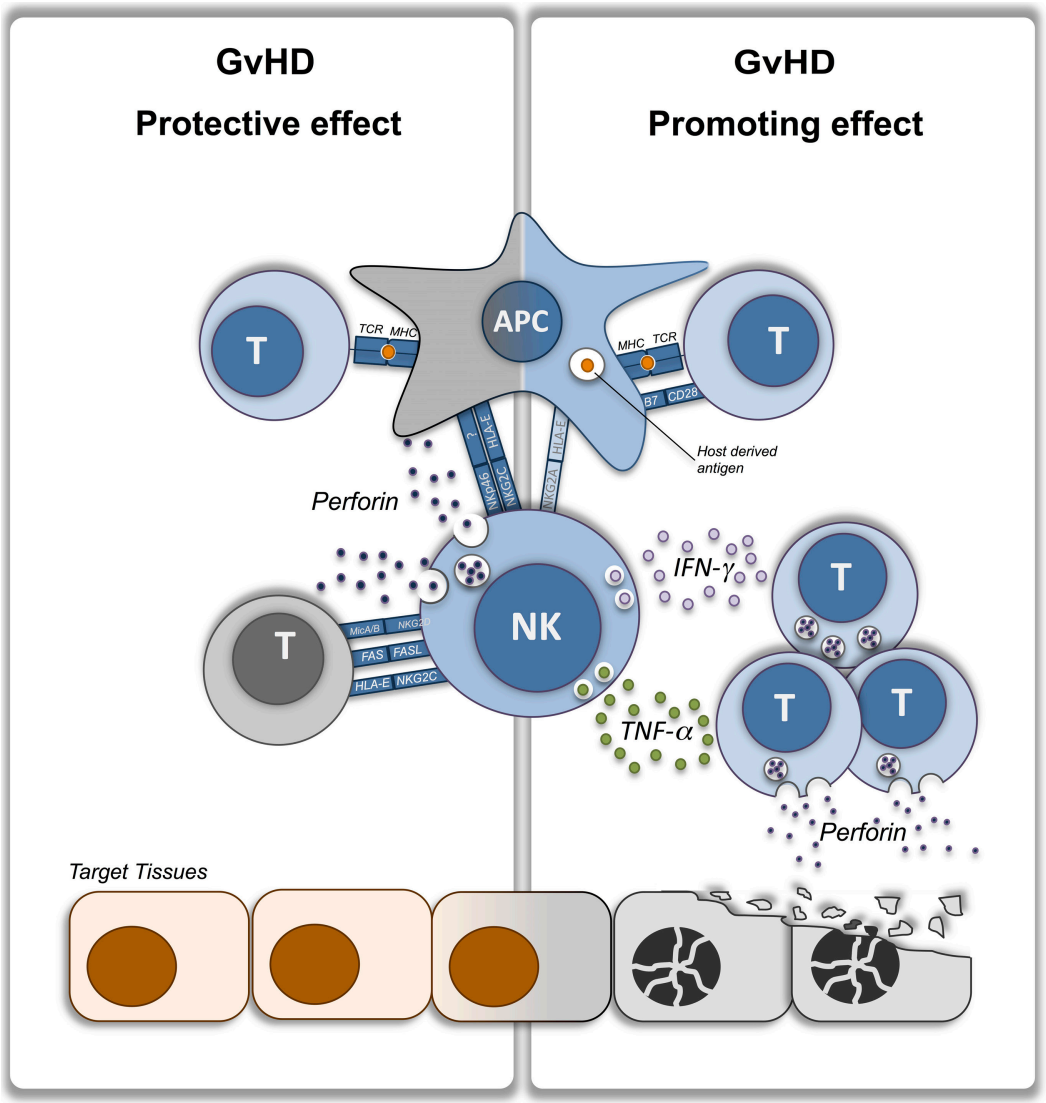


FIGURE 1 | NK cells contribution to graft-versus-host-disease (GvHD) protection or induction. Immunological interactions leading to GvHD protective (left panel) or promoting (right panel) effect of NK cells. Donor immune cells are depicted in blue while host target cells are depicted in orange. NK, natural killer; T, T lymphocyte; APC, antigen-presenting cell; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

by increasing T cell-mediated tissue damage through their well-known property to increase MHC expression (**Figure 1**, right panel). This model can be useful in the interpretation of the otherwise surprising results recently reported by Shah and coworkers (67). Most studies involving adoptive transfer of NK cells into HCT recipients failed to observe GvHD induction after infusion (68–70) (**Table 1**). Similarly, studies assessing the potential of adoptively transferred allogeneic haploidentical NK cells into lymphodepleted patients in non-allogeneic HCT settings did not observe any cases of acute GvHD (71–75) (**Table 1**). Few studies reporting the development of acute GvHD after allogeneic NK cell adoptive transfer (76–78) were unable to establish a causative relationship between the NK cell infusion and GvHD development because of other potential contributing factors including immune-suppression discontinuation (76) or residual T cell contamination of the administered cell product (77). Conversely, the report by Shah and coworkers provide some evidence for an NK cell involvement in GvHD development. The authors reported the development of GvHD in five out of nine recipients of HLA-matched, T-cell-depleted peripheral blood HCT upon adoptive transfer of donor-derived IL-15/4-1BBL-activated NK cells (67). The direct involvement of donor NK cells in GvHD was suggested by their presence in the lymphoid infiltrate found in biopsies of GvHD involved tissues (67). However, despite the fact that grafts contained very low numbers of T cells as a result of T cell depletion by CD34 positive selection, several issues suggested that the NK-cell-promoting role on GvHD could have been mediated by an indirect effect on T cells. First, a higher proportion of patients developing GvHD received grafts from unrelated donors, therefore, were provided

TABLE 1 | Acute graft-versus-host-disease (GvHD) development reported in published natural killer (NK) cells adoptive transfer clinical trials.

Reference	*N*	Age	Disease	Donor type	Conditioning	Time from allo-hematopoietic cell transplantation (HCT)	Cell isolation	NK cells preparation	Cell dose (10^6/kg)	Combined therapy	Acute GvHD
Passweg et al. (68)	5	Adult	AML CML	Haploidentical	Etoposide Cy/TBI/ATG	Post allo-HCT (day +3 to +26)	CD3 depletion CD56 selection	Fresh	6.9–14.1	–	0/5 (0%)
Miller et al. (71)	19	Adult	AML Solid tumors	Haploidentical	Cy/Flu	No allo-HCT	CD3 depletion	Interleukin-2 (IL-2) activated	0.1–20	IL-2	0/43 (0%)
Rubnitz et al. (72)	10	Ped	AML	Haploidentical	Cy/Flu	No allo-HCT	CD3 depletion CD56 selection	Fresh	5–8	IL-2	0/10 (0%)
Yoon et al. (76)	14	Adult	AML MDS ALL	Haploidentical HLA-mismatched	Bu/Flu/ATG	Post allo-HCT (day +43 to +50)	CD34 selection *In vitro* differentiation	*In vitro* differentiated	N/A	–	1/14 (7%) (1 grade II)
Curti et al. (73)	13	Adult	AML	Haploidentical	Cy/Flu	No allo-HCT	CD3 depletion CD56 selection	Fresh	1.11–5	IL-2	0/13 (0%)
Stern et al. (77)	16	Adult Ped	AML ALL Solid tumors	Haploidentical	MAC/ATG or OKT3	Post allo-HCT (day +3 to +40)	CD3 depletion CD56 selection	Fresh Cryopreserved	8–76	–	4/16 (25%) (1 grade II, 2 grade III, 1 grade IV)
Klingemann et al. (74)	13	Adult	HL NHL MM	Haploidentical	None	No allo-HCT	CD3 depletion	IL-2 activated	0.1–20	–	0/13 (0%)
Bachanova et al. (75)	57	Adult Ped	AML	Haploidentical	Cy/Flu	No allo-HCT (n = 53) Post allo-HCT (n = 4)	CD3 depletion ±CD19 depletion ±CD56 selection	IL-2 activated	3.4–15	IL-2 IL2DT	0/57 (0%)
Choi et al. (69)	41	Adult	AML/MDS ALL Lymphoma	Haploidentical	Bu/Flu/ATG	Post allo-HCT (day +14 to +21)	CD3 depletion	*Ex vivo* expanded	20–500	–	9/41 (21%) (2 grade I, 2 grade II, 5 grade III–IV)
Shah et al. (67)	9	Adult Ped	Sarcomas	HLA-matched sibling/unrelated donor	Cy/Flu/Melph	Post allo-HCT (day +7 to +35)	CD3 depletion CD56 selection	IL-15/4-1BBL activated	0.1–1	–	5/9 (55%) (1 grade II, 3 grade IV, 1 non-gradable)
Lee et al. (78)	21	Adult Ped	AML MDS CML	Haploidentical	Bu/Flu	Post allo-HCT (day −8)	CD3 depletion CD56 selection	IL-2 activated	0.02–8.32	IL-2	7/21 (33%) (5 grade II, 2 grade III)
Jaiswal et al. (70)	10	Adult Ped	AML CML	Haploidentical	Treo/Flu/TBI PTCy	Post allo-HCT (day +7)	CD56 selection	Fresh	1.7–17.7	–	0/10 (0%)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, antithymocyte globulin; BM, bone marrow; Bu, busulfan; CML, chronic myeloid leukemia; Cy, cyclophosphamide; Flu, fludarabine; HL, Hodgkin's lymphoma; MDS, myelodysplastic syndrome; Melph, melphalan; NHL, non-Hodgkin lymphoma; MAC, myeloablative conditioning; MM, multiple myeloma; PTCy, posttransplant cyclophosphamide; TBI, total body irradiation; Treo, treosulfan.

with a higher alloreactive potential, compared to patients not developing GvHD (67). Second, patients developing GvHD displayed more rapid T-cell engraftment, as revealed by day 14 and day 28 CD3-chimerism, compared with patients not developing GvHD (67). Moreover, it should be noted that patients were free of T-cell directed immune-suppressive treatment at the time of adoptive transfer. Importantly, the timing of the administration of the NK cells could have been another factor pushing the balance toward GvHD induction. Patients from the aforementioned report (67) received the pre-activated NK cells around the time of engraftment. Murine studies have demonstrated the importance of the timing of NK cell administration on GvHD prevention, showing no benefit of delayed treatment (37) and even a potential for GvHD exacerbation when NK cells were administered at later time points (34), although in these latter experiments IL-2 was administered at the same time as the NK cells and could have contributed to the phenomenon. This opposing effect can be related with the production of IFN-γ that has been shown to inhibit GVHD when provided early after HCT and to exacerbate GVHD when acting at a later time (79). Considering all of these factors, it can be speculated that the administration of highly pre-activated NK cells can enhance clinically undetectable T-cell alloreactivity through the production of pro-inflammatory cytokines (**Figure 1**, right panel) and that this functional aspect can, therefore, prevail on their GvHD-protective cytotoxic activity (**Figure 1**, left panel), thus promoting GvHD development.

GvHD MODULATION OF NK CELLS

While NK cells may positively or negatively participate in GvHD development, the GvHD process can in turn affect NK cell biology. Pattengale and coworkers were the first to demonstrate in murine models that acute but not chronic GvHD induce a marked decrease in NK cell activity associated with an impaired production of IFN-γ (80). NK cell reconstitution appears to be significantly delayed by acute GvHD in mice (81) and by acute and chronic GvHD in humans (42, 82–85). Recent evidence from a murine model of GvHD suggest that activated T cells could limit NK cell access to IL-15 through direct competition for this cytokine necessary for NK cell development and homeostasis, administration of exogenous IL-15 being able to restore NK cell reconstitution (81). In addition to its quantitative effect, GvHD induces qualitative defects on NK cells ultimately leading to impaired function. Bunting and coworkers recently showed in mice that, during GvHD, donor NK cells display a hyperactivated phenotype associated with increased signs of apoptosis and autophagy (81). Importantly, they showed that GVHD-induced alterations in NK cells resulted in defective *in vivo* cytotoxicity resulting in a reduction of graft-versus-leukemia effect and an impaired control of cytomegalovirus infection (81). This dysfunctional status induced by GvHD is reminiscent of the NK cell exhaustion phenomenon we observed upon chronic proliferation, characterized by an impaired transcriptional machinery as revealed by the downregulation of the Tbox transcription factors Eomesodermin and Tbet (86). Accordingly, we reported in humans that exhaustion is increased in NK cells after HCT and is further exacerbated in NK cells from patients with acute GvHD (87).

CONCLUDING REMARKS

Despite major efforts undertaken during many years to better understand NK cells biology in the context of HCT, the role of NK cells during GvHD remained elusive because of conflicting evidence coming from different experimental approaches. NK cells are capable of both effector and regulatory functions. This pleiotropic nature of NK cells is likely responsible for the variable and even conflicting roles that NK cells can play during GvHD. We hope our model (**Figure 1**) will help interpret this apparent contradiction. Importantly, clarifying the impact of NK cell activation status on their GvHD induction potential will hopefully contribute to the optimization of cell manufacturing procedures to maximize allogeneic NK cell antitumor potential while preventing GvHD induction.

AUTHOR CONTRIBUTIONS

FS wrote the manuscript and designed the figure. MA critically revised the work for important intellectual content and edited the manuscript. RN edited the manuscript and provided overall guidance.

FUNDING

This work was supported by the Program Project Grant CA49605 from the National Institute of Health. FS is supported by the Geneva University Hospitals, the Swiss Cancer League, the Dubois-Ferrière-Dinu-Lipatti Foundation, and the Fondation Genevoise de bienfaisance Valeria Rossi di Montelera. MA is supported by the American Society for Blood and Marrow Transplantation and the American Association for Cancer Research.

REFERENCES

1. Murphy WJ, Kumar V, Bennett M. Rejection of bone marrow allografts by mice with severe combined immune deficiency (SCID). Evidence that natural killer cells can mediate the specificity of marrow graft rejection. *J Exp Med* (1987) 165(4):1212–7. doi:10.1084/jem.165.4.1212
2. Murphy WJ, Bennett M, Kumar V, Longo DL. Donor-type activated natural killer cells promote marrow engraftment and B cell development during allogeneic bone marrow transplantation. *J Immunol* (1992) 148(9):2953–60.
3. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* (2002) 295(5562):2097–100. doi:10.1126/science.1068440
4. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* (2007) 110(1):433–40. doi:10.1182/blood-2006-07-038687
5. Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood* (2000) 95(9):2754–9.
6. Lopez C, Sorell M, Kirkpatrick D, O'Reilly R, Ching C; The Bone Marrow Transplant Unit. Association between pre-transplant natural kill and

graft-versus-host disease after stem-cell transplantation. *Lancet* (1979) 314(8152):1103–7. doi:10.1016/S0140-6736(79)92506-6
7. Livnat S, Seigneuret M, Storb R, Prentice RL. Analysis of cytotoxic effector cell function in patients with leukemia or aplastic anemia before and after marrow transplantation. *J Immunol* (1980) 124(1):481–90.
8. Dokhelar M-C, Wiels J, Lipinski M, Tetaud C, Devergie A, Gluckman E, et al. Natural killer cell activity in human bone marrow recipients: early reappearance of peripheral natural killer activity in graft-versus-host disease. *Transplantation* (1981) 31(1):61–5. doi:10.1097/00007890-198101000-00014
9. Roy C, Ghayur T, Kongshavn PA, Lapp WS. Natural killer activity by spleen, lymph node, and thymus cells during the graft-versus-host reaction1. *Transplantation* (1982) 34(3):144–6. doi:10.1097/00007890-198209000-00006
10. Borland A, Mowat AMI, Parrott DM. Augmentation of intestinal and peripheral natural killer cell activity during the graft-versus-host reaction in mice. *Transplantation* (1983) 36(5):513–9. doi:10.1097/00007890-198311000-00009
11. Ghayur T, Seemayer TA, Lapp WS. Kinetics of natural killer cellcytotoxicity reaction: relationship between natural killer cell activity, T and B cell activity, and development of histopathological alterations. *Transplantation* (1987) 44(2):254–60. doi:10.1097/00007890-198708000-00016
12. Ghayur T, Seemayer TA, Lapp WS. Association between the degree of thymic dysplasia and the kinetics of thymic NK cell activity during the graft-versus-host reaction. *Clin Immunol Immunopathol* (1988) 48(1):19–30. doi:10.1016/0090-1229(88)90153-5
13. Guillen F, Ferrara J, Hancock W, Messadi D, Fonferko E, Burakoff S, et al. Acute cutaneous graft-versus-host disease to minor histocompatibility antigens in a murine model. Evidence that large granular lymphocytes are effector cells in the immune response. *Lab Invest* (1986) 55(1):35–42.
14. Ferrara JL, Guillen FJ, van Duken PJ, Marion A, Murphy GF, Burakoff SJ. Evidence that large granular lymphocytes of donor origin mediate acute graft-versus-host disease. *Transplantation* (1989) 47(1):50–4. doi:10.1097/00007890-198901000-00012
15. Acevedo A, Aramburu J, López J, Fernández-Herrera J, Fern JM, López-Botet M. Identification of natural killer (NK) cells in lesions of human cutaneous graft-versus-host disease: expression of a novel NK-associated surface antigen (Kp43) in mononuclear infiltrates. *J Invest Dermatol* (1991) 97(4):659–66. doi:10.1111/1523-1747.ep12483724
16. Horn TD, Haskell J. The lymphocytic infiltrate in acute cutaneous allogeneic graft-versus-host reactions lacks evidence for phenotypic restriction in donor-derived cells. *J Cutan Pathol* (1998) 25(4):210–4. doi:10.1111/j.1600-0560.1998.tb01721.x
17. Takata M, Imai T, Hirone T. Immunoelectron microscopy of acute graft versus host disease of the skin after allogeneic bone marrow transplantation. *J Clin Pathol* (1993) 46(9):801–5. doi:10.1136/jcp.46.9.801
18. Dilly S, Sloane J. An immunohistological study of human hepatic graft-versus-host disease. *Clin Exp Immunol* (1985) 62(3):545.
19. Tanaka M, Umihara J, Shimmoto K, Cui S, Sata H, Ishikawa T, et al. The pathogenesis of graft-versus-host reaction in the intrahepatic bile duct. *Pathol Int* (1989) 39(10):648–55. doi:10.1111/j.1440-1827.1989.tb02412.x
20. Roy J, PLATT JL, Weisdorf DJ. The immunopathology of upper gastrointestinal acute graft-versus-host disease lymphoid cells and endothelial adhesionmolecules. *Transplantation* (1993) 55(3):572–7. doi:10.1097/00007890-199303000-00022
21. Charley M, Mikhael A, Bennett M, Gilliam J, Sontheimer R. Prevention of lethal, minor-determinate graft-host disease in mice by the *in vivo* administration of anti-asialo GM1. *J Immunol* (1983) 131(5):2101–3.
22. Varkila K. Depletion of asialo-GM1 + cells from the F1 recipient mice prior to irradiation and transfusion of parental spleen cells prevents mortality to acute graft-versus-host disease and induction of anti-host specific cytotoxic T cells. *Clin Exp Immunol* (1987) 69(3):652.
23. Ghayur T, Seemayer TA, Lapp WS. Prevention of murine graft-versus-host disease by inducing and eliminating ASGM1 + cells of donor origin. *Transplantation* (1988) 45(3):586–90. doi:10.1097/00007890-198803000-00017
24. Varkila K, Hurme M. Natural killer (NK) cells and graft-versus-host disease (GVHD): no correlation between the NK cell levels and GVHD in the murine P—F1 model. *Immunology* (1985) 54(1):121.
25. Blazar BR, Soderling CC, Koo GC, Vallera DA. Absence of a facilitory role for NK 1.1-positive donor cells in engraftment across a major histocompatibility barrier in mice1, 2. *Transplantation* (1988) 45(5):876–82. doi:10.1097/00007890-198805000-00007
26. Johnson BD, Truitt RL. A decrease in graft-vs.-host disease without loss of graft-vs.-leukemia reactivity after MHC-matched bone marrow transplantation by selective depletion of donor NK cells *in vivo*. *Transplantation* (1992) 54(1):104–12. doi:10.1097/00007890-199207000-00019
27. Xenocostas A, Ghayur T, Setrakian J, Lapp W, Osmond D. A donor-derived asialo-GM1 + cell induces depression of B-cell genesis during systemic graft-versus-host disease. *Blood* (1994) 84(11):3965–73.
28. Charley MR, Mikhael A, Hoot G, Hackett J, Bennett M. Studies addressing the mechanism of anti-asialo GM 1 prevention of graft-versus-host disease due to minor histocompatibility antigenic differences. *J Invest Dermatol* (1985) 85:121s–3s. doi:10.1111/1523-1747.ep12275630
29. Charley MR, Mikhael A, Hackett J, Kumar V, Bennett M. Mechanism of anti-asialo GM1 prevention of graft-vs-host disease: identification of allo-antigen activated T cells. *J Invest Dermatol* (1988) 91(3):202–6. doi:10.1111/1523-1747.ep12464858
30. Zeng D, Lewis D, Dejbakhsh-Jones S, Lan F, García-Ojeda M, Sibley R, et al. Bone marrow NK1. 1− and NK1. 1 + T cells reciprocally regulate acute graft versus host disease. *J Exp Med* (1999) 189(7):1073–81. doi:10.1084/jem.189.7.1073
31. Ghayur T, Seemayer TA, Kongshavn PA, Gartner JG, Lapp WS. Graft-versus-host reactions in the beige mouse: an investigation of the role of host and donor natural killer cells in the pathogenesis of graft-versus-host disease. *Transplantation* (1987) 44(2):261–6. doi:10.1097/00007890-198708000-00017
32. Carlson GA, Marshall ST, Truesdale AT. Adaptive immune defects and delayed rejection of allogeneic tumor cells in beige mice. *Cell Immunol* (1984) 87(2):348–56. doi:10.1016/0008-8749(84)90004-2
33. Halle-Pannenko O, Bruley-Rosset M. Decreased graft-versus-host reaction and T cell cytolytic potential of beige mice. *Transplantation* (1985) 39(1):85–7. doi:10.1097/00007890-198501000-00013
34. Asai O, Longo DL, Tian Z-G, Hornung RL, Taub DD, Ruscetti FW, et al. Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation. *J Clin Invest* (1998) 101(9):1835. doi:10.1172/JCI1268
35. Lundqvist A, McCoy JP, Samsel L, Childs R. Reduction of GVHD and enhanced antitumor effects after adoptive infusion of alloreactive Ly49-mismatched NK cells from MHC-matched donors. *Blood* (2007) 109(8):3603–6. doi:10.1182/blood-2006-05-024315
36. Chen G, Wu D, Wang Y, Cen J, Feng Y, Sun A, et al. Expanded donor natural killer cell and IL-2, IL-15 treatment efficacy in allogeneic hematopoietic stem cell transplantation. *Eur J Haematol* (2008) 81(3):226–35. doi:10.1111/j.1600-0609.2008.01108.x
37. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood* (2010) 115(21):4293–301. doi:10.1182/blood-2009-05-222190
38. Meinhardt K, Kroeger I, Bauer R, Ganss F, Ovsiy I, Rothamer J, et al. Identification and characterization of the specific murine NK cell subset supporting graft-versus-leukemia- and reducing graft-versus-host-effects. *Oncoimmunology* (2015) 4(1):e981483. doi:10.4161/2162402X.2014.981483
39. Huber CM, Doisne JM, Colucci F. IL-12/15/18-preactivated NK cells suppress GvHD in a mouse model of mismatched hematopoietic cell transplantation. *Eur J Immunol* (2015) 45(6):1727–35. doi:10.1002/eji.201445200
40. Soiffer RJ, Gonin R, Murray C, Robertson MJ, Cochran K, Chartier S, et al. Prediction of graft-versus-host disease by phenotypic analysis of early immune reconstitution after CD6-depleted allogeneic bone marrow transplantation. *Blood* (1993) 82(7):2216–23.
41. Yamasaki S, Henzan H, Ohno Y, Yamanaka T, Iino T, Itou Y, et al. Influence of transplanted dose of CD56 + cells on development of graft-versus-host disease in patients receiving G-CSF-mobilized peripheral blood progenitor cells from HLA-identical sibling donors. *Bone Marrow Transplant* (2003) 32(5):505–10. doi:10.1038/sj.bmt.1704165
42. Abrahamsen IW, Somme S, Heldal D, Egeland T, Kvale D, Tjonnfjord G. Immune reconstitution after allogeneic stem cell transplantation: the impact of stem cell source and graft-versus-host disease. *Haematologica* (2005) 90(1):86–93.

43. Kim DH, Sohn SK, Lee NY, Baek JH, Kim JG, Won DI, et al. Transplantation with higher dose of natural killer cells associated with better outcomes in terms of non-relapse mortality and infectious events after allogeneic peripheral blood stem cell transplantation from HLA-matched sibling donors. *Eur J Haematol* (2005) 75(4):299–308. doi:10.1111/j.1600-0609.2005.00514.x
44. Larghero J, Rocha V, Porcher R, Filion A, Ternaux B, Lacassagne MN, et al. Association of bone marrow natural killer cell dose with neutrophil recovery and chronic graft-versus-host disease after HLA identical sibling bone marrow transplants. *Br J Haematol* (2007) 138(1):101–9. doi:10.1111/j.1365-2141.2007.06623.x
45. Savani BN, Mielke S, Adams S, Uribe M, Rezvani K, Yong AS, et al. Rapid natural killer cell recovery determines outcome after T-cell-depleted HLA-identical stem cell transplantation in patients with myeloid leukemias but not with acute lymphoblastic leukemia. *Leukemia* (2007) 21(10):2145–52. doi:10.1038/sj.leu.2404892
46. Tanaka M, Kobayashi S, Numata A, Tachibana T, Takasaki H, Maruta A, et al. The impact of the dose of natural killer cells in the graft on severe acute graft-versus-host disease after unrelated bone marrow transplantation. *Leuk Res* (2012) 36(6):699–703. doi:10.1016/j.leukres.2011.11.009
47. Kim SY, Lee H, Han M-S, Shim H, Eom H-S, Park B, et al. Post-transplantation natural killer cell count: a predictor of acute graft-versus-host disease and survival outcomes after allogeneic hematopoietic stem cell transplantation. *Clin Lymphoma Myeloma Leuk* (2016) 16(9):527.–535. doi:10.1016/j.clml.2016.06.013
48. Ludajic K, Balavarca Y, Bickeböller H, Rosenmayr A, Fae I, Fischer G, et al. KIR genes and KIR ligands affect occurrence of acute GVHD after unrelated, 12/12 HLA matched, hematopoietic stem cell transplantation. *Bone Marrow Transplant* (2009) 44(2):97–103. doi:10.1038/bmt.2008.432
49. Clausen J, Kircher B, Auberger J, Schumacher P, Ulmer H, Hetzenauer G, et al. The role of missing killer cell immunoglobulin-like receptor ligands in T cell replete peripheral blood stem cell transplantation from HLA-identical siblings. *Biol Blood Marrow Transplant* (2010) 16(2):273–80. doi:10.1016/j.bbmt.2009.10.021
50. Sivori S, Carlomagno S, Falco M, Romeo E, Moretta L, Moretta A. Natural killer cells expressing the KIR2DS1-activating receptor efficiently kill T-cell blasts and dendritic cells: implications in haploidentical HSCT. *Blood* (2011) 117(16):4284–92. doi:10.1182/blood-2010-10-316125
51. Cao D, Hu L, Wang Y, Wang L, Zheng W, Ma W. Suppression of graft-versus-host disease after adoptive infusion of alloreactive NK cells induced by silencing Ly49C gene in mice. *Transpl Immunol* (2009) 20(4):243–8. doi:10.1016/j.trim.2008.10.011
52. Kordelas L, Steckel N-K, Horn PA, Beelen DW, Rebmann V. The activating NKG2C receptor is significantly reduced in NK cells after allogeneic stem cell transplantation in patients with severe graft-versus-host disease. *Int J Mol Sci* (2016) 17(11):1797. doi:10.3390/ijms17111797
53. Ghadially H, Horani A, Glasner A, Elboim M, Gazit R, Shoseyov D, et al. NKp46 regulates allergic responses. *Eur J Immunol* (2013) 43(11):3006–16. doi:10.1002/eji.201343388
54. Ghadially H, Ohana M, Elboim M, Gazit R, Gur C, Nagler A, et al. NK cell receptor NKp46 regulates graft-versus-host disease. *Cell Rep* (2014) 7(6):1809–14. doi:10.1016/j.celrep.2014.05.011
55. Rabinovich BA, Li J, Shannon J, Hurren R, Chalupny J, Cosman D, et al. Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. *J Immunol* (2003) 170(7):3572–6. doi:10.4049/jimmunol.170.7.3572
56. Cerboni C, Zingoni A, Cippitelli M, Piccoli M, Frati L, Santoni A. Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK-cell lysis. *Blood* (2007) 110(2):606–15. doi:10.1182/blood-2006-10-052720
57. Ardolino M, Zingoni A, Cerboni C, Cecere F, Soriani A, Iannitto ML, et al. DNAM-1 ligand expression on Ag-stimulated T lymphocytes is mediated by ROS-dependent activation of DNA-damage response: relevance for NK–T cell interaction. *Blood* (2011) 117(18):4778–86. doi:10.1182/blood-2010-08-300954
58. Noval Rivas M, Hazzan M, Weatherly K, Gaudray F, Salmon I, Braun MY. NK cell regulation of CD4 T cell-mediated graft-versus-host disease. *J Immunol* (2010) 184(12):6790–8. doi:10.4049/jimmunol.0902598
59. Olson JA, Zeiser R, Beilhack A, Goldman JJ, Negrin RS. Tissue-specific homing and expansion of donor NK cells in allogeneic bone marrow transplantation. *J Immunol* (2009) 183(5):3219–28. doi:10.4049/jimmunol.0804268
60. Nalle SC, Kwak HA, Edelblum KL, Joseph NE, Singh G, Khramtsova GF, et al. Recipient NK cell inactivation and intestinal barrier loss are required for MHC-matched graft-versus-host disease. *Science translational medicine* (2014) 6(243):243ra87. doi:10.1126/scitranslmed.3008941
61. Alvarez M, Bouchlaka MN, Sckisel GD, Sungur CM, Chen M, Murphy WJ. Increased antitumor effects using IL-2 with anti-TGF-β reveals competition between mouse NK and CD8 T cells. *J Immunol* (2014) 193(4):1709–16. doi:10.4049/jimmunol.1400034
62. Xun C, Brown SA, Jennings CD, Henslee-Downey PJ, Thompson JS. Acute graft-versus-host-like disease induced by transplantation of human activated natural killer cells into SCID mice. *Transplantation* (1993) 56(2):409–16. doi:10.1097/00007890-199308000-00031
63. Xun CQ, Thompson JS, Jennings CD, Brown SA. The effect of human IL-2-activated natural killer and T cells on graft-versus-host disease and graft-versus-leukemia in SCID mice bearing human leukemic cells. *Transplantation* (1995) 60(8):821–7. doi:10.1097/00007890-199510000-00011
64. MacDonald GC, Gartner JG. Prevention of acute lethal graft-versus-host disease in F1 hybrid mice by pretreatment of the graft with anti-NK-1.1 and complement. *Transplantation* (1992) 54(1):147–51. doi:10.1097/00007890-199207000-00026
65. Ellison CA, HayGlass KT, Fischer JM, Rector ES, MacDonald GC, Gartner JG. Depletion of natural killer cells from the graft reduces interferon-γ levels and lipopolysaccharide-induced tumor necrosis factor-α release in F1 hybrid mice with acute graft-versus-host disease1. *Transplantation* (1998) 66(3):284–94. doi:10.1097/00007890-199808150-00002
66. Cooley S, McCullar V, Wangen R, Bergemann TL, Spellman S, Weisdorf DJ, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. *Blood* (2005) 106(13):4370–6. doi:10.1182/blood-2005-04-1644
67. Shah NN, Baird K, Delbrook CP, Fleisher TA, Kohler ME, Rampertaap S, et al. Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell–depleted stem cell transplantation. *Blood* (2015) 125(5):784–92. doi:10.1182/blood-2014-07-592881
68. Passweg JR, Tichelli A, Meyer-Monard S, Heim D, Stern M, Kühne T, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. *Leukemia* (2004) 18(11):1835–8. doi:10.1038/sj.leu.2403524
69. Choi I, Yoon SR, Park S-Y, Kim H, Jung S-J, Jang YJ, et al. Donor-derived natural killer cells infused after human leukocyte antigen–haploidentical hematopoietic cell transplantation: a dose-escalation study. *Biol Blood Marrow Transplant* (2014) 20(5):696–704. doi:10.1016/j.bbmt.2014.01.031
70. Jaiswal SR, Zaman S, Nedunchezhian M, Chakrabarti A, Bhakuni P, Ahmed M, et al. CD56-enriched donor cell infusion after post-transplantation cyclophosphamide for haploidentical transplantation of advanced myeloid malignancies is associated with prompt reconstitution of mature natural killer cells and regulatory T cells with reduced incidence of acute graft versus host disease: a pilot study. *Cytotherapy* (2017) 19(4):531–42. doi:10.1016/j.jcyt.2016.12.006
71. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* (2005) 105(8):3051–7. doi:10.1182/blood-2004-07-2974
72. Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *J Clin Oncol* (2010) 28(6):955–9. doi:10.1200/jco.2009.24.4590
73. Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. *Blood* (2011) 118(12):3273–9. doi:10.1182/blood-2011-01-329508
74. Klingemann H, Grodman C, Cutler E, Duque M, Kadidlo D, Klein AK, et al. Autologous stem cell transplant recipients tolerate haploidentical related-donor natural killer cell–enriched infusions. *Transfusion* (2013) 53(2):412–8. doi:10.1111/j.1537-2995.2012.03764.x
75. Bachanova V, Cooley S, Defor TE, Verneris MR, Zhang B, McKenna DH, et al. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood* (2014) 123(25):3855–63. doi:10.1182/blood-2013-10-532531

76. Yoon S, Lee Y, Yang S, Ahn K, Lee J-H, Lee J-H, et al. Generation of donor natural killer cells from CD34+ progenitor cells and subsequent infusion after HLA-mismatched allogeneic hematopoietic cell transplantation: a feasibility study. *Bone Marrow Transplant* (2010) 45(6):1038–46. doi:10.1038/bmt.2009.304
77. Stern M, Passweg JR, Meyer-Monard S, Esser R, Tonn T, Soerensen J, et al. Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers. *Bone Marrow Transplant* (2013) 48(3):433–8. doi:10.1038/bmt.2012.162
78. Lee DA, Denman CJ, Rondon G, Woodworth G, Chen J, Fisher T, et al. Haploidentical natural killer cells infused before allogeneic stem cell transplantation for myeloid malignancies: a phase I trial. *Biol Blood Marrow Transplant* (2016) 22(7):1290–8. doi:10.1016/j.bbmt.2016.04.009
79. Yang Y-G, Dey BR, Sergio JJ, Pearson DA, Sykes M. Donor-derived interferon gamma is required for inhibition of acute graft-versus-host disease by interleukin 12. *J Clin Invest* (1998) 102(12):2126. doi:10.1172/JCI4992
80. Pattengale PK, Ramstedt U, Gidlund M, Örn A, Axberg I, Wigzell H. Natural killer activity in (C57BL/6 × DBA/2) F1 hybrids undergoing acute and chronic graft-vs.-host reaction. *Eur J Immunol* (1983) 13(11):912–9. doi:10.1002/eji.1830131110
81. Bunting MD, Varelias A, Souza-Fonseca-Guimaraes F, Schuster IS, Lineburg KE, Kuns RD, et al. GVHD prevents NK-cell-dependent leukemia and virus-specific innate immunity. *Blood* (2017) 129(5):630–42. doi:10.1182/blood-2016-08-734020
82. Kheav VD, Busson M, Scieux C, de Latour RP, Maki G, Haas P, et al. Favorable impact of natural killer cell reconstitution on chronic graft-versus-host disease and cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation. *Haematologica* (2014) 99(12):1860–7. doi:10.3324/haematol.2014.108407
83. Podgorny PJ, Liu Y, Dharmani-Khan P, Pratt LM, Jamani K, Luider J, et al. Immune cell subset counts associated with graft-versus-host disease. *Biol Blood Marrow Transplant* (2014) 20(4):450–62. doi:10.1016/j.bbmt.2014.01.002
84. Ullrich E, Salzmann-Manrique E, Bakhtiar S, Bremm M, Gerstner S, Herrmann E, et al. Relation between acute GVHD and NK cell subset reconstitution following allogeneic stem cell transplantation. *Front Immunol* (2016) 7:595. doi:10.3389/fimmu.2016.00595
85. Huenecke S, Cappel C, Esser R, Pfirrmann V, Salzmann-Manrique E, Betz S, et al. Development of three different NK cell subpopulations during immune reconstitution after pediatric allogeneic hematopoietic stem cell transplantation: prognostic markers in GvHD and viral infections. *Front Immunol* (2017) 8:109. doi:10.3389/fimmu.2017.00109
86. Gill S, Vasey AE, De Souza A, Baker J, Smith AT, Kohrt HE, et al. Rapid development of exhaustion and down-regulation of eomesodermin limit the antitumor activity of adoptively transferred murine natural killer cells. *Blood* (2012) 119(24):5758–68. doi:10.1182/blood-2012-03-415364
87. Simonetta F, Pradier A, Bosshard C, Masouridi-Levrat S, Chalandon Y, Roosnek E. NK cell functional impairment after allogeneic hematopoietic stem cell transplantation is associated with reduced levels of T-bet and eomesodermin. *J Immunol* (2015) 195(10):4712–20. doi:10.4049/jimmunol.1501522

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 24 April 2017
doi: 10.3389/fimmu.2017.00467

Uterine Natural Killer Cells: Functional Distinctions and Influence on Pregnancy in Humans and Mice

Louise M. Gaynor[1,2] *and Francesco Colucci*[1,2]*

[1] Centre for Trophoblast Research, University of Cambridge, Cambridge, UK, [2] Department of Obstetrics and Gynaecology, National Institute for Health Research Cambridge Biomedical Research Centre, University of Cambridge School of Clinical Medicine, Cambridge, UK

Our understanding of development and function of natural killer (NK) cells has progressed significantly in recent years. However, exactly how uterine NK (uNK) cells develop and function is still unclear. To help investigators that are beginning to study tissue NK cells, we summarize in this review our current knowledge of the development and function of uNK cells, and what is yet to be elucidated. We compare and contrast the biology of human and mouse uNK cells in the broader context of the biology of innate lymphoid cells and with reference to peripheral NK cells. We also review how uNK cells may regulate trophoblast invasion and uterine spiral arterial remodeling in human and murine pregnancy.

Keywords: uterine natural killer cells, uterine innate lymphoid cells, placenta, pregnancy, arterial remodeling, trophoblast

OPEN ACCESS

Edited by:
Sandra Laurence Lopez-Verges, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama

Reviewed by:
Jacques Zimmer, Centre de Recherche Public de la Santé, Luxembourg
Veronika Sexl, University of Veterinary Medicine, Austria

****Correspondence:***
Francesco Colucci
fc287@medschl.cam.ac.uk

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *30 January 2017*
Accepted: *05 April 2017*
Published: *24 April 2017*

Citation:
Gaynor LM and Colucci F (2017) Uterine Natural Killer Cells: Functional Distinctions and Influence on Pregnancy in Humans and Mice. Front. Immunol. 8:467. doi: 10.3389/fimmu.2017.00467

INTRODUCTION

CD56^{superbright} uterine natural killer (uNK) cells are present in human endometrium prior to the initiation of pregnancy, and markedly expand and become progressively more granulated during the progesterone-dominated secretory phase after ovulation and throughout the first trimester (1–3). uNK cells within the decidua have a distinct phenotype compared to peripheral blood NK (pbNK) cells and share features of both CD56^{bright} and CD56^{dim} pbNK subsets (**Table 1**). Similarly to CD56^{bright} pbNK cells, uNK preferentially produce cytokines and are poorly cytotoxic, despite their abundant intracellular granules containing granzymes, granulysin, and perforin (4–9). Killer-cell immunoglobulin-like receptors (KIR) and natural killer group 2 (NKG2)A/C/E receptors, which recognize trophoblast MHC class I human leukocyte antigen (HLA)-C and HLA-E, respectively, are expressed at higher levels among uNK than their pbNK cell counterparts, and are skewed toward recognition of their respective ligands (8, 10, 11). All human decidual uNK cells are CD49a^{+}, also known as very late antigen-1 (VLA-1) or integrin $\alpha_1\beta_1$, and express CD69 (2, 12). uNK cells peak in frequency during the first trimester, before becoming progressively less granular and beginning to diminish in numbers midway through gestation, so that only small numbers are present at term (13, 14).

In comparison to humans, two functionally disparate populations of uNK cells have been identified in mice, which are distinguished in most studies to date by their reactivity to *Dolichos biflorus agglutinin* (DBA). Gene expression studies show that DBA^{+} uNK cells predominantly express transcripts for angiogenic factors, whereas interferon (IFN)-γ transcripts dominate in the DBA^{-} subset (27). Murine uNK do not begin to mature into large, granulated lymphocytes

Abbreviations: cNK, conventional natural killer cells; NKR, natural killer receptor; pbNK, peripheral blood natural killer cells; pNK, peripheral natural killer cells; trNK, tissue-resident natural killer cells; uNK, uterine natural killer cells.

TABLE 1 | Characterization of human natural killer (NK) cells in peripheral blood and decidua.

Characteristic	Peripheral blood NK (pbNK) cells		Uterine NK (uNK) cells
	$CD56^{bright}$ $CD16^{-}$	$CD56^{dim}$ $CD16^{+}$	$CD56^{superbright}$ $CD16^{-}$
% of total	~ 5–30% circulating lymphocytes (15)		≥70% leukocytes in first trimester (2)
% of total NK cells	10% (16)	90% (16)	80% (2)
CD94	$CD94^{bright}$ (17)	50% $CD94^{dim}$ (18)	$CD94^{bright}$ (18)
Natural killer group 2 (NKG2)A/C/E	+ (8)	+ (8)	++ (8, 11)
NKG2D	+ (19)	+ (19)	++ (20)
Killer-cell immunoglobulin-like receptors (KIR)	– (21)	+ (21)	++ (8, 22)
NKp46	+ (23)	+ (23)	+ (4)
CD9	– (8)	– (8)	+ (8)
CD49a	– (12)	– (12)	+ (12)
CD57	– (24)	60%+ (24)	– (2)
CD69	– (25)	– (25)	40%+ (2)
Cytokine production	+++ (26)	+ (26)	+++ (4, 5)
Cytotoxicity	– (21, 24)	+++ (21, 24)	– (6)

until blastocyst implantation, and they acquire reactivity to DBA after g.d. 5 alongside their increase in granularity (28, 29). As in humans, murine uNK cells are poorly cytotoxic despite containing granules encasing perforin and granzymes (30–32). At the mesometrial pole of the implantation site and adjacent to the decidua basalis, a lymphocyte-rich accretion of leukocytes composed largely of uNK cells, macrophages, and dendritic cells develops (29, 33, 34). This mesometrial lymphoid aggregate of pregnancy (MLAp) is a feature of pregnancy unique to rodents, which is established by g.d. 8. Mature uNK cells are most abundant throughout the decidua basalis and MLAp approximately halfway through gestation (**Figure 1**) (28, 29, 35). uNK undergoing apoptosis begin to appear from mid-gestation onwards and are highly prevalent by g.d. 12 (28, 35). Expression of lectin-like Ly49 receptors, which recognize MHC class I, is higher among uNK than peripheral (pNK) cells and, as in humans, some receptors are mildly skewed toward recognition of trophoblast MHC ligands (36, 37). uNK in mice also express killer-cell lectin-like receptor G1 (KLRG1) more highly than their pNK cell counterparts, indicating a more mature phenotype (36, 38). The features of murine uNK cells are summarized in **Table 2**.

The relatively recent designation of CD49a as a marker of tissue residency and its inclusion in the cytometric analysis of uterine lymphocytes alongside common NK cell markers such as CD49b (DX5) has enabled the redefinition of murine uNK subsets (33, 48). uNK cells in mice can now be classified as $CD49a^{+}$ $DX5^{+/-}$ uterine tissue-resident NK (trNK) cells and $CD49a^{-}$ $DX5^{+}$ uterine conventional NK (cNK) cell populations (33, 48, 58, 59), which will be described in greater depth later in this review. DBA reactivity is strongest on uterine $CD49a^{+}$ trNK, and is weak on $DX5^{+}$ uterine cNK (40, 58). As in DBA^{+} uNK, decidual $CD49a^{+}$ $DX5^{+/-}$ trNK cells produce less total IFN-γ at mid-gestation than $CD49a^{-}$ $DX5^{+}$ cNK cells, which further supports the correlation between CD49a and DBA reactivity (27, 58, 59). Although the correlation between CD49a and DBA co-expression is not sufficiently clear-cut to consider DBA as a specific marker of uterine trNK cells, it does enable some reconsideration of historical histological studies.

Despite numerous anatomical and physiological differences between murine and human pregnancies, the functions and regulation of uNK cells are reasonably comparable between these species. In both species, uNK contribute to fundamental physiological processes of pregnancy within the decidua, but there are key differences in how these effects are mediated (**Figure 2**). Human uNK assist in the initial stages of decidua-associated vascular remodeling and control the depth of invasion of extravillous trophoblast (EVT), which are responsible for the majority of arterial transformation in human pregnancy. Comparatively, murine uNK are composed of two subsets, with largely differing roles. uNK-derived IFN-γ is essential for remodeling of the decidual vasculature in mice, whereas the contribution of trophoblast is relatively insignificant and, indeed, rodent uNK predominantly suppress trophoblast invasion. In both species, uNK produce angiogenic factors, but in mice this is predominantly mediated by the DBA^{+} subset. As such, considering the broader themes of the decidual adaptations to pregnancy, mice provide a useful animal model in which to study reproductive immunology.

INNATE LYMPHOID CELLS

Natural killer cells are the most abundant and well-characterized subset of innate lymphoid cells (ILCs), which comprise lymphocytes belonging to the innate arm of the immune system exhibiting features of both innate and adaptive immunity (60, 61). ILCs are an important component in the immune response to a wide range of pathogens, particularly at epithelial barrier surfaces. They also contribute to tissue and metabolic homeostasis and have been implicated in the pathogenesis of cancer and inflammatory diseases. Features common to all ILCs are the absence of recombination activating gene (RAG)-dependent antigen-specific receptors, absence of myeloid lineage markers, and a lymphoid cellular morphology. Based upon this classification, ILCs can be broadly categorized into three major groups according to their development, cell surface markers, and functions (62, 63).

Our understanding of the origins and functions of ILCs is rapidly evolving, and the identities of disparate ILC populations are becoming increasingly apparent (64–67). While the pathways of ILC development in mice have been well defined, ILC differentiation in humans has yet to be determined. There is also a degree of phenotypic plasticity among ILCs, indicating that ILCs are capable of adapting their identities and functions *in vivo* in response to other immune cells and secreted factors in the local environment.

Functional comparisons have been made between ILCs and T cells, as the stimuli and cytokine profiles of ILC1s, ILC2s, and ILC3s are analogous to those of the T_H cell subsets T_H1, T_H2, and T_H17, respectively. Group 1 ILCs comprise *bona fide* helper-like ILC1s and NK cells. NK cells can be further subdivided into cNK and trNK subsets that differ in their phenotype, function, and development. cNK cells are the only ILC population to

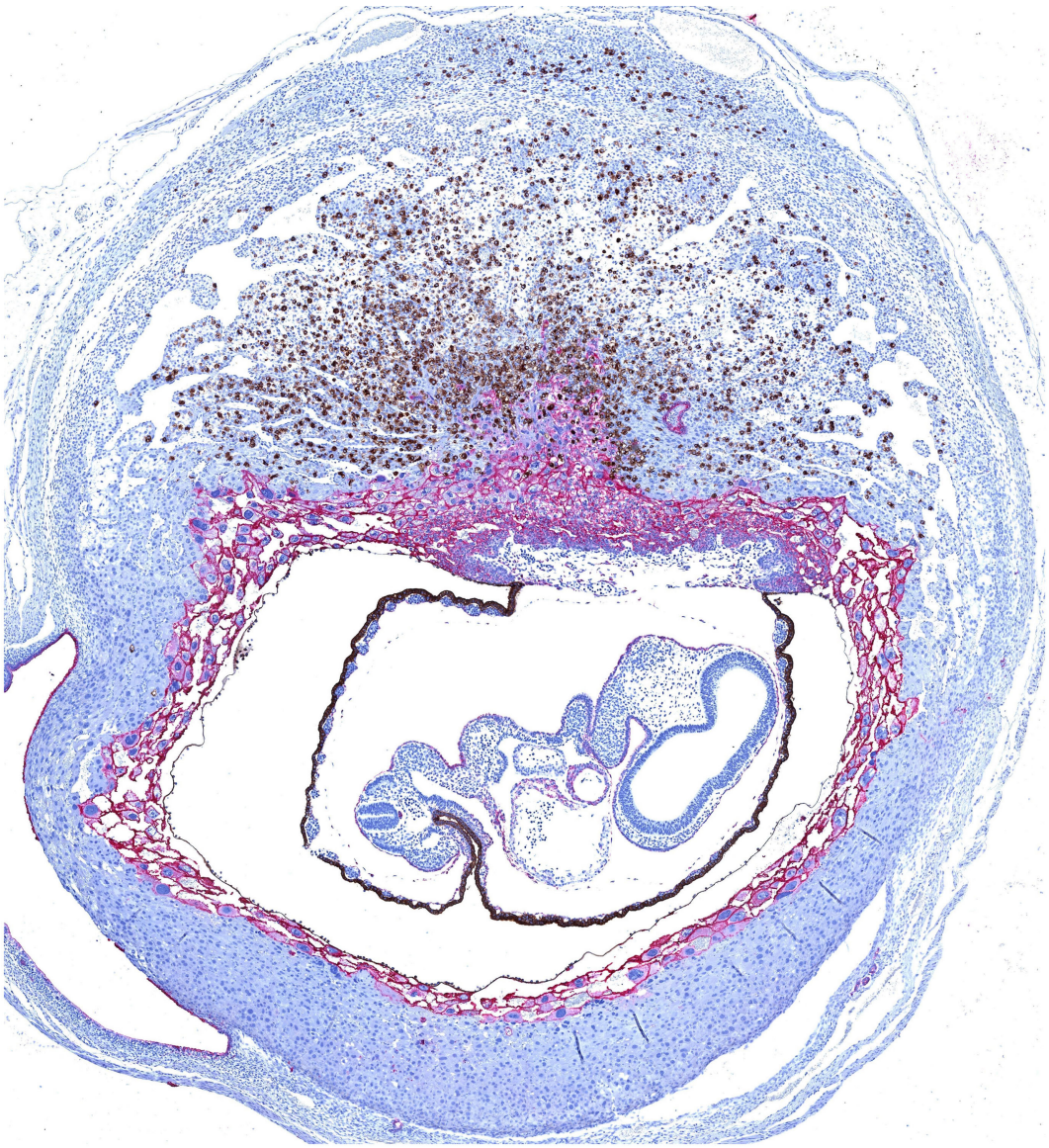


FIGURE 1 | Dual immunohistochemical staining of *Dolichos biflorus* agglutinin (DBA)$^+$ uterine natural killer (uNK) cells and trophoblast in a mouse implantation site at mid-gestation. Trophoblast (shown in *pink*) migrate centrally into the decidua to form an ectoplacental cone, which is surrounded by a layer of moderately invasive trophoblast giant cells expressing MHC class I. Mature uNK are abundant throughout the decidua basalis, and in a lymphocyte-rich accretion at the uppermost pole of the implantation site, known as the mesometrial lymphoid aggregate of pregnancy. Interactions between uNK Ly49 receptors and trophoblast MHC class I can modulate the activity of DBA$^+$ uNK (shown in brown) and DBA$^-$ uNK cells, and impact on their production of angiogenic factors and interferon (IFN)-γ respectively. Reproduced from Moffett and Colucci (39) with permission.

exhibit cytotoxicity mediated by exocytosis of cytotoxic granules containing perforin and granzymes, similarly to CD8$^+$ cytotoxic T-lymphocytes (60).

UTERINE ILCs

Since the amalgamation of diverse innate lymphocyte populations into the ILC family, there has been considerable focus on determining the distribution and biological significance of these cells *in vivo* (68). Other than uNK cells, first defined in 1991, ILC1s and ILC3s have also been identified in human decidua (2, 33, 69, 70). Uterine ILC3s (uILC3s) were initially classified as stage 3 uNK cell progenitors based upon their CD34$^-$ CD117$^+$ CD94$^-$ CD56$^+$ KIR$^-$ phenotype. These cells produced interleukin (IL)-22 and expressed *RORC* and *LTA*, encoding the transcription factor RORγ and lymphotoxin (LT)-α, respectively, which makes them indistinguishable from uILC3s (62, 69). The presence of human uILC3s and lymphoid tissue inducer (LTi)-like uILC3s has since been confirmed in accordance with currently accepted ILC definitions (33, 70). However, it has since been proposed

TABLE 2 | Characterization of murine natural killer (NK) cells in spleen and decidua.

Characteristic	Peripheral NK (pNK) cells	Uterine natural killer (uNK) cells
% of total lymphocytes	~ 2%	~ 30% at mid-gestation (40)
CD94	+ (41)	No published evidence
NKG2A/C/E	+ (41, 42)	No published evidence
NKG2D	+ (43)	+ (44)
Ly49s	+ (45)	++ (36)
NKp46	+ (46)	+ *Dolichos biflorus* agglutinin (DBA)$^-$, ++ DBA$^+$ (47)
CD49a	– (48)	~ 75% CD49a$^+$ CD49b$^{+/-}$ (33)
CD49b	+ (49)	~ 25% CD49a$^-$ CD49b$^+$ (33)
CD69	– (50)	++ (38, 51)
Killer-cell lectin-like receptor G1	+ (52)	++ (36)
Cytokine production	+ (53)	+ (54, 55)
Cytotoxicity	+ (56)	– (57)

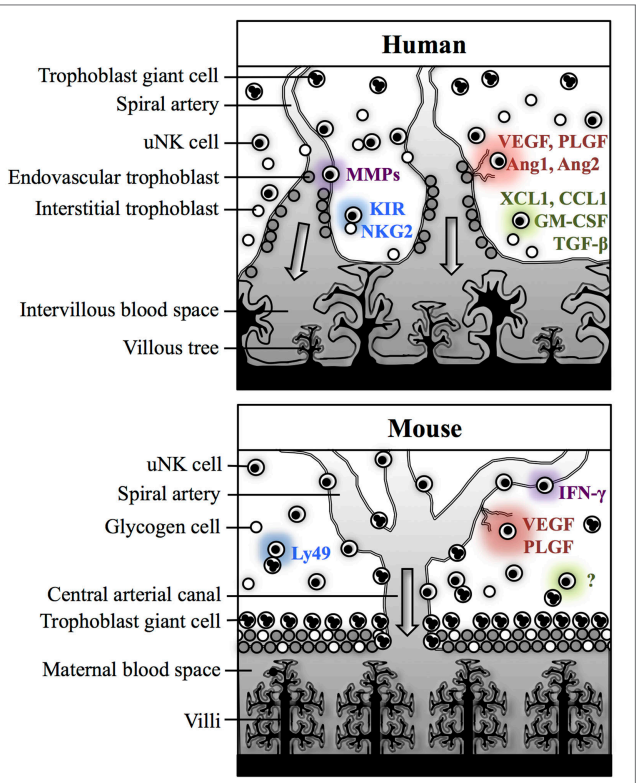


FIGURE 2 | Physiological processes of pregnancy within the decidua in human and mice. In both humans and mice, uterine natural killer (uNK) cells are thought to contribute to spiral arterial remodeling (*purple*), angiogenesis (*red*), and control of trophoblast invasion (*green*). Interactions between uNK receptors and trophoblast MHC class I antigens may modulate uNK cell activity (*blue*). In humans, uNK cells may contribute directly to decidua-associated vascular remodeling through secretion of matrix metalloproteinases (MMPs). Human uNK may also influence trophoblast-mediated vascular remodeling through secreting factors which enhance extravillous trophoblast (EVT) invasion [XCL1, CCL1, granulocyte-macrophage colony-stimulating factor (GM-CSF)] or suppress EVT migration [transforming growth factor (TGF)-β]. uNK cells in humans also secrete several angiogenic factors including vascular endothelial growth factor (VEGF), placental growth factor (PLGF), Ang1, and Ang2. Their activity may be modulated by killer-cell immunoglobulin-like receptors (KIR) and natural killer group 2 (NKG2) receptors, which recognize human leukocyte antigen class I ligands expressed by EVT. In mice, IFN-γ secreted by *Dolichos biflorus* agglutinin (DBA)$^-$ uNK cells is essential for decidual vascular remodeling. DBA$^+$ uNK cells are predominantly responsible for producing angiogenic factors, including VEGF and PLGF. Evidence from studies in rats and mice suggests that uNK cells primarily suppress trophoblast motility, but the mechanisms for this are not currently understood. Murine uNK cell function can be modulated by Ly49 receptors, which recognize MHC class I expressed by trophoblast giant cells.

that a population of CD34$^+$ CD122$^+$ CD309$^-$ lymphoid-like cells in human decidua represent NK-committed decidual hematopoietic progenitor cells (HPCs) (71). If these cells can be more definitively characterized as such, through detection of multiple co-expressed cell surface markers and transcription factors, it is likely that the population described by Male et al. were a heterogeneous mix of stage 3 uNK cell precursors and uILC3s. A proportion of uILC3s have been shown to differentiate to stage 3-like CD117$^+$ CD56$^+$ CD94$^+$ uNK cells upon *in vitro* culture with IL-15 (70). A similar report indicates that uILCs can differentiate into stage 4 CD117$^-$ CD56$^+$ CD94$^+$ uNK cells *in vitro*, further suggesting that uNK cell precursors were present (69). In view of the recent finding that tonsillar ILC3s can differentiate to stage 4 CD94$^+$ CD56^{bright} NK cells upon aryl hydrocarbon receptor (AhR) silencing *in vitro*, it would also be interesting to ascertain whether AhR is expressed by uILC3s and whether its manipulation is similarly able to induce differentiation to an NK cell phenotype (72).

All groups of ILCs are present in the uteri of virgin and pregnant mice (33). Uterine trNK cells were initially considered to develop independently of the transcription factors nuclear factor, interleukin-3 regulated (Nfil3) and T-box transcription factor Tbx21 (T-bet), but their dependency on Eomesodermin (Eomes) was not ascertained. As such, it was not possible to deduce their identity as belonging to an NK cell or *bona fide* ILC1 lineage (48). Similarly to those in the salivary gland, trNK cells in the uterus do express Eomes and, together with uterine cNK cells and Eomes$^-$ uILC1s, they are found throughout the decidua and myometrium during pregnancy. uILC1s can produce tumor necrosis factor (TNF)-α and IFN-γ but, owing to the fact that uILC1-sufficient *Nfil3*$^{-/-}$ females exhibit poor decidual vascular remodeling, the contribution of uILC1-derived IFN-γ to vascular modification is seemingly negligible (33, 58, 59). uILC2s, uILC3s, and LTi-like uILC3s are found only in the myometrium and in the MLAp (33). The MLAp is of unknown function but, since it is traversed by branches of the uterine artery, it is possible that it exerts some effect on the perfusion of individual implantation sites through leukocyte-mediated modification of vessels proximal to the spiral arteries (73, 74). Unlike lymph nodes, MLAp formation does not depend on LTα and LTβ-receptor signaling, making a role for LTi-like uILC3s in MLAp development unlikely (35, 75). Whether uILC2s and uILC3s and their derived cytokines, IL-5, IL-13, IL-17, and IL-22 participate in local immune regulation or tissue remodeling is currently unknown (33).

NK CELL DEVELOPMENT

Murine cNK cells arise from NK progenitors (NKP), which represent the first of six defined stages of murine NK cell

development (76–78). In contrast to helper-like ILCs, cNK cells develop independently of IL-7 and become CD127$^-$ CD122$^+$ (IL-2Rβ; IL-15Rβ) at the NKP stage. IL-15 signaling is essential for the differentiation of NKPs to immature NK (iNK) cells (79–81). Subsequently, iNK cells acquire functionally modulatory receptors such as NK1.1, NKp46, Ly49, and NKG2 receptors. Expression of the integrin CD49b (DX5) denotes the transition of iNK cells to a mature phenotype, which correlates with the development of functional competence such as IFN-γ production and cytolytic potential (82, 83). Three further stages of maturation can be defined by the differential expression of CD27 and CD11b, which culminate in the development of terminally mature NK cells expressing KLRG1 and CD43 (52, 84).

However, NK cell development can occur *via* alternative pathways. NK cells of thymic origin have been identified, which depend upon the GATA-binding protein-3 (GATA-3) transcription factor and IL-7 signaling. These cells appear phenotypically immature compared to cNK cells, but are more effective cytokine producers (85). More recently, Nfil3-independent NK cells have been described in skin, uterus, and salivary glands, which all express the integrin CD49a as a marker of tissue residency but which differ in their dependency on T-bet (33, 48, 86–88). However, since the population originally classified as CD49a$^+$ DX5$^-$ hepatic trNK cells does not express the transcription factor Eomes, it is more appropriate to consider these as hepatic ILC1s. These exhibit a broader cytokine profile than cNK and highly express TNF-related apoptosis-inducing ligands (TRAIL), which confer potential to induce apoptosis (48, 89, 90).

Ontogenesis of human NK cells is broadly analogous to that in mice, but there are notable differences in the sequence and anatomical sites of each developmental stage. Human NK cells arise from bone marrow (BM)-derived CD34$^+$ HPCs. Although elusive until recently, NK lineage-restricted progenitors have been identified in adult and fetal bone marrow, fetal liver, and adult tonsils (91). Evidence suggests that CD34^{dim} pro-NK cells are exported from BM comparatively early and home to secondary lymphoid tissues where they continue to differentiate (92). Five continuous stages of human NK cell development have been characterized in lymph node and tonsil. IL-15 acts on stage two pre-NK cells to support their transition to stage three. Human NK cells do not begin to express receptors for class I HLA antigens, including KIR and CD94/NKG2 dimers, until they reach a mature CD56^{bright} phenotype (17). At this stage, human NK cells are competent cytokine producers, which either remain *in situ* or terminally differentiate in peripheral blood to acquire cytotoxic potential as CD56^{dim} CD16$^+$ NK cells (21, 26, 93).

The transcriptional control of human NK cell development has not been delineated clearly, but *GATA-3* transcripts are abundant in stage 3 NK cells, and T-bet and Eomes are highly expressed in stage four and stage five NK cells (17, 94). However, as in mouse, subpopulations of CD49a$^+$ trNK cells have been identified in uterine endometrium and liver (59, 95). A subpopulation of CD127$^-$ CD56$^+$ Eomes$^+$ tonsillar and intestinal intraepithelial ILC1s are phenotypically and functionally resemblant of NK cells, but their murine counterparts develop independently of IL-15 (96). As such, it is possible that these, and perhaps other ILC1s, arise from pre-NK cells, and are more closely developmentally linked to NK cells than we currently appreciate.

ORIGIN OF uNK CELLS

Uterine natural killer cells account for over 70% of decidual leukocytes in the first trimester of human pregnancy and for approximately 30% of lymphocytes in murine decidua at mid-gestation (2, 40). The origin of these distinct and specialized NK cells has been a subject of investigation for over 30 years, but it is becoming increasingly accepted that uNK cells are likely to be a heterogeneous population arising from *in situ* progenitors and from homing of NKPs and/or pNK cells (97).

When mice were lethally irradiated in the presence of a protective lead shield covering one uterine horn, and subsequently rat BM cells were adoptively transferred, only uNK cells of rat origin could be identified in the irradiated uterine tissue, indicating that peripherally derived NKPs contribute to the generation of uNK cells (98). This is supported by observations that uteri from NK-sufficient mice are devoid of uNK cells when engrafted into NK-deficient hosts (99). Leukocytes of donor origin can be found in both murine and human decidua, following experimental transgenic labeling of BM cells and hematopoietic stem cell transplantation (HSCT) respectively, which suggests that decidual leukocytes are derived, at least in part, from BM HPCs *in vivo* (100, 101). A very small population of stage 3 NK precursors in peripheral blood, which are capable of maturing to stage 4 cells in the presence of IL-15, also raises the possibility that NK precursors home to the uterus and differentiate to mature uNK cells *in situ* (69). As pbNK cells can be induced to acquire phenotypic and functional attributes of uNK cells under the influence of hypoxia, transforming growth factor (TGF)-β, and demethylating agents, it is also possible that some uNK cells develop as a result of pbNK cell recruitment (102).

However, in the study by Peel and Stewart, no uNK cells could be detected in the irradiated uterine horn in half of the mice which had retained functional BM as a result of lead shielding of their legs during irradiation. This suggests that uNK cell precursors present in uterine tissue prior to irradiation were either destroyed or rendered incapable of proliferation, and that recruitment of circulating NKPs was insufficient to restore the uNK cell population (98). The proposed NK-committed decidual HPCs identified by Vacca et al. can differentiate to mature uNK cells in the presence of IL-15, which is expressed abundantly in first trimester decidua and placenta (71, 103). The presence of *in situ* HPCs would also account for the CD56$^+$ NK cells detected in human endometrial tissue which had been xenografted into hormone-treated immunodeficient mice (104). NK cell development from resident hematopoietic progenitors has also been documented in mice (105). Taken together, it seems probable that uNK cells arise from proliferation of peripherally derived HPCs and/or NK precursors which have homed to the pregravid uterus, but a potential contribution by pbNK cells which undergo phenotypic adaptation *in situ* cannot be discounted.

EFFECTOR FUNCTIONS OF NK CELLS

Soluble factors secreted by other leukocytes can stimulate cytokine production by NK cells, which provides a means by which these immune cells can indirectly interact with each other and reciprocally induce effector functions. NK cells are responsive to a number of cytokines released by monocytes, including IL-1, IL-10, IL-12, IL-15, and IL-18, and T_H lymphocytes, including IL-2 and IL-21. These induce production of key NK cell-mediated cytokines such as IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), TNF-α, and macrophage inflammatory protein (MIP)-1 (106). Of these, IFN-γ has the most diverse immunomodulatory roles and promotes T_H1 cell differentiation, activation of macrophages and enhancement of antigen presentation *via* upregulation of class I and class II MHC molecules; all of which cumulatively contribute to antimicrobial, antiviral, and anti-tumor immunity (107).

All mature murine cNK cells have the capacity to produce cytokines and mediate perforin-dependent cytotoxicity. Distinct tissue-specific NK cell subpopulations display variation in functionality, such that salivary gland trNK cells only induce TRAIL-dependent cytolysis and uNK cells are weakly cytotoxic under physiological conditions (57, 87). In humans, $CD56^{dim}$ $CD16^{+}$ NK cells contain lytic granules, and are less effective cytokine producers and express KIR at far higher frequencies than their $CD56^{bright}$ $CD16^{-}$ counterparts (21, 26). That the $CD56^{dim}$ $CD16^{+}$ subset accounts for 90% of circulating NK cells emphasizes the importance of HLA class I recognition as a means of immunosurveillance by pNK cells. Indeed, the absence of NK cells *in vivo* enhances susceptibility to viral infections and metastatic progression of malignant tumors (108–110).

Natural killer cells express a broad repertoire of modulatory receptors, of which many are common to both human and mouse. Among these are the activating receptors NKp46, which recognizes viral hemagglutinins, NKG2D which binds cellular stress-induced ligands, and CD16, which mediates antibody-dependent cellular cytotoxicity (ADCC) in response to immunoglobulin G (IgG) (111, 112). The induction of cytotoxic effector responses is tightly regulated and, with the exception of CD16, requires the synergistic input of signaling *via* two activating receptors, reduced inhibition and/or the presence of stimulatory cytokines (113). As many inhibitory NK cell receptors recognize MHC class I ligands, reduced inhibition predominantly occurs in the context of downregulation of MHC class I molecules by virally infected and malignant cells.

The probable roles of uNK in both human and mouse are the production of cytokines, chemokines and angiogenic factors, which may mediate the key physiological processes required for successful pregnancy, discussed in greater depth later in this review (**Figure 2**). Comprehensive gene expression analyzes have demonstrated the extent to which human NK cells in the uterus functionally and phenotypically differ from those in peripheral blood (4, 8). Although uNK cells are phenotypically and functionally distinct from pNK cells, their activity can be similarly modulated through interactions with soluble factors and cell-bound ligands, including MHC class I.

NK CELL RECOGNITION OF MHC MOLECULES

Recognition of class I MHC is mediated by KIR in humans, Ly49 receptors in mice, and by CD94/NKG2 heterodimers in both species (114). KIRs are highly polymorphic receptors encoded within the leukocyte receptor complex (LRC) on chromosome 19, which bind to HLA class I molecules (115). Sixteen *KIR* genes have been identified and, for each, between 18 and 112 alleles are currently known (116, 117). Fourteen of these genes encode functional receptors for classical HLA, of which six are inhibitory and eight are activating.

KIR genes can be grouped into two main haplotypes, termed *A* and *B*. With the exception of *KIR2DS4*, which is most commonly truncated, haplotype *A* encodes only inhibitory receptors whereas haplotype *B* contains genes for both inhibitory and activating KIR. The majority of KIR2D receptors exhibit binding specificity for one of two epitopes of all HLA-C allotypes, C1 and C2, which differ due to diallelic polymorphism at positions 77 and 80 of the α1 chain (116, 118). Binding affinities between KIR2DL and HLA-C molecules also influence functional responses, such that weak interactions induce less inhibition. *KIR A* haplotypes are typified by KIR2DL1, which binds C2 epitopes with high avidity, and KIR2DL3, which weakly binds C1 epitopes. Comparatively, *KIR B* haplotypes are characterized by an allotype of KIR2DL1 which binds C2 epitopes with low affinity, and KIR2DL2, which binds more strongly than KIR2DL3 to C1 epitopes (119).

The functionally analogous receptors for classical MHC class I molecules in the mouse are polymorphic lectin-like Ly49 receptors. These are encoded within the natural killer complex (NKC) on chromosome 6 and bind classical H-2 antigens. *Ly49* gene content varies considerably between strains, ranging from eight in BALB/c mice to 22 in non-obese diabetic (NOD) mice (120, 121). Ly49 receptors use the same signaling pathways as KIR, including intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) for inhibitory receptors and signaling through DAP12 for activating receptors (122–124). Nomenclature of *Ly49* genes, which are synonymous with killer cell lectin-like receptor subfamily A (*Klra*) genes, is non-descriptive and each receptor is designated a letter between A and X (125). The major Ly49 receptors in C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) strains and their respective ligands are summarized in **Table 3**. Of particular

TABLE 3 | Ly49 receptors and their respective ligands in C57BL/6 and BALB/c mice.

Ly49 receptor	Ligands	Notes
Ly49A	$H\text{-}2D^d$, $H\text{-}2D^b$, H2-M3 (128–130)	
Ly49C	$H\text{-}2K^b$, $H\text{-}2D^b$, $H\text{-}2K^d$, $H\text{-}2D^d$ (128, 129)	
Ly49D	$H\text{-}2D^d$ (131)	Absent in BALB/c
Ly49G2	$H\text{-}2D^d$ (128, 129)	
Ly49H	Murine cytomegalovirus m157 glycoprotein (127)	Absent in BALB/c
Ly49I	$H\text{-}2K^b$, $H\text{-}2K^d$ (128, 129)	Pseudogene in BALB/c

Inhibitory receptors denoted in red, and activating receptors denoted in blue.

functional significance is the activating Ly49H receptor, which recognizes the murine cytomegalovirus (MCMV) m157 glycoprotein. BALB/c mice notably lack this receptor and, as such, are highly susceptible to MCMV, with high viral titers and increased mortality following infection (126, 127).

In both humans and mice, recognition of non-classical MHC class I molecules is predominantly mediated by CD94/NKG2 heterodimers. Inhibitory CD94/NKG2A dimers signal *via* an ITIM-dependent pathway, whereas activating CD94/NKG2C and CD94/NKG2E associate with DAP12 (132–136). CD94/NKG2 dimers recognize HLA-E in humans and Qa-1^b in mice, which are expressed in complex with peptides derived from leader sequences of other MHC class I molecules (41, 42, 137, 138). As such, HLA-E and Qa-1^b provide an additional means by which aberrant MHC class I expression in diseased cells can be detected by NK cells. There are potentially some species-related differences in the expression profiles of these receptors, as human CD94/NKG2E exists only in an intracellular form, and cell surface expression of neither CD94/NKG2C nor CD94/NKG2E has been definitively detected in mice (41, 42, 134).

It has long been considered that acquisition of individual KIR and Ly49 receptors occurs stochastically, such that the co-expression frequencies of individual receptors do not deviate markedly from the product rule (139). This generates subsets of NK cells expressing anywhere between zero and the full complement of NK cell receptors for MHC class I herein referred to as NKRs. However, deviations in the NKR repertoire in accordance with the MHC environment indicate that there are some selective influences (140–143). Specific NKRs are downregulated in the presence of their cognate MHC ligands in a manner that is both MHC dose-dependent and reflective of receptor–ligand binding avidity (142, 144, 145). Refinement of the NKR repertoire is an important aspect of the adaptation of NK cells to their host environment, and is complementary to a process referred to as NK cell education, during which interactions with self-MHC calibrate NK cell responsiveness. Taken together, these processes may allow for selection of the most biologically useful and least self-reactive NK cell subsets *in vivo*.

NK CELL EDUCATION

The concept of NK cell education, or "licensing," was first proposed in 2005 when it was observed that cells expressing inhibitory NKRs for self-MHC are functionally more responsive than those that do not, both in terms of cytotoxicity and IFN-γ production. This was proposed as a mechanism for NK cell self-tolerance, so that uneducated cells lacking NKRs for self-MHC respond poorly to activating stimuli, such as cross-linking of activating receptors and MHC class I deficient cells. This negates the requirement for NKR-mediated counter-inhibition and reduces the potential for autoreactivity (140, 146). However, NK cell education is by no means an essential requirement for functionality, since responsiveness can be at least partially restored among uneducated NK cells in the presence of pro-inflammatory cytokines (140, 146–148).

The outcome of the educative process is that NK cells attain the capacity to respond to aberrant MHC class I expression. This occurs through "missing-self" recognition, which may result from the absence of self-MHC class I or in the presence of allogeneic MHC class I ligands. The latter effect can be harnessed for therapeutic benefit in HSCT, used in the treatment of hematological malignancies. NK cells from HLA haplotype-mismatched donors enhance graft tolerance in patients with acute myeloid leukemia and induce disease remission with protection against relapse (149). The only physiological situation in which allogeneic class I MHC is presented to a host is during pregnancy. uNK cells are a sufficiently distinct subset that their behavior cannot be effectively modeled on pNK cells, owing to significant phenotypic and functional differences. However, a wealth of evidence from human genetic association studies and mouse models suggests that uNK cell activity can be modulated through interactions with class I MHC from both parents, and that this has the potential to significantly impact on reproductive outcome (150).

REGULATION OF SPIRAL ARTERIAL REMODELING BY uNK CELLS

The placenta was originally thought to provide the means of "anatomical separation of fetus from mother," enabling development of the semi-allogeneic fetus without maternal immune rejection (151). A modern view of immunogenetics of pregnancy proposes that in fact maternal NK cells regulate placentation and vascular remodeling through direct interactions with fetal trophoblast cells (150). Despite numerous anatomical and physiological differences between human and murine pregnancy, mice can provide a useful model in which to study trophoblast differentiation and immune regulation of placental development because both species exhibit hemochorial placentation, where placental trophoblast cells invade the maternal decidua and come into direct contact with maternal blood (152–154). Key features of human and murine pregnancy are summarized in **Table 4**.

Remodeling of the maternal spiral arteries is an essential local vascular adaptation to pregnancy, which transforms the arteries supplying the feto-placental unit to large bore, high conductance vessels with non-turbulent flow (158). The initial stages of

TABLE 4 | Features of pregnancy in humans and mice (154–157).

Feature	Human	Mouse
Decidualization	Cyclical (approx. 28 days) Prior to implantation	Post-implantation
Implantation	~ 6 days post-conception (p.c.)	Gestation day (g.d.) 4.5
Type of placentation	Discoid Hemochorial Villous	Discoid Hemochorial Labyrinthine
Placental development	First functional at 10 weeks p.c. Growth continues until term	First functional at g.d. 10.5 Maximal size at g.d. 16.5
Fetal growth	Disproportionate to placental growth in third trimester	Disproportionate to placental growth from g.d. 14.5
Fetal:placental weight ratio	Approx. 7.5:1	Approx. 15:1
Duration of gestation	~ 40 weeks	19–21 days

vascular transformation in humans occur during the secretory phase of the menstrual cycle and become more pronounced in early pregnancy independently of trophoblast invasion (159). The maternal vessels during these stages are closely apposed by leukocytes, particularly macrophages and uNK cells, which may contribute to this decidua-associated remodeling through secretion of proteolytic matrix metalloproteinases (MMPs). $CD56^+$ cells have been shown histologically to express MMP-7, MMP-9, MMP-19, and MMP-23 (160–162). However, since CD56 is also expressed by endovascular EVT, it cannot be asserted from dual immunohistochemical staining alone that intramural and endovascular $CD56^+$ MMP^+ cells are uNK cells (159). Subsequent stages of remodeling in humans are dependent upon the deep invasion of EVT by interstitial and endovascular routes. Interstitial EVT migrate through the decidua and are thought to intravasate into the walls of the maternal spiral arteries to contribute to disorganization of the vascular smooth muscle (159, 163). Perivascular trophoblast may intravasate further into the vascular lumen to account for some of the endovascular trophoblast which migrates retrogradely along the lumen of the arteries (164). It is considered that EVT from both interstitial and endovascular routes become incorporated into the vascular wall and replace vascular smooth muscle cells (VSMCs) with fibrinoid material, which maintains the vessel in a dilated state and renders it incapable of vasoconstriction (165).

The contribution of trophoblast to decidual vascular transformation in mice is less well defined. Moderately invasive trophoblast giant cells (TGCs) associate with decidual vessels in their more distal segments, and line the arterial canals which supply the feto-placental unit (166). It seems likely that vascular modification in mice is predominantly mediated by other decidual cells, including uNK cells which become integrated into the vascular media (167). Indeed, it is well established that IFN-γ of uNK cell origin is essential for spiral arterial remodeling in murine pregnancy (54). NK cell-deficient mice consistently have defective decidual vascular remodeling, characterized by narrow vascular lumens, thick vascular walls, and retention of vascular smooth muscle actin (58, 168–170). Through utilizing alymphoid mice which were engrafted with BM from IFN-$\gamma^{-/-}$ mice or severe combined immunodeficient (SCID) mice, which lack T- and B-lymphocytes, it has been elegantly and conclusively demonstrated that IFN-γ of uNK cell origin is essential for murine spiral arterial remodeling (54). NK cell-deficient mice also exhibit IFN-γ-dependent morphological abnormalities such as decidual hypocellularity and failure of MLAp formation, which can be restored through adoptive transfer of BM from C57BL/6 or SCID mice (54, 169, 171). The mechanisms by which IFN-γ mediates vascular remodeling have not been elucidated. Although murine uNK cells have not been reported to produce MMPs, decidual macrophages do produce MMP-9, and MMP-2 and MMP-9 expression can be observed throughout the decidua basalis and in close proximity of decidual arteries (172, 173). Since uNK cells produce IFN-γ and MIP-1α, which are key cytokines involved in macrophage activation, it is possible that uNK cells mediate vascular remodeling through stimulation of MMP production by macrophages (36, 107). Indeed, MMP-2 and macrophage-derived MMP-9 are essential in the pathogenesis of murine abdominal aortic aneurysms, in which pathological destruction of the aortic vascular media leads to extensive dilatation and risk of rupture (174).

More similarly to humans, modification of the spiral arteries in rats involves initial medial disorganization by uNK cells and subsequent destruction of the smooth muscle layer by interstitial and endovascular trophoblast, which invade deep into the decidua and myometrium (175, 176). This demonstrates that, even among species that exhibit hemochorial placentation, there is significant variability in the dependence upon trophoblast and uNK cells for transformation of the spiral arteries supplying the feto-placental unit.

REGULATION OF TROPHOBLAST INVASION BY uNK CELLS

Uterine natural killer cells may also contribute to modification of spiral arteries indirectly, through their influence on EVT. A recent study shows that human uNK produce the chemokines XCL1 and CCL1. The receptor for XCL1, XCR1, is expressed by several cell types in the placenta, including fetal endothelial cells and EVT. XCR1 is also expressed by decidual cells, including a small population of $CD14^+$ macrophages. The CCL1 receptor, CCR8, has been identified on all decidual macrophages and on a small proportion of uNK (177). It has been determined by intracellular cytometry that uNK secrete GM-CSF, and the chemokines IL-8 and interferon-inducible protein (IP)-10 have been detected in supernatants of uNK cells *in vitro*. All of these factors enhance motility of primary trophoblast in cell migration and invasion assays (4, 177–180). Intracellular cytometry has since shown that macrophages are probably the predominant source of IL-8 among decidual leukocytes, although activated uNK cells were not assessed in this study (181). IL-8 stimulates production of MMP-2 and MMP-9 in a first trimester EVT cell line, which is suggestive of a mechanism by which leukocyte-derived factors may promote EVT-induced vascular remodeling (180). However, uNK also secrete TGF-β, which impairs the invasive properties of primary trophoblast *in vitro* (5, 182). As such, human uNK seemingly mediate a balance between enhancing and inhibiting EVT invasion, and alterations in their function may lead to placental pathology and associated disorders of pregnancy. Supernatants from IL-15-activated uNK cell isolates from women with high uterine artery resistance, which denotes incomplete arterial remodeling, do not effectively induce motility of a first trimester EVT cell line and apoptosis of VSMC and endothelial cell lines *in vitro* (183). While this likely indicates that uNK cell-derived factors contribute to vascular remodeling and modulating trophoblast migration *in vivo*, uNK cells were harvested in this study at 9–14 weeks gestation, when transformation of the decidual sections of the spiral arteries is advanced and uNK cell function is declining (184). Assessment of uNK function at an earlier gestational time-point would be more informative for understanding the relative contribution of uNK cells to these physiological processes.

Whereas human uNK cells have been demonstrated to both enhance and inhibit EVT invasion, evidence from studies in rats

and mice suggests that uNK cells primarily suppress trophoblast motility. The onset of trophoblast invasion in both rats and mice was observed to correlate with the demise of uNK cells, at around g.d. 14 and was accelerated in NK cell-deficient and IFN-$\gamma^{-/-}$ mice (176). This is seemingly dependent upon a profound deficit in uNK cell number and/or function, as no effect on depth of trophoblast invasion could be determined in a model of more subtle, MHC-dependent uNK inhibition (36). It has also been suggested that, through contributing to decidual angiogenesis, uNK cells contribute to increased oxygen tensions at the maternal–fetal interface, which prevents trophoblast adopting an invasive phenotype (175). This would most likely be mediated by murine DBA^+ uNK cells, which are known to produce angiogenic factors including vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) (27, 55, 185).

Human uNK also secrete several angiogenic factors including VEGF, PLGF, angiopoietin (Ang)1, and Ang2 (4, 5, 179). Production of all factors mentioned can be modulated through KIR/HLA interactions and by the activating receptors NKG2D, NKp30, NKp44, and NKp46 (4, 178, 179). Human trophoblast express ligands for NKp44, but not for NKG2D (4, 20, 179). However, since decidual stromal cells express ligands for NKp30 and NKG2D, it is likely that uNK cell function is also modulated through interactions with maternal tissues (4, 186). There is some evidence to suggest that ligation of NKp30 also induces production of IFN-γ, TNF-α, MIP-1α, and MIP-1β, but since uNK cells were stimulated in the presence of IL-2, the physiological significance of these results is questionable (187). Moreover, recent work suggests that the decidual microenvironment influences the expression of NKp30 and NKp44 splicing variants that may be responsible for decreased cytotoxicity and altered cytokine secretion of uNK cells compared to pbNK cells (188).

SEQUELAE OF DEFECTIVE PLACENTATION

Defective vascular remodeling, characterized by the absence of intramural EVT and retention of VSMCs, particularly within the myometrial segments of the spiral arteries, is a common pathologic feature in cases of pre-eclampsia, early miscarriage, unexplained stillbirth, and fetal growth restriction (FGR) (189–191). As such, these conditions may reasonably be considered as a spectrum of disorders that can arise from a common primary pathology and, collectively, they are often referred to as the Great Obstetric Syndromes. Some cases of recurrent miscarriage (RM) may also be caused by insufficient trophoblast invasion (192).

To date, many of the studies investigating impaired decidual vascular transformation in mice have focused on the causative mechanisms and histological features. Defective remodeling of spiral arteries does not spontaneously induce systemic hypertension in mice, but is linked to poor fetal growth, indicating that pre-eclampsia only occurs as a response to placental stress from underperfusion in humans (36, 193).

Human EVT invasion may also occur excessively when a blastocyst implants in poorly or non-decidualized tissue. Placenta accreta occurs due to pathological trophoblastic invasion of the myometrium, which most commonly occurs as a result of implantation at the site of uterine scar tissue from previous intrauterine surgery (194). Similar pathological features are observed in ectopic pregnancies, in which the thin wall of the Fallopian tube is commonly entirely infiltrated by EVT in the absence of decidual tissue (195). Given that trophoblast migration is enhanced in mice and rats depleted of NK cells, excessive invasion of EVT in non-decidualized tissue in humans is highly suggestive of a fundamental role for human uNK cells in regulating trophoblast invasion (175, 176).

IMMUNOGENETICS OF TROPHOBLAST AND uNK CELL INTERACTIONS

Uterine natural killer cell activity can be directly modulated through interactions with decidual stromal cells, uterine leukocytes, and invasive trophoblast. Of these, the regulation of uNK cell function by trophoblast has been particularly well explored, owing to the association between certain KIR/HLA interactions and disorders of pregnancy. Trophoblast cells express a distinct repertoire of HLA ligands in comparison to somatic cells. Syncytiotrophoblast, which directly contacts maternal blood, and villous cytotrophoblast are HLA negative (196). Invasive EVT express a unique combination of polymorphic HLA-C and oligomorphic HLA-E and HLA-G, but not HLA-A, HLA-B, or MHC class II (197). Each of the EVT HLA class I ligands is able to interact with uNK cell receptors (uNKRs), as outlined in **Table 5**. Soluble HLA-G is reportedly produced by trophoblast, and is suggested to modulate uNK cell activity (198, 199). However, assessment of the crystal structure of KIR2DL4 and its potential interaction with HLA-G has revealed no evidence of direct receptor–ligand binding (200). In view of this, and in the absence of functional

TABLE 5 | Uterine natural killer (uNK) cell receptors and respective trophoblast ligands in human and mouse.

uNK cell receptor	Trophoblast ligand
Human	
KIR2DL1 (A and B haplotypes)	Human leukocyte antigen (HLA)-C2 (116, 119)
KIR2DL2 (B haplotype)	HLA-C1 (116, 119)
KIR2DL3 (A haplotype)	HLA-C1 (116, 119)
KIR2DL4 (A and B haplotypes)	HLA-G? (201)
KIR2DS1 (B haplotype)	HLA-C2 (116, 119)
KIR2DS4 (A haplotype)	HLA-C1, HLA-C2 (202)
LILRB1	HLA-G (197, 203)
CD94:NKG2A	HLA-E (197)
CD94:NKG2C; CD94:NKG2E	HLA-E (197)
NKp44	Unidentified (4, 179)
NKp46	Unidentified (4, 204)
NKG2D	Not expressed (20)
Mouse	
Ly49A, Ly49G2	$H\text{-}2D^d$ (BALB/c) (36)
Ly49C, Ly49I	$H\text{-}2K^b$ (C57BL/6) (37)
Ly49D	$H\text{-}2D^d$ (BALB/c) (36)
Ly49H	Not expressed
CD94:NKG2A	Not expressed (205)
CD94:NKG2C; CD94:NKG2E	Not expressed (205)
NKG2D	Rae1 (44)

Inhibitory receptors denoted in red, and activating receptors denoted in blue. KIR2DL4 can be both inhibitory and activating.

data using non-preactivated uNK cells, hypotheses regarding the role of uNK-expressed KIR2DL4 remain unsubstantiated.

As in humans, murine trophoblast cells at the site of physiological exchange are MHC negative, whereas invasive trophoblast does express MHC class I antigens (36, 37, 206). Invasive TGCs from C57BL/6 mice have been shown to express H-2K^b ligands at far greater intensity than H-2D^b, which is only detectable at very low levels (37). Trophoblast expression of transgenic H-2D^d epitopes in C57BL/6 mice has also been demonstrated by immunofluorescence staining (36). uNK cells interact with trophoblast class I MHC through Ly49 receptors (**Table 5**), but since trophoblast do not express Qa-1^b, any functional modulation of uNK cells through NKG2A/C/E is most likely mediated by decidual stromal cells (205, 207).

TROPHOBLAST AND uNK CELL INTERACTIONS IN DISORDERS OF PREGNANCY

Immunogenetic associations between maternal KIR/fetal HLA variants and disorders of pregnancy show that combinations of a maternal *KIR AA* genotype and fetal *C2* epitopes are present at significantly higher frequencies in pregnancies complicated by pre-eclampsia (208). Extension of this work later showed that this increased risk of developing pre-eclampsia is highest when trophoblast *C2* epitopes are paternally inherited (209). An increased frequency of maternal *KIR AA* and paternally derived *C2* epitopes has also been observed in cases of RM (209, 210). There is an additional weaker correlation, between maternal *KIR AA* and FGR (209). Within the *KIR A* haplotype are two genes for inhibitory KIR, *KIR2DL1* and *KIR2DL3*, which encode receptors for C2 and C1 epitopes, respectively (**Table 5**). It may be reasonably considered that KIR2DL1/C2 interactions are particularly detrimental to uNK cell function, as binding between KIR2DL1 and C2 epitopes is stronger and more specific than that between KIR2DL3 and C1 epitopes (211). Furthermore, interaction between KIR2DL1 and its cognate HLA-C ligand significantly reduces production of chemokines and angiogenic factors by IL-2-activated uNK cells *in vitro* (4).

Conversely, the presence of the telomeric region of the *KIR B* haplotype (*Tel-B*) was shown to be protective against RM and pre-eclampsia, particularly when trophoblast expressed C2 epitopes (208–210). The *Tel-B* region contains *KIR2DS1*, which encodes an activating KIR that binds C2 epitopes (**Table 5**). Maternal *KIR2DS1* predisposes to high birth weights, above the 90th centile, when the fetus expresses paternally derived C2 epitopes (212). Interaction between uNK cell KIR2DS1 and C2-expressing target cells *in vitro* induces GM-CSF production, which enhances trophoblast migration in a Transwell assay (178). Taken together, these data strongly indicate that uNK cell activity is modulated through KIR/HLA interactions and further support the hypothesis that imbalance in uNK cell function, potentially leading to dysregulation of physiological processes essential to pregnancy, can lead to undesirable reproductive outcomes in humans.

Most studies investigating murine uNK cell function to date have assessed the contribution of uNK cell deficiency or uNK cell-derived factors to reproductive success. Only more recently have mouse models been used to examine the impact of more subtle variations in uNK cell activity, such as that mediated by parental MHC disparity. Allogeneic, paternally inherited MHC class I is sufficient in isolation to modulate uNK cell function, and to directly impact on spiral arterial remodeling and fetal growth (36, 37). BALB/c females mated with BALB.B males, which express the C57BL/6 H-2^b MHC allotype, exhibit enhanced decidual vascular remodeling and increased fetal growth (37). However, the underlying mechanisms for this remain unclear. Paternally derived trophoblast H-2D^d has been convincingly demonstrated to inhibit uNK cell function in C57BL/6 females. Reduced production of IFN-γ by uNK cells was mediated by inhibitory interactions between H-2D^d with Ly49A and Ly49G2, which resulted in incomplete spiral arterial remodeling and reduced fetal growth. Maternally expressed H-2D^d was also disadvantageous for vascular transformation and fetal growth, which suggests that murine uNK cell education does not confer protective benefits during pregnancy (36).

The relative impact of maternal NKR variability as a determinant of pregnancy outcome in mice has been less well investigated. *Ncr1*$^{-/-}$ mice, which lack NKp46, exhibit impaired decidual vascular remodeling and disrupted angiogenesis, which suggests that NKp46-mediated activation of uNK is important for optimal reproductive outcome in mice (47). Expression of Ly49 receptors can also influence pregnancy, as Ly49 knockdown (Ly49^{KD}) mice, in which Ly49s are expressed by only 50% of DX5$^+$ and 6% of DBA$^+$ uNK, exhibit subfertility, impaired angiogenesis, reduced vascular remodeling and, unexpectedly, enhanced fetal growth (213). However, given the incongruence of the fetal phenotype in this model, it is feasible that extraneous factors are contributing to the outcomes observed. As the transcriptionally silenced region in Ly49^{KD} mice spans approximately 10.2 megabase pairs (Mbp), encompassing the ~2 Mbp NKC region, the potential impact of genes irrelevant to NK and/or leukocyte function cannot be discounted (214, 215). In keeping with defective angiogenesis, VEGF expression within the decidua and MLAp was reduced in Ly49^{KD} females. The total concentration of IFN-γ within these tissues was unaffected but, as uNK function was not specifically assessed in this study, it is not possible to determine whether uNK dysregulation is responsible for the vascular phenotype observed (213). As such, the strongest data relating to the consequences of NKR-MHC interactions in murine pregnancy are from studies investigating parental MHC disparity.

CONCLUDING REMARKS

Results from many studies using mouse models to date have substantiated data from human genetic association studies, which strongly suggest that reduced uNK cell activation is disadvantageous for reproductive outcome. However, it is apparent that the success of pregnancy depends upon a highly complex network of interactions between trophoblast, uNK cells, decidual stromal cells, and other decidual leukocytes. New technology, including improved mouse models, high-throughput

genotyping, mass cytometry, and single-cell RNA sequencing should help to define the role of immune cells in pregnancy, including tissue NK cells and other ILCs in human and mouse uterus.

AUTHOR CONTRIBUTIONS

The manuscript was written by LMG and edited by FC.

ACKNOWLEDGMENTS

The authors would like to thank Ashley Moffett and Jens Kieckbusch for critically reading the manuscript and all members of the laboratory which is funded by the Wellcome Trust and the Centre for Trophoblast Research. LMG was funded by fellowships from the British Heart Foundation (FS/12/4/29254) and the Centre for Trophoblast Research.

REFERENCES

1. King A, Wellings V, Gardner L, Loke YW. Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Hum Immunol* (1989) 24:195–205. doi:10.1016/0198-8859(89)90060-8
2. King A, Balendran N, Wooding P, Carter NP, Loke YW. CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Dev Immunol* (1991) 1:169–90. doi:10.1155/1991/83493
3. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol* (2002) 2:656–63. doi:10.1038/nri967
4. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* (2006) 12:1065–74. doi:10.1038/nm1452
5. Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, et al. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *J Leukoc Biol* (2006) 80:572–80. doi:10.1189/jlb.0406250
6. Kopcow HD, Allan DS, Chen X, Rybalov B, Andzelm MM, Ge B, et al. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc Natl Acad Sci U S A* (2005) 102:15563–8. doi:10.1073/pnas.0507835102
7. King A, Wooding P, Gardner L, Loke YW. Immunology: expression of perforin, granzyme A and Tia-1 by human uterine CD56+ NK cells implies they are activated and capable of effector functions. *Hum Reprod* (1993) 8:2061–7. doi:10.1093/oxfordjournals.humrep.a137982
8. Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med* (2003) 198:1201–12. doi:10.1084/jem.20030305
9. Vujaklija DV, Gulic T, Sucic S, Nagata K, Ogawa K, Laskarin G, et al. First trimester pregnancy decidual natural killer cells contain and spontaneously release high quantities of granulysin. *Am J Reprod Immunol* (2011) 66:363–72. doi:10.1111/j.1600-0897.2011.01015.x
10. Sharkey AM, Gardner L, Hiby S, Farrell L, Apps R, Masters L, et al. Killer Ig-like receptor expression in uterine NK cells is biased toward recognition of HLA-C and alters with gestational age. *J Immunol* (2008) 181:39–46. doi:10.4049/jimmunol.181.1.39
11. King A, Allan DS, Bowen M, Powis SJ, Joseph S, Verma S, et al. Hla-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *Eur J Immunol* (2000) 30:1623–31. doi:10.1002/1521-4141(200006)30:6<1623::AID-IMMU1623>3.0.CO;2-M
12. Dietl J, Ruck P, Marzusch K, Horny HP, Kaiserling E, Handgretinger R. Uterine granular lymphocytes are activated natural killer cells expressing VLA-1. *Immunol Today* (1992) 13:236. doi:10.1016/0167-5699(92)90161-Y
13. Bulmer JN, Williams PJ, Lash GE. Immune cells in the placental bed. *Int J Dev Biol* (2010) 54:281–94. doi:10.1387/ijdb.082763jb
14. Williams PJ, Searle RF, Robson SC, Innes BA, Bulmer JN. Decidual leucocyte populations in early to late gestation normal human pregnancy. *J Reprod Immunol* (2009) 82:24–31. doi:10.1016/j.jri.2009.08.001
15. Bisset LR, Lung TL, Kaelin M, Ludwig E, Dubs RW. Reference values for peripheral blood lymphocyte phenotypes applicable to the healthy adult population in Switzerland. *Eur J Haematol* (2004) 72:203–12. doi:10.1046/j.0902-4441.2003.00199.x
16. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* (1986) 136:4480–6.
17. Freud AG, Yokohama A, Becknell B, Lee MT, Mao HC, Ferketich AK, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med* (2006) 203:1033–43. doi:10.1084/jem.20052507
18. King A, Hiby SE, Verma S, Burrows T, Gardner L, Loke YW. Uterine NK cells and trophoblast HLA class I molecules. *Am J Reprod Immunol* (1997) 37:459–62. doi:10.1111/j.1600-0897.1997.tb00260.x
19. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* (1999) 285:727–9. doi:10.1126/science.285.5428.727
20. Apps R, Gardner L, Traherne J, Male V, Moffett A. Natural-killer cell ligands at the maternal-fetal interface: UL-16 binding proteins, MHC class-I chain related molecules, HLA-F and CD48. *Hum Reprod* (2008) 23:2535–48. doi:10.1093/humrep/den223
21. Jacobs R, Hintzen G, Kemper A, Beul K, Kempf S, Behrens G, et al. CD56bright cells differ in their Kir repertoire and cytotoxic features from CD56dim NK cells. *Eur J Immunol* (2001) 31:3121–7. doi:10.1002/1521-4141(2001010)31:10<3121::AID-IMMU3121>3.0.CO;2-4
22. Verma S, King A, Loke YW. Expression of killer cell inhibitory receptors on human uterine natural killer cells. *Eur J Immunol* (1997) 27:979–83. doi:10.1002/eji.1830270426
23. Sivori S, Vitale M, Morelli L, Sanseverino L, Augugliaro R, Bottino C, et al. p46, a novel natural killer cell–specific surface molecule that mediates cell activation. *J Exp Med* (1997) 186:1129–36. doi:10.1084/jem.186.7.1129
24. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. *J Immunol* (1989) 143:3183–91.
25. Lanier LL, Buck DW, Rhodes L, Ding A, Evans E, Barney C, et al. Interleukin 2 activation of natural killer cells rapidly induces the expression and phosphorylation of the Leu-23 activation antigen. *J Exp Med* (1988) 167:1572–85. doi:10.1084/jem.167.5.1572
26. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56bright subset. *Blood* (2001) 97:3146–51. doi:10.1182/blood.V97.10.3146
27. Chen Z, Zhang J, Hatta K, Lima PD, Yadi H, Colucci F, et al. DBA-lectin reactivity defines mouse uterine natural killer cell subsets with biased gene expression. *Biol Reprod* (2012) 87:81. doi:10.1095/biolreprod.112.102293
28. Paffaro VA Jr, Bizinotto MC, Joazeiro PP, Yamada AT. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. *Placenta* (2003) 24:479–88. doi:10.1053/plac.2002.0919
29. Croy BA, Esadeg S, Chantakru S, Van Den Heuvel M, Paffaro VA, He H, et al. Update on pathways regulating the activation of uterine natural killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. *J Reprod Immunol* (2003) 59:175–91. doi:10.1016/S0165-0378(03)00046-9
30. Parr EL, Young LH, Parr MB, Young JD. Granulated metrial gland cells of pregnant mouse uterus are natural killer-like cells that contain perforin and serine esterases. *J Immunol* (1990) 145:2365–72.
31. Zheng LM, Ojcius DM, Liu CC, Kramer MD, Simon MM, Parr EL, et al. Immunogold labeling of perforin and serine esterases in granulated metrial gland cells. *FASEB J* (1991) 5:79–85.
32. Croy BA, Reed N, Malashenko BA, Kim K, Kwon BS. Demonstration of YAC target cell lysis by murine granulated metrial gland cells. *Cell Immunol* (1991) 133:116–26. doi:10.1016/0008-8749(91)90184-D

33. Doisne JM, Balmas E, Boulenouar S, Gaynor LM, Kieckbusch J, Gardner L, et al. Composition, development, and function of uterine innate lymphoid cells. *J Immunol* (2015) 195:3937–45. doi:10.4049/jimmunol.1500689
34. Tagliani E, Shi C, Nancy P, Tay CS, Pamer EG, Erlebacher A. Coordinate regulation of tissue macrophage and dendritic cell population dynamics by CSF-1. *J Exp Med* (2011) 208:1901–16. doi:10.1084/jem.20110866
35. Kather A, Chantakru S, He H, Minhas K, Foster R, Markert UR, et al. Neither lymphotoxin alpha nor lymphotoxin beta receptor expression is required for biogenesis of lymphoid aggregates or differentiation of natural killer cells in the pregnant mouse uterus. *Immunology* (2003) 108:338–45. doi:10.1046/j.1365-2567.2003.01586.x
36. Kieckbusch J, Gaynor LM, Moffett A, Colucci F. MHC-dependent inhibition of uterine NK cells impedes fetal growth and decidual vascular remodelling. *Nat Commun* (2014) 5:3359. doi:10.1038/ncomms4359
37. Madeja Z, Yadi H, Apps R, Boulenouar S, Roper SJ, Gardner L, et al. Paternal MHC expression on mouse trophoblast affects uterine vascularization and fetal growth. *Proc Natl Acad Sci U S A* (2011) 108:4012–7. doi:10.1073/pnas.1005342108
38. Mallidi TV, Craig LE, Schloemann SR, Riley JK. Murine endometrial and decidual NK1.1+ natural killer cells display a B220+CD11c+ cell surface phenotype. *Biol Reprod* (2009) 81:310–8. doi:10.1095/biolreprod.109.076448
39. Moffett A, Colucci F. Co-evolution of NK receptors and HLA ligands in humans is driven by reproduction. *Immunol Rev* (2015) 267:283–97. doi:10.1111/imr.12323
40. Yadi H, Burke S, Madeja Z, Hemberger M, Moffett A, Colucci F. Unique receptor repertoire in mouse uterine NK cells. *J Immunol* (2008) 181:6140–7. doi:10.4049/jimmunol.181.9.6140
41. Vance RE, Kraft JR, Altman JD, Jensen PE, Raulet DH. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J Exp Med* (1998) 188:1841–8. doi:10.1084/jem.188.10.1841
42. Vance RE, Jamieson AM, Raulet DH. Recognition of the class Ib molecule Qa-1b by putative activating receptors Cd94/NKg2c and Cd94/NKg2e on mouse natural killer cells. *J Exp Med* (1999) 190:1801–12. doi:10.1084/jem.190.12.1801
43. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol* (2000) 1:119–26. doi:10.1038/77793
44. Carayannopoulos LN, Barks JL, Yokoyama WM, Riley JK. Murine trophoblast cells induce NK cell interferon-gamma production through KLRK1. *Biol Reprod* (2010) 83:404–14. doi:10.1095/biolreprod.110.084509
45. Yokoyama WM. Natural killer cell receptors specific for major histocompatibility complex class I molecules. *Proc Natl Acad Sci U S A* (1995) 92:3081–5. doi:10.1073/pnas.92.8.3081
46. Biassoni R, Pessino A, Bottino C, Pende D, Moretta L, Moretta A. The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur J Immunol* (1999) 29:1014–20. doi:10.1002/(SICI)1521-4141(199903)29:03<1014::AID-IMMU1014>3.0.CO;2-O
47. Felker AM, Chen Z, Foster WG, Croy BA. Receptors for non-MHC ligands contribute to uterine natural killer cell activation during pregnancy in mice. *Placenta* (2013) 34:757–64. doi:10.1016/j.placenta.2013.06.004
48. Sojka DK, Plougastel-Douglas B, Yang L, Pak-Wittel MA, Artyomov MN, Ivanova Y, et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *Elife* (2014) 3:e01659. doi:10.7554/eLife.01659
49. Arase H, Saito T, Phillips JH, Lanier LL. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 moncoclonal antibody is CD49b (α2 integrin, very late antigen-2). *J Immunol* (2001) 167:1141–4. doi:10.4049/jimmunol.167.3.1141
50. Karlhofer FM, Yokoyama WM. Stimulation of murine natural killer (NK) cells by a monoclonal antibody specific for the NK1.1 antigen. IL-2-activated NK cells possess additional specific stimulation pathways. *J Immunol* (1991) 146:3662–73.
51. Croy BA, Chen Z, Hofmann AP, Lord EM, Sedlacek AL, Gerber SA. Imaging of vascular development in early mouse decidua and its association with leukocytes and trophoblasts. *Biol Reprod* (2012) 87:125. doi:10.1095/biolreprod.112.102830
52. Huntington ND, Tabarias H, Fairfax K, Brady J, Hayakawa Y, Degli-Esposti MA, et al. NK Cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. *J Immunol* (2007) 178:4764–70. doi:10.4049/jimmunol.178.8.4764
53. Yokoyama WM, Kim S, French AR. The dynamic life of natural killer cells. *Annu Rev Immunol* (2004) 22:405–29. doi:10.1146/annurev.immunol.22.012703.104711
54. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med* (2000) 192:259–70. doi:10.1084/jem.192.2.259
55. Wang C, Umesaki N, Nakamura H, Tanaka T, Nakatani K, Sakaguchi I, et al. Expression of vascular endothelial growth factor by granulated metrial gland cells in pregnant murine uteri. *Cell Tissue Res* (2000) 300:285–93. doi:10.1007/s004410000198
56. Ljunggren HG, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J Exp Med* (1985) 162:1745–59. doi:10.1084/jem.162.6.1745
57. Stewart IJ, Peel S. Mouse metrial gland cells do not kill Yac-1 myeloma cells. *J Reprod Immunol* (1993) 24:165–71. doi:10.1016/0165-0378(93)90018-D
58. Boulenouar S, Doisne JM, Sferruzzi-Perri A, Gaynor LM, Kieckbusch J, Balmas E, et al. The residual innate lymphoid cells in NFIL3-deficient mice support suboptimal maternal adaptations to pregnancy. *Front Immunol* (2016) 7:43. doi:10.3389/fimmu.2016.00043
59. Montaldo E, Vacca P, Chiossone L, Croxatto D, Loiacono F, Martini S, et al. Unique Eomes+ NK cell subsets are present in uterus and decidua during early pregnancy. *Front Immunol* (2016) 6:646. doi:10.3389/fimmu.2015.00646
60. Eberl G, Colonna M, Di Santo JP, Mckenzie AN. Innate lymphoid cells: a new paradigm in immunology. *Science* (2015) 348:aaa6566. doi:10.1126/science.aaa6566
61. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science* (2011) 331:44–9. doi:10.1126/science.1198687
62. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells – a proposal for uniform nomenclature. *Nat Rev Immunol* (2013) 13:145–9. doi:10.1038/nri3365
63. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* (2015) 517:293–301. doi:10.1038/nature14189
64. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol* (2016) 17:765–74. doi:10.1038/ni.3489
65. Tait Wojno ED, Artis D. Emerging concepts and future challenges in innate lymphoid cell biology. *J Exp Med* (2016) 213:2229–48. doi:10.1084/jem.20160525
66. Juelke K, Romagnani C. Differentiation of human innate lymphoid cells (ILCs). *Curr Opin Immunol* (2016) 38:75–85. doi:10.1016/j.coi.2015.11.005
67. Simoni Y, Fehlings M, Kloverpris HN, Mcgovern N, Koo SL, Loh CY, et al. Human innate lymphoid cell subsets possess tissue-type based heterogeneity in phenotype and frequency. *Immunity* (2017) 46:148–61. doi:10.1016/j.immuni.2016.11.005
68. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol* (2011) 12:21–7. doi:10.1038/ni.1962
69. Male V, Hughes T, Mcclory S, Colucci F, Caligiuri MA, Moffett A. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *J Immunol* (2010) 185:3913–8. doi:10.4049/jimmunol.1001637
70. Vacca P, Montaldo E, Croxatto D, Loiacono F, Canegallo F, Venturini PL, et al. Identification of diverse innate lymphoid cells in human decidua. *Mucosal Immunol* (2015) 8:254–64. doi:10.1038/mi.2014.63
71. Vacca P, Vitale C, Montaldo E, Conte R, Cantoni C, Fulcheri E, et al. CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proc Natl Acad Sci U S A* (2011) 108:2402–7. doi:10.1073/pnas.1016257108
72. Hughes T, Briercheck EL, Freud AG, Trotta R, Mcclory S, Scoville SD, et al. The transcription Factor Ahr prevents the differentiation of a stage 3 innate lymphoid cell subset to natural killer cells. *Cell Rep* (2014) 8:150–62. doi:10.1016/j.celrep.2014.05.042

73. Croy BA. Hasn't the time come to replace the term metrial gland? *J Reprod Immunol* (1999) 42:127–9; discussion 131–4.
74. Croy BA, Van Den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol Rev* (2006) 214:161–85. doi:10.1111/j.1600-065X.2006.00447.x
75. Cupedo T, Mebius RE. Cellular interactions in lymph node development. *J Immunol* (2005) 174:21–5. doi:10.4049/jimmunol.174.1.21
76. Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) 157:340–56. doi:10.1016/j.cell.2014.03.030
77. Male V, Nisoli I, Kostrzewski T, Allan DS, Carlyle JR, Lord GM, et al. The transcription factor E4bp4/Nfil3 controls commitment to the NK lineage and directly regulates Eomes and Id2 expression. *J Exp Med* (2014) 211:635–42. doi:10.1084/jem.20132398
78. Colucci F, Caligiuri MA, Di Santo JP. What does it take to make a natural killer? *Nat Rev Immunol* (2003) 3:413–25. doi:10.1038/nri1088
79. Fathman JW, Bhattacharya D, Inlay MA, Seita J, Karsunky H, Weissman IL. Identification of the earliest natural killer cell–committed progenitor in murine bone marrow. *Blood* (2011) 118:5439–47. doi:10.1182/blood-2011-04-348912
80. Rosmaraki EE, Douagi I, Roth C, Colucci F, Cumano A, Di Santo JP. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* (2001) 31:1900–9. doi:10.1002/1521-4141(200106)31:6<1900::AID-IMMU1900>3.0.CO;2-M
81. Vosshenrich CA, Ranson T, Samson SI, Corcuff E, Colucci F, Rosmaraki EE, et al. Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J Immunol* (2005) 174:1213–21. doi:10.4049/jimmunol.174.3.1213
82. Kim S, Iizuka K, Kang H-SP, Dokun A, French AR, Greco S, et al. In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol* (2002) 3:523–8. doi:10.1038/ni796
83. Vosshenrich CA, Samson-Villeger SI, Di Santo JP. Distinguishing features of developing natural killer cells. *Curr Opin Immunol* (2005) 17:151–8. doi:10.1016/j.coi.2005.01.005
84. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. *Blood* (2009) 113:5488–96. doi:10.1182/blood-2008-10-187179
85. Vosshenrich CAJ, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Goff OR-L, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol* (2006) 7:1217–24. doi:10.1038/ni1395
86. Seillet C, Huntington ND, Gangatirkar P, Axelsson E, Minnich M, Brady HJ, et al. Differential requirement for Nfil3 during NK cell development. *J Immunol* (2014) 192:2667–76. doi:10.4049/jimmunol.1302605
87. Cortez VS, Fuchs A, Cella M, Gilfillan S, Colonna M. Cutting edge: salivary gland NK cells develop independently of Nfil3 in steady-state. *J Immunol* (2014) 192:4487–91. doi:10.4049/jimmunol.1303469
88. Peng H, Jiang X, Chen Y, Sojka DK, Wei H, Gao X, et al. Liver-resident NK cells confer adaptive immunity in skin-contact inflammation. *J Clin Invest* (2013) 123:1444–56. doi:10.1172/JCI66381
89. Takeda K, Cretney E, Hayakawa Y, Ota T, Akiba H, Ogasawara K, et al. TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood* (2005) 105:2082–9. doi:10.1182/blood-2004-08-3262
90. Warren HS, Smyth MJ. NK cells and apoptosis. *Immunol Cell Biol* (1999) 77:64–75. doi:10.1046/j.1440-1711.1999.00790.x
91. Renoux VM, Zriwil A, Peitzsch C, Michaelsson J, Friberg D, Soneji S, et al. Identification of a human natural killer cell lineage-restricted progenitor in fetal and adult tissues. *Immunity* (2015) 43:394–407. doi:10.1016/j.immuni.2015.07.011
92. Freud AG, Becknell B, Roychowdhury S, Mao HC, Ferketich AK, Nuovo GJ, et al. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* (2005) 22:295–304. doi:10.1016/j.immuni.2005.01.013
93. Freud AG, Caligiuri MA. Human natural killer cell development. *Immunol Rev* (2006) 214:56–72. doi:10.1111/j.1600-065X.2006.00451.x
94. Knox JJ, Cosma GL, Betts MR, Mclane LM. Characterization of T-bet and eomes in peripheral human immune cells. *Front Immunol* (2014) 5:217. doi:10.3389/fimmu.2014.00217
95. Marquardt N, Beziat V, Nystrom S, Hengst J, Ivarsson MA, Kekalainen E, et al. Cutting edge: identification and characterization of human intrahepatic CD49a+ NK cells. *J Immunol* (2015) 194:2467–71. doi:10.4049/jimmunol.1402756
96. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity* (2013) 38:769–81. doi:10.1016/j.immuni.2013.02.010
97. Manaster I, Mandelboim O. The unique properties of uterine NK cells. *Am J Reprod Immunol* (2010) 63:434–44. doi:10.1111/j.1600-0897.2009.00794.x
98. Peel S, Stewart I. The differentiation of granulated metrial gland cells in chimeric mice and the effect of uterine shielding during irradiation. *J Anat* (1984) 139(Pt 4):593–8.
99. Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC, et al. Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *J Immunol* (2002) 168:22–8. doi:10.4049/jimmunol.168.1.22
100. Lysiak JJ, Lala PK. In situ localization and characterization of bone marrow-derived cells in the decidua of normal murine pregnancy. *Biol Reprod* (1992) 47:603–13. doi:10.1095/biolreprod47.4.603
101. Taylor HS. Endometrial cells derived from donor stem cells in bone marrow transplant recipients. *JAMA* (2004) 292:81–5. doi:10.1001/jama.292.1.81
102. Cerdeira AS, Rajakumar A, Royle CM, Lo A, Husain Z, Thadhani RI, et al. Conversion of peripheral blood NK cells to a decidual NK-like phenotype by a cocktail of defined factors. *J Immunol* (2013) 190:3939–48. doi:10.4049/jimmunol.1202582
103. Verma S, Hiby SE, Loke YW, King A. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. *Biol Reprod* (2000) 62:959–68. doi:10.1095/biolreprod62.4.959
104. Matsuura-Sawada R, Murakami T, Ozawa Y, Nabeshima H, Akahira J, Sato Y, et al. Reproduction of menstrual changes in transplanted human endometrial tissue in immunodeficient mice. *Hum Reprod* (2005) 20:1477–84. doi:10.1093/humrep/deh783
105. Chiossone L, Vacca P, Orecchia P, Croxatto D, Damonte P, Astigiano S, et al. In vivo generation of decidual natural killer cells from resident hematopoietic progenitors. *Haematologica* (2014) 99:448–57. doi:10.3324/haematol.2013.091421
106. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* (2001) 22:633–40. doi:10.1016/S1471-4906(01)02060-9
107. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* (2004) 75:163–89. doi:10.1189/jlb.0603252
108. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* (1989) 320:1731–5. doi:10.1056/NEJM198906293202605
109. Orange JS. Natural killer cell deficiency. *J Allergy Clin Immunol* (2013) 132:515–26. doi:10.1016/j.jaci.2013.07.020
110. Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci U S A* (2000) 97:2731–6. doi:10.1073/pnas.050588297
111. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* (2001) 409:1055–60. doi:10.1038/35059110
112. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol* (2013) 31:413–41. doi:10.1146/annurev-immunol-032712-095951
113. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol* (2013) 31:227–58. doi:10.1146/annurev-immunol-020711-075005
114. Kelley J, Walter L, Trowsdale J. Comparative genomics of natural killer cell receptor gene clusters. *PLoS Genet* (2005) 1(2):e27. doi:10.1371/journal.pgen.0010027
115. Beziat V, Hilton HG, Norman PJ, Traherne JA. Deciphering the killer-cell immunoglobulin-like receptor system at super-resolution for natural killer and T-cell biology. *Immunology* (2016) 150:248–264. doi:10.1111/imm.12684
116. Carrington M, Norman P. The Kir Gene Cluster. *The KIR Gene Cluster [Internet]*. Bethesda, MD: National Center for Biotechnology Information (US) (2003). Available from: http://www.ncbi.nlm.nih.gov/books/NBK10134

117. Robinson J, Halliwell JA, Hayhurst JD, Flicek P, Parham P, Marsh SG. The IPD and IMGT/HLA database: allele variant databases. *Nucleic Acids Res* (2015) 43:D423–31. doi:10.1093/nar/gku1161
118. Colonna M, Spies T, Strominger JL, Ciccone E, Moretta A, Moretta L, et al. Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci U S A* (1992) 89:7983–5. doi:10.1073/pnas.89.17.7983
119. Hilton HG, Guethlein LA, Goyos A, Nemat-Gorgani N, Bushnell DA, Norman PJ, et al. Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. *J Immunol* (2015) 195:3160–70. doi:10.4049/jimmunol.1501358
120. Proteau MF, Rousselle E, Makrigiannis AP. Mapping of the BALB/c Ly49 cluster defines a minimal natural killer cell receptor gene repertoire. *Genomics* (2004) 84:669–77. doi:10.1016/j.ygeno.2004.05.004
121. Belanger S, Tai LH, Anderson SK, Makrigiannis AP. Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome. *Genes Immun* (2008) 9:509–21. doi:10.1038/gene.2008.43
122. Colucci F, Di Santo JP, Leibson PJ. Natural killer cell activation in mice and men: different triggers for similar weapons? *Nat Immunol* (2002) 3:807–13. doi:10.1038/ni0902-807
123. Mason LH, Gosselin P, Anderson SK, Fogler WE, Ortaldo JR, Mcvicar DW. Differential tyrosine phosphorylation of inhibitory versus activating Ly-49 receptor proteins and their recruitment of SHP-1 phosphatase. *J Immunol* (1997) 159:4187–96.
124. Smith KM, Wu J, Bakker AB, Phillips JH, Lanier LL. Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. *J Immunol* (1998) 161:7–10.
125. Schenkel AR, Kingry LC, Slayden RA. The ly49 gene family. A brief guide to the nomenclature, genetics, and role in intracellular infection. *Front Immunol* (2013) 4:90. doi:10.3389/fimmu.2013.00090
126. Daniels KA, Devora G, Lai WC, O'Donnell CL, Bennett M, Welsh RM. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J Exp Med* (2001) 194:29–44. doi:10.1084/jem.194.1.29
127. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* (2002) 296:1323–6. doi:10.1126/science.1070884
128. Hanke T, Takizawa H, Mcmahon CW, Busch DH, Pamer EG, Miller JD, et al. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* (1999) 11:67–77. doi:10.1016/S1074-7613(00)80082-5
129. Scarpellino L, Oeschger F, Guillaume P, Coudert JD, Levy F, Leclercq G, et al. Interactions of Ly49 family receptors with MHC class I ligands in trans and cis. *J Immunol* (2007) 178:1277–84. doi:10.4049/jimmunol.178.3.1277
130. Andrews DM, Sullivan LC, Baschuk N, Chan CJ, Berry R, Cotterell CL, et al. Recognition of the nonclassical MHC class I molecule H2-M3 by the receptor Ly49A regulates the licensing and activation of NK cells. *Nat Immunol* (2012) 13:1171–7. doi:10.1038/ni.2468
131. George TC, Mason LH, Ortaldo JR, Kumar V, Bennett M. Positive recognition of MHC class I molecules by the Ly49D receptor of murine NK cells. *J Immunol* (1999) 162:2035–43.
132. Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J Immunol* (1996) 157:4741–5.
133. Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity* (1998) 8:693–701. doi:10.1016/S1074-7613(00)80574-9
134. Orbelyan GA, Tang F, Sally B, Solus J, Meresse B, Ciszewski C, et al. Human NKG2E is expressed and forms an intracytoplasmic complex with CD94 and DAP12. *J Immunol* (2014) 193:610–6. doi:10.4049/jimmunol.1400556
135. Lohwasser S, Hande P, Mager DL, Takei F. Cloning of murine NKG2A, B and C: second family of C-type lectin receptors on murine NK cells. *Eur J Immunol* (1999) 29:755–61. doi:10.1002/(SICI)1521-4141(199903)29:03<755::AID-IMMU755>3.3.CO;2-O
136. Fang M, Orr MT, Spee P, Egebjerg T, Lanier LL, Sigal LJ. CD94 is essential for NK cell-mediated resistance to a lethal viral disease. *Immunity* (2011) 34:579–89. doi:10.1016/j.immuni.2011.02.015
137. Braud VM, Allan DSJ, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* (1998) 391:795–9. doi:10.1038/35869
138. Kaiser BK, Barahmand-Pour F, Paulsene W, Medley S, Geraghty DE, Strong RK. Interactions between NKG2x immunoreceptors and HLA-E ligands display overlapping affinities and thermodynamics. *J Immunol* (2005) 174:2878–84. doi:10.4049/jimmunol.174.5.2878
139. Raulet DH, Vance RE, Mcmahon CW. Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol* (2001) 19:291–330. doi:10.1146/annurev.immunol.19.1.291
140. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song Y-J, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* (2005) 436:709–13. doi:10.1038/nature03847
141. Yawata M, Yawata N, Draghi M, Partheniou F, Little AM, Parham P. MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. *Blood* (2008) 112:2369–80. doi:10.1182/blood-2008-03-143727
142. Brodin P, Lakshmikanth T, Karre K, Hoglund P. Skewing of the NK cell repertoire by MHC class I via quantitatively controlled enrichment and contraction of specific Ly49 subsets. *J Immunol* (2012) 188:2218–26. doi:10.4049/jimmunol.1102801
143. Sternberg-Simon M, Brodin P, Pickman Y, Onfelt B, Karre K, Malmberg KJ, et al. Natural killer cell inhibitory receptor expression in humans and mice: a closer look. *Front Immunol* (2013) 4:65. doi:10.3389/fimmu.2013.00065
144. Johansson S, Johansson M, Rosmaraki E, Vahlne G, Mehr R, Salmon-Divon M, et al. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J Exp Med* (2005) 201:1145–55. doi:10.1084/jem.20050167
145. Brodin P, Lakshmikanth T, Johansson S, Kärre K, Höglund P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* (2008) 113:2434–41. doi:10.1182/blood-2008-05-156836
146. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* (2005) 105:4416–23. doi:10.1182/blood-2004-08-3156
147. Champsaur M, Beilke JN, Ogasawara K, Koszinowski UH, Jonjic S, Lanier LL. Intact NKG2D-independent function of NK cells chronically stimulated with the NKG2D ligand Rae-1. *J Immunol* (2010) 185:157–65. doi:10.4049/jimmunol.1000397
148. Fauriat C, Ivarsson MA, Ljunggren H-G, Malmberg K-J, Michaëlsson J. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* (2010) 115:1166–74. doi:10.1182/blood-2009-09-245746
149. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* (2002) 295:2097–100. doi:10.1126/science.1068440
150. Moffett A, Colucci F. Uterine NK cells: active regulators at the maternal-fetal interface. *J Clin Invest* (2014) 124:1872–9. doi:10.1172/JCI68107
151. Medawar PB. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symp Soc Exp Biol* (1953) 7:320–38.
152. Watson ED, Cross JC. Development of structures and transport functions in the mouse placenta. *Physiology* (2005) 20:180–93. doi:10.1152/physiol.00001.2005
153. Croy BA, He H, Esadeg S, Wei Q, Mccartney D, Zhang J, et al. Uterine natural killer cells: insights into their cellular and molecular biology from mouse modelling. *Reproduction* (2003) 126:149–60. doi:10.1530/rep.0.1260149
154. Georgiades P, Ferguson-Smith AC, Burton GJ. Comparative developmental anatomy of the murine and human definitive placentae. *Placenta* (2002) 23:3–19. doi:10.1053/plac.2001.0738
155. Benirschke K, Kaufmann P, Baergen RN. Characterization of the developmental stages. 5th ed. In: Benirschke K, Kaufmann P, Baergen RN, editors. *Pathology of the Human Placenta*. New York, NY: Springer (2006). p. 174–90.
156. Benirschke K, Kaufmann P, Baergen RN. Early development of the human placenta. 5th ed. In: Benirschke K, Kaufmann P, Baergen RN, editors. *Pathology of the Human Placenta*. New York, NY: Springer (2006). p. 42–50.
157. Mu J, Adamson SL. Developmental changes in hemodynamics of uterine artery, utero- and umbilicoplacental, and vitelline circulations in mouse

throughout gestation. *Am J Physiol Heart Circ Physiol* (2006) 291:H1421–8. doi:10.1152/ajpheart.00031.2006
158. Burton GJ, Woods AW, Jauniaux E, Kingdom JC. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. *Placenta* (2009) 30:473–82. doi:10.1016/j.placenta.2009.02.009
159. Kam EPY, Gardner L, Loke YW, King A. The role of trophoblast in the physiological change in decidual spiral arteries. *Hum Reprod* (1999) 14:2131–8. doi:10.1093/humrep/14.8.2131
160. Smith SD, Dunk CE, Aplin JD, Harris LK, Jones RL. Evidence for immune cell involvement in decidual spiral arteriole remodeling in early human pregnancy. *Am J Pathol* (2009) 174:1959–71. doi:10.2353/ajpath.2009.080995
161. Hazan AD, Smith SD, Jones RL, Whittle W, Lye SJ, Dunk CE. Vascular-leukocyte interactions: mechanisms of human decidual spiral artery remodeling in vitro. *Am J Pathol* (2010) 177:1017–30. doi:10.2353/ajpath.2010.091105
162. Anacker J, Segerer SE, Hagemann C, Feix S, Kapp M, Bausch R, et al. Human decidua and invasive trophoblasts are rich sources of nearly all human matrix metalloproteinases. *Mol Hum Reprod* (2011) 17:637–52. doi:10.1093/molehr/gar033
163. Pijnenborg R, Bland JM, Robertson WB, Brosens I. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta* (1983) 4:397–413. doi:10.1016/S0143-4004(83)80043-5
164. Lyall F. The human placental bed revisited. *Placenta* (2002) 23:555–62. doi:10.1053/plac.2002.0850
165. Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta* (2006) 27:939–58. doi:10.1016/j.placenta.2005.12.006
166. Adamson SL, Lu Y, Whiteley KJ, Holmyard D, Hemberger M, Pfarrer C, et al. Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol* (2002) 250:358–73. doi:10.1006/dbio.2002.0773
167. Zhang J, Chen Z, Smith GN, Croy BA. Natural killer cell-triggered vascular transformation: maternal care before birth? *Cell Mol Immunol* (2011) 8:1–11. doi:10.1038/cmi.2010.38
168. Guimond MJ, Luross JA, Wang B, Terhorst C, Danial S, Croy BA. Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice. *Biol Reprod* (1997) 56:169–79. doi:10.1095/biolreprod56.1.169
169. Greenwood JD, Minhas K, Di Santo JP, Makita M, Kiso Y, Croy BA. Ultrastructural studies of implantation sites from mice deficient in uterine natural killer cells. *Placenta* (2000) 21:693–702. doi:10.1053/plac.2000.0556
170. Ashkar AA, Black GP, Wei Q, He H, Liang L, Head JR, et al. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. *J Immunol* (2003) 171:2937–44. doi:10.4049/jimmunol.171.6.2937
171. Ashkar AA, Croy BA. Interferon-gamma contributes to the normalcy of murine pregnancy. *Biol Reprod* (1999) 61:493–502. doi:10.1095/biolreprod61.2.493
172. Kaitu'u TJ, Shen J, Zhang J, Morison NB, Salamonsen LA. Matrix metalloproteinases in endometrial breakdown and repair: functional significance in a mouse model. *Biol Reprod* (2005) 73:672–80. doi:10.1095/biolreprod.105.042473
173. Fontana V, Coll TA, Sobarzo CM, Tito LP, Calvo JC, Cebral E. Matrix metalloproteinase expression and activity in trophoblast-decidual tissues at organogenesis in CF-1 mouse. *J Mol Histol* (2012) 43:487–96. doi:10.1007/s10735-012-9429-8
174. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest* (2002) 110:625–32. doi:10.1172/JCI0215334
175. Chakraborty D, Rumi MA, Konno T, Soares MJ. Natural killer cells direct hemochorial placentation by regulating hypoxia-inducible factor dependent trophoblast lineage decisions. *Proc Natl Acad Sci U S A* (2011) 108:16295–300. doi:10.1073/pnas.1109478108
176. Ain R, Canham LN, Soares MJ. Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Dev Biol* (2003) 260:176–90. doi:10.1016/S0012-1606(03)00210-0
177. Kennedy PR, Chazara O, Gardner L, Ivarsson MA, Farrell LE, Xiong S, et al. Activating KIR2DS4 is expressed by uterine NK cells and contributes to successful pregnancy. *J Immunol* (2016) 197:4292–300. doi:10.4049/jimmunol.1601279
178. Xiong S, Sharkey AM, Kennedy PR, Gardner L, Farrell LE, Chazara O, et al. Maternal uterine NK cell-activating receptor KIR2DS1 enhances placentation. *J Clin Invest* (2013) 123:4264–72. doi:10.1172/JCI68991
179. Vacca P, Cantoni C, Prato C, Fulcheri E, Moretta A, Moretta L, et al. Regulatory role of NKp44, NKp46, Dnam-1 and NKG2D receptors in the interaction between NK cells and trophoblast cells. Evidence for divergent functional profiles of decidual versus peripheral NK cells. *Int Immunol* (2008) 20:1395–405. doi:10.1093/intimm/dxn105
180. Jovanović M, Stefanoska I, Radojčić L, Vićovac L. Interleukin-8 (CXCL8) stimulates trophoblast cell migration and invasion by increasing levels of matrix metalloproteinase (MMP)2 and MMP9 and integrins α5 and β1. *Reproduction* (2010) 139:789–98. doi:10.1530/REP-09-0341
181. Apps R, Sharkey A, Gardner L, Male V, Kennedy P, Masters L, et al. Ex vivo functional responses to HLA-G differ between blood and decidual NK cells. *Mol Hum Reprod* (2011) 17:577–86. doi:10.1093/molehr/gar022
182. Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Inhibition of trophoblast cell invasion by TGFB1, 2, and 3 is associated with a decrease in active proteases. *Biol Reprod* (2005) 73:374–81. doi:10.1095/biolreprod.105.040337
183. Fraser R, Whitley GS, Johnstone AP, Host AJ, Sebire NJ, Thilaganathan B, et al. Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *J Pathol* (2012) 228:322–32. doi:10.1002/path.4057
184. Lash GE, Otun HA, Innes BA, Percival K, Searle RF, Robson SC, et al. Regulation of extravillous trophoblast invasion by uterine natural killer cells is dependent on gestational age. *Hum Reprod* (2010) 25:1137–45. doi:10.1093/humrep/deq050
185. Tayade C, Hilchie D, He H, Fang Y, Moons L, Carmeliet P, et al. Genetic deletion of placenta growth factor in mice alters uterine NK cells. *J Immunol* (2007) 178:4267–75. doi:10.4049/jimmunol.178.7.4267
186. Croxatto D, Vacca P, Canegallo F, Conte R, Venturini PL, Moretta L, et al. Stromal cells from human decidua exert a strong inhibitory effect on NK cell function and dendritic cell differentiation. *PLoS One* (2014) 9:e89006. doi:10.1371/journal.pone.0089006
187. El Costa H, Casemayou A, Aguerre-Girr M, Rabot M, Berrebi A, Parant O, et al. Critical and differential roles of NKp46- and NKp30-activating receptors expressed by uterine NK cells in early pregnancy. *J Immunol* (2008) 181:3009–17. doi:10.4049/jimmunol.181.5.3009
188. Siewiera J, Gouilly J, Hocine HR, Cartron G, Levy C, Al-Daccak R, et al. Natural cytotoxicity receptor splice variants orchestrate the distinct functions of human natural killer cell subtypes. *Nat Commun* (2015) 6:10183. doi:10.1038/ncomms10183
189. Jauniaux E, Zaidi J, Jurkovic D, Campbell S, Hustin J. Comparison of colour Doppler features and pathological findings in complicated early pregnancy. *Hum Reprod* (1994) 9:2432–7. doi:10.1093/oxfordjournals.humrep.a138465
190. Khong TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. *Br J Obstet Gynaecol* (1986) 93:1049–59. doi:10.1111/j.1471-0528.1986.tb07830.x
191. Lyall F, Robson SC, Bulmer JN. Spiral artery remodeling and trophoblast invasion in preeclampsia and fetal growth restriction: relationship to clinical outcome. *Hypertension* (2013) 62:1046–54. doi:10.1161/HYPERTENSIONAHA.113.01892
192. Pijnenborg R. The human decidua as a passage-way for trophoblast invasion: a review. *Placenta* (1998) 19(Suppl 1):229–41. doi:10.1016/S0143-4004(98)80017-9
193. Burke SD, Barrette VF, Bianco J, Thorne JG, Yamada AT, Pang SC, et al. Spiral arterial remodeling is not essential for normal blood pressure regulation in pregnant mice. *Hypertension* (2010) 55:729–37. doi:10.1161/HYPERTENSIONAHA.109.144253
194. Jauniaux E, Jurkovic D. Placenta accreta: pathogenesis of a 20th century iatrogenic uterine disease. *Placenta* (2012) 33:244–51. doi:10.1016/j.placenta.2011.11.010
195. Kemp B, Kertschanska S, Handt S, Funk A, Kaufmann P, Rath W. Different placentation patterns in viable compared with nonviable tubal pregnancy suggest a divergent clinical management. *Am J Obstet Gynecol* (1999) 181:615–20. doi:10.1016/S0002-9378(99)70501-6

196. Hunt JS, Fishback JL, Chumbley G, Loke YW. Identification of class I MHC mRNA in human first trimester trophoblast cells by in situ hybridization. *J Immunol* (1990) 144:4420–5.
197. Apps R, Murphy SP, Fernando R, Gardner L, Ahad T, Moffett A. Human leucocyte antigen (Hla) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology* (2009) 127:26–39. doi:10.1111/j.1365-2567.2008.03019.x
198. Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K, Marquardt H, et al. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. *J Immunol* (2003) 171:1376–84. doi:10.4049/jimmunol.171.3.1376
199. Van Der Meer A, Lukassen HG, Van Cranenbroek B, Weiss EH, Braat DD, Van Lierop MJ, et al. Soluble HLA-G promotes Th1-type cytokine production by cytokine-activated uterine and peripheral natural killer cells. *Mol Hum Reprod* (2007) 13:123–33. doi:10.1093/molehr/gal100
200. Moradi S, Berry R, Pymm P, Hitchen C, Beckham SA, Wilce MC, et al. The structure of the atypical killer cell immunoglobulin-like receptor, KIR2DL4. *J Biol Chem* (2015) 290:10460–71. doi:10.1074/jbc.M114.612291
201. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med* (1999) 189:1093–100. doi:10.1084/jem.189.7.1093
202. Graef T, Moesta AK, Norman PJ, Abi-Rached L, Vago L, Older Aguilar AM, et al. KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for Hla-A*11 while diminishing avidity for HLA-C. *J Exp Med* (2009) 206:2557–72. doi:10.1084/jem.20091010
203. Apps R, Gardner L, Sharkey AM, Holmes N, Moffett A. A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1. *Eur J Immunol* (2007) 37:1924–37. doi:10.1002/eji.200737089
204. Vacca P, Pietra G, Falco M, Romeo E, Bottino C, Bellora F, et al. Analysis of natural killer cells isolated from human decidua: evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. *Blood* (2006) 108:4078–85. doi:10.1182/blood-2006-04-017343
205. Erlebacher A, Lukens AK, Glimcher LH. Intrinsic susceptibility of mouse trophoblasts to natural killer cell-mediated attack in vivo. *Proc Natl Acad Sci U S A* (2002) 99:16940–5. doi:10.1073/pnas.222652199
206. Philpott KL, Rastan S, Brown S, Mellor AL. Expression of H-2 class I genes in murine extra-embryonic tissues. *Immunology* (1988) 64:479–85.
207. Rodgers JR, Cook RG. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* (2005) 5:459–71. doi:10.1038/nri1635
208. Hiby SE, Walker JJ, O'Shaughnessy KM, Redman CW, Carrington M, Trowsdale J, et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* (2004) 200:957–65. doi:10.1084/jem.20041214
209. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, et al. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest* (2010) 120:4102–10. doi:10.1172/JCI43998
210. Hiby SE, Regan L, Lo W, Farrell L, Carrington M, Moffett A. Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. *Hum Reprod* (2008) 23:972–6. doi:10.1093/humrep/den011
211. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* (2005) 5:201–14. doi:10.1038/nri1570
212. Hiby SE, Apps R, Chazara O, Farrell LE, Magnus P, Trogstad L, et al. Maternal KIR in combination with paternal HLA-C2 regulate human birth weight. *J Immunol* (2014) 192:5069–73. doi:10.4049/jimmunol.1400577
213. Lima PD, Tu MM, Rahim MM, Peng AR, Croy BA, Makrigiannis AP. Ly49 receptors activate angiogenic mouse DBA(+) uterine natural killer cells. *Cell Mol Immunol* (2014) 11:467–76. doi:10.1038/cmi.2014.44
214. Belanger S, Tu MM, Rahim MM, Mahmoud AB, Patel R, Tai LH, et al. Impaired natural killer cell self-education and "missing-self" responses in Ly49-deficient mice. *Blood* (2012) 120:592–602. doi:10.1182/blood-2012-02-408732
215. Brown MG, Fulmek S, Matsumoto K, Cho R, Lyons PA, Levy ER, et al. A 2-Mb YAC contig and physical map of the natural killer gene complex on mouse chromosome 6. *Genomics* (1997) 42:16–25. doi:10.1006/geno.1997.4721

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on