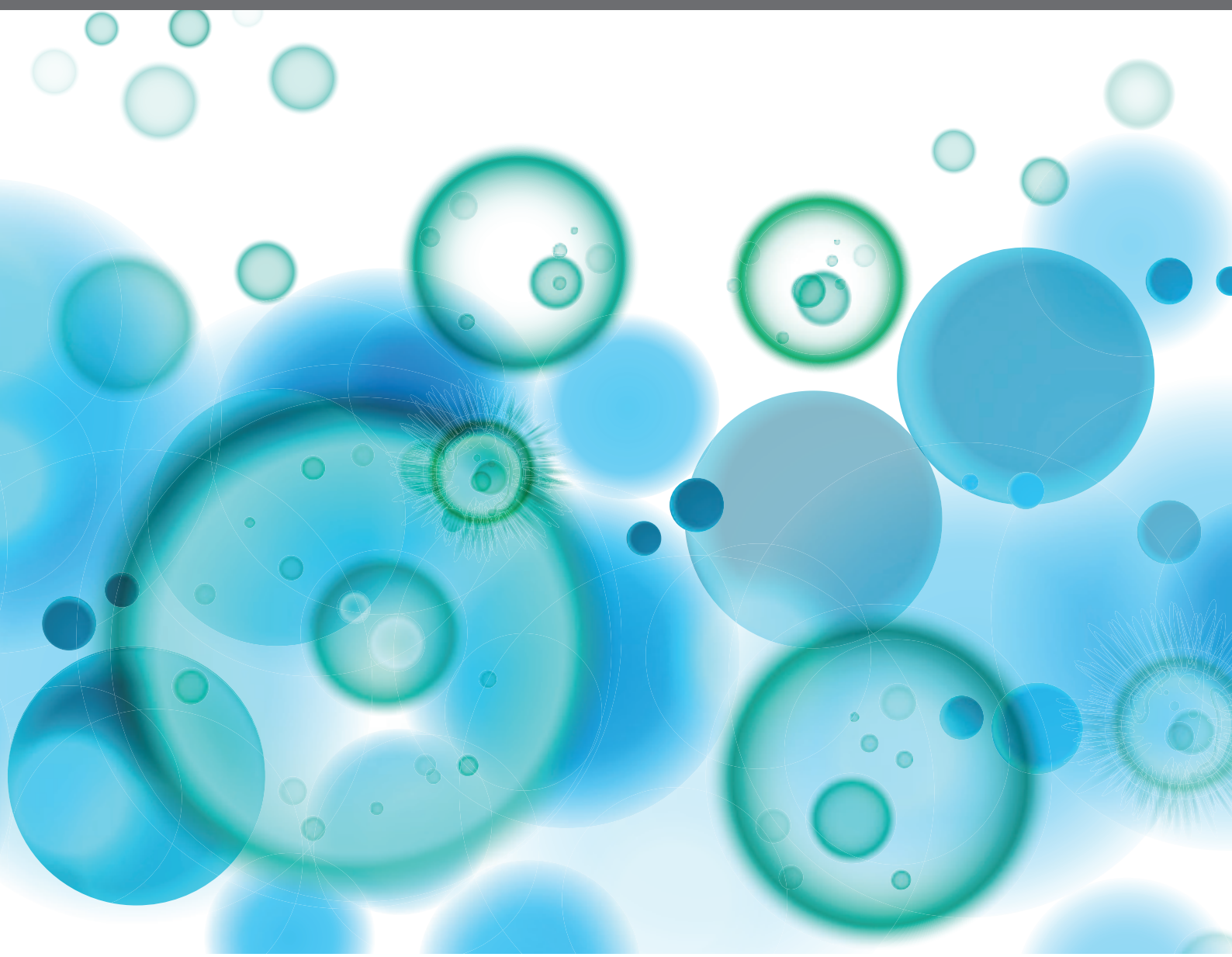
# EMERGING CONCEPTS ON THE NKG2D RECEPTOR- NKG2D LIGAND AXIS IN HEALTH AND DISEASES

EDITED BY: Nadia Guerra and Lewis L. Lanier
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88963-745-4
DOI 10.3389/978-2-88963-745-4

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# EMERGING CONCEPTS ON THE NKG2D RECEPTOR- NKG2D LIGAND AXIS IN HEALTH AND DISEASES

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**Citation:** Guerra, N., Lanier, L. L., eds. (2020). Emerging Concepts on the NKG2D Receptor- NKG2D Ligand Axis in Health and Diseases. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-745-4

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frontiers in Immunology

EDITORIAL
published: 07 April 2020
doi: 10.3389/fimmu.2020.00562

# Editorial: Emerging Concepts on the NKG2D Receptor-Ligand Axis in Health and Diseases

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**Keywords: NKG2D, natural killer cells, tumor immunology, autoimmunity, cytomegalovirus (CMV)**

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

***Received:*** *07 February 2020*
***Accepted:*** *12 March 2020*
***Published:*** *07 April 2020*

***Citation:***
*Guerra N and Lanier LL (2020) Editorial: Emerging Concepts on the NKG2D Receptor-Ligand Axis in Health and Diseases. Front. Immunol. 11:562. doi: 10.3389/fimmu.2020.00562*

## Editorial on the Research Topic

## Emerging Concepts on the NKG2D Receptor-Ligand Axis in Health and Diseases

The immune activating receptor NKG2D and its cognate ligands represent a fascinating immune recognition system, essential to the activation of innate and adaptive effector cells. It is a potent axis for the detection of danger recognized as MHC I-like self-molecules induced under stress conditions or during rapid proliferation. As such, it complements the recognition of non-self-antigens presented by classic MHC molecules, ensuring an early and efficient elimination of threats. The strength of the NKG2D pathway relies on its capacity to: (i) activate several immune effector cells due to the wide expression of the NKG2D receptor on lymphocytes, and (ii) distinguish diverse types of stress via multiple ligands displaying distinct affinities and levels of regulation.

The central dogma states that NKG2D engagement initiates or strengthens immune responses against infected and tumor cells by means of cytokine and chemokine secretion and direct cytotoxic activity. However, recently we have started to appreciate the multifaceted involvement of this pathway in immunity and how genetic polymorphism of the NKG2D-NKG2D ligand (NKG2DL) axis might explain the different and even contradictory outcome of NKG2D-driven responses observed across patients in certain disease context. Still, important gaps in knowledge remain regarding the regulation of human and mouse NKG2DL expression in space and time. The nature of ligand (binding affinity, membrane bound vs. soluble), the amount of ligand expressed and distribution at the membrane, and the cell types displaying ligands (tumor vs. normal tissue vs. stroma vs. immune cells) are likely to differentially impact on disease as it progresses and reciprocally be affected by it. Importantly, NKG2D ligand expression in healthy tissues is no longer anecdotal and it has become clear that NKG2D can contribute to the development of autoimmunity, highlighting the paradoxical outcome of NKG2D-NKG2DL engagement on disease progression (protective vs. detrimental), including in cancer.

This Research Topic gathers research articles and literature reviews from leaders in the NK and T cell biology fields highlighting recent advances on the expression and function of NKG2D and its

ligands. Together, these 17 articles provide a timely survey of this recognition pathway as a critical immunologic component of health and disease to engage in further discussion and developments in basic and translational immunology.

## EXPRESSION AND REGULATION OF NKG2D AND NKG2D LIGANDS

There are eight human ligands for NKG2D with differential expression, affinity, and ability to be released as soluble and exosomes-bound forms, accounting for the difficulty to fully understand their unique and concomitant role in complex pathologies. These ligands are the MHC class I-related chain A and B (MICA, MICB) proteins and the six unique long 16 (UL-16)-binding proteins (ULBP1-6). Their counterparts in mice are the retinoic acid early inducible gene-1 (RAE-1α-ε), minor histocompatibility H60a-c, and murine UL16-binding protein-like transcript 1 (MULT1) proteins. The multiplicity of ligands warrants an efficient immune recognition of cell extrinsic and intrinsic danger signals.

In the last two decades, extensive knowledge uncovered the different layers of regulation of most ligands at the transcriptional, post-transcriptional, and post-translational levels. Yet, the lack of a holistic characterization of the mechanisms driving the heterogeneous expression of one or multiple ligands in different cell types, tissues, and disease contexts hampers their manipulation for therapeutic purposes. Zingoni et al. provide a comprehensive overview of the various levels of regulation of human and mouse NKG2D ligands, focusing on the specificity and redundancy of the molecular pathways able to simultaneously control the expression of several NKG2D ligands in normal and pathological settings. Further, they discuss the functional relevance of these distinct ligands or allelic variants in their ability to modulate NKG2D-mediated signaling consequently affecting target cell killing.

NKG2D polymorphism has not been extensively studied despites the rising number of allelic variants identified to date, including over 100 known allelic variants for MICA and MICB. Zuo et al. provide a state-of-the-art review on the polymorphism and functional features of human NKG2D ligands and their connections with different diseases. This comprehensive review compares and contrasts polymorphisms within MICA and MICB to the ULBP genes family and discusses the evolutionary pressures that have driven NKG2DL polymorphism. Notably, the authors discuss how viral infections may have acted as a selective force toward differential tissue-specific regulation of the ligands, allowing effective anti-viral control while limiting autoimmunity. The influence of polymorphisms on the NKG2DL expression, describing how MICA variants dictate not only the amount of protein expressed but also its subcellular location and shedding; the binding affinity to NKG2D and how it impacts effector functions are discussed. The clinical relevance of NKG2DL polymorphisms is considered in the context of autoimmune diseases, viral infections, cancer, and transplantation with the goal to translate that knowledge to target the NKG2D-NKG2DL axis for therapeutic purpose.

ULBP4 is the most polymorphic member of the ULBP family, yet the least characterized ligand for NKG2D. Zöller et al. discuss inconsistencies between the biochemical features of ULBP4 in the literature and publicly available databases and undertook to perform further biochemical and expression analyses in three tumor cell lines: C1R, HepG32, and HeLa. The authors confirmed several features of the mature ULBP protein in line with the prediction by Uniprot, e.g., histidine 31 occupies the first position in the mature ULBP4 glycoprotein, not as previously assumed with glycine 29, providing evidence of a recessed β1 strand of the ULBP4 α1 domain that may impact protein folding and function compared with other ULBP molecules. They characterized isoform 0 as a variant with a divergent carboxy-terminus that results in a shortened mature glycoprotein. To further study isoform 0, they generated a novel ULBP4 mAb and compared the specificity of staining with commercially available antibodies, raising concerns for potential artifactual staining with the latter. Interestingly, the authors show that the cervical carcinoma cell line HeLa does not express membrane bound ULBP4s yet contains a large amount of ULBP4 retained intracellularly. Finally, the authors show that the release of soluble ULBP4 is partly due to metalloproteases and possibly due to alternative splicing of the full-length isoform 0. These novel interesting findings will stimulate future efforts to address ULBP4 expression *in situ* and its physiological relevance in cancer immunity.

The presence of soluble NKG2D ligands in cord blood plasma (CBP) has been shown to be significantly higher than in adult plasma from healthy individuals. Here, Cox et al. examined the expression of different allelic variants for MICA and MICB in the coding and promoter regions in CBP samples and observed that common MICB alleles, such as MICB*005:02, resulted in increased concentration of soluble MICB (sMICB) with the promoter polymorphisms P2, leading to elevated expression of soluble MICB while the opposite was observed with promoter polymorphisms P9. *In vitro* assays showed that increasing concentration of soluble ULBP1 strongly associated with lower percentage of IFNγ+ NK cells among stimulated PBMC and moderately associated with CD107a+ CBP-derived NK cells. In contrast, the concentration of neither sMICA nor sMICB affected expression of CD107a whatever cell type studied. Yet, when samples were stratified according to homozygous MICB allele or promoter type, MICB*005:02 with MICB-P2, induced a lower % of IFN-γ+ NK cells compared with MICB*008 with MICB-P9 type indicating differential amount and inhibitory potential for sMICB. While sMICB and sULBP1 were detected in all CBP samples, sMICA was detected in only a third of the samples. Most sMICA detected in CBP corresponded to the Val/Val dimorphism known to be associated with a weak binding affinity for NKG2D. Cells exposed to plasma from UCB samples homozygous for MICA-129val showed significantly increased percentage of IFN-γ and CD107a compared with

those from Met/Met or Val/Met samples indicating enhanced NK cell function. The authors propose a model that could contribute to fetal-maternal tolerance based on the presence of soluble NKG2D ligands in CBP. Future studies will need to address important pending questions including identifying the cell type(s) responsible for the presence of soluble ligands in CBP. It will be key to also demonstrate if and how various soluble ligand harboring different affinity (sMICB, sULBP1 vs. sMICA) may compete for NKG2D binding on fetal NK cells.

## NKG2D IN CYTOMEGALOVIRUS INFECTION

Cytomegalovirus (CMV) infection is a serious cause of morbidity in immuno-compromised transplant recipients with the majority of patients experiencing CMV re-infection or reactivation in the absence of anti-viral prophylactic treatment. Rohn et al. address this important topic in NK and T cell biology by determining the association of MICA and NKG2D polymorphisms with the clinical outcome of CMV. Both univariate and multivariate analyses of three functional SNPs in a cohort of 181 kidney donor-recipients' pairs revealed that the MICA rs2596538 GG donor genotype—known to be associated with elevated MICA expression- is significantly less frequent in patients displaying CMV disease during the first year after kidney transplant. Hence, they identify the G allele status as a protective prognostic determinant for CMV disease and the control of CMV viremia. Along with NK cells, CMV-specific $CD8^+$T and $CD4^+$T cells may be involved in controlling viral replication in an NKG2D-dependent manner. Future studies should aim to address the functional relevance and collaborative nature of these cell types, as well as the influence of soluble ligands during CMV re-infection or reactivation in transplanted patients.

With the goal to induce a strong and persistent immune response against CMV, Hiršl et al. explored the idea of developing a CMV vaccine vector expressing NKG2D ligands to generate protective T cell Immunity. The present studies are built upon previous work from the Jonjic lab where mouse CMV (MCMV) engineered to express RAE-1 in place of immune evasion gene m152 was proven to be highly attenuated yet retained the ability to induce a strong $CD8^+$ T cell response *in vivo*. Here, they replaced m145 with MULT-1, a higher affinity ligand for NKG2D than RAE-1, and showed that MULT-1-MCMV replication in various infected tissues was attenuated in an NK cell- and NKG2D-dependent manner. It could elicit a robust adaptive response when transferred into immunocompromised mice. Interestingly, the authors detected the presence of anti-CMV antibodies in infected mice and showed that protective immunity can be transferred to the offspring of immunized females. Hence, the ability for such attenuated virus to induce a durable $CD8^+$T cell memory response and a humoral response that can cross the placental barrier are critical features for its use as a vector vaccine. Safety concerns require further testing addressing whether attenuated virus can persist in the host and potentially be reactivated in immune-compromised patients, such as transplant recipients.

## NKG2D-NKG2D LIGANDS IN INFLAMMATION AND AUTOIMMUNITY

Various sources of stress can induce or upregulate NKG2D ligands in healthy tissues, including immune cells, in the context of inflammation. Four complementary review articles address an emerging topic in the function of NKG2D-NKG2DL interactions as a regulatory axis of immune responses broadening our views on the potential mechanisms accounting for the conflicting role for NKG2D reported in disease settings.

Trembath and Markiewicz review the growing literature regarding NKG2D ligand expression on healthy immune cells, including on activated T cells, B cells, NK cells, and myeloid cells. The discussion encompasses studies reporting opposing effects of ligand induction on immune cells, either killing, suppressing, and/or stimulating the proliferation and functional activities of NKG2D-expressing cells, with evidence of reciprocal effects during cell-cell interactions. They discuss how the "immunological multi-purposing" of NKG2D-NKG2DL interactions can fine-tune immune responses toward different biological outcome in immune homeostasis, tumor immunity, and autoimmune diseases. More recently, the authors further reported the constitutive expression of ULBP-4 on healthy monocytes and resulting effect in downregulating NKG2D expression on NK cells (1).

Wensveen et al. further discuss the role of NKG2D in controlling the activation thresholds of T cells, NK cells, and B cells. They examine evidence supporting that depending on the intensity and duration of ligand engagement, NKG2D can mediate both stimulatory and inhibitory signals impacting on NK cell development and education. The authors further discuss the anergy in T cells and NK cells induced by chronic NKG2D stimulation and how it can be overcome in a sufficiently inflammatory environment. Finally, they discuss unexpected findings by their group demonstrating that the lack of NKG2D alters mouse B1a cell development at an early pro-B progenitors and reduce the anti-microbial activity of this subset, which are mainly present in the peritoneal and pleural cavities of mice. Further studies are required to support the hypothetical existence of NKG2D signaling in an early progenitor common to NK cells and B cells and consequent impact on gene expression relevant to lineage commitment at later stages of B cell development.

Stojanovic et al. provide an extensive review on NKG2D ligand expression on immune cells with a particular focus on myeloid cells, discussing evidence of NK cell crosstalk with dendritic cells and macrophages. The authors examine the data generated from different types of infectious agents including bacteria, viruses, and parasites that cause NKG2DL expression in myeloid cells, likely through TLR signaling. They highlight several *in vitro* studies reporting that other factors produced by stromal, parenchymal, and immune cells in response to infection upregulate NKG2D and its ligands, contributing to a crosstalk between myeloid cells and NK cells. Besides infections,

the authors also discuss evidence for NKG2DL expression on myeloid cells infiltrating tumors and inflamed normal tissues under autoimmune attack from several clinical studies, as well as in experimental models. The nature of the NKG2DL that functionally impact an NKG2D-mediated crosstalk of myeloid cells and NKG2D+ lymphocytes and its outcome *in vivo* remains nonetheless understudied. Whether ILC1 and ILC3 via NKG2D also display the ability to communicate with myeloid cells under cellular stress is not known.

Besides immune cells, ligands for NKG2D are detected in healthy tissues under stress. Babic and Romagnani compare and contrast the literature on the role of the NKG2D-NKG2DL pathway in chronic inflammation and autoimmunity, discussing Type 1 diabetes, multiple sclerosis, Experimental Autoimmune Encephalomyelitis (EAE), Rheumatoid Arthritis (RA), celiac and Inflammatory Bowel diseases (IBD). They underline the presence of IL-15 as a common feature in most of these pathologies in priming $CD4^+$T cells and $CD8^+$T cells into cytotoxic cells and in driving the upregulation of NKG2D on those cells, as well as increasing NKG2DL expression on epithelial cells. Of importance, they discuss the enrichment of IL-17-producing cells among $NKG2D^+CD4^+$ T cells when compared to $NKG2D^-$ $CD4^+$ T cells in patients with Crohn's disease and in a model of colitis. With regards to intestinal inflammation, Hosomi et al. discuss novel findings from their group that uncovered the implication of endoplasmic reticulum (ER) stress as a novel mechanism for selective expression of the mouse NKG2D ligand MULT-1 on epithelial cells. Whether this is also the case in other autoimmune disease is not known, yet Babic and Romagnani highlight that the expression of MULT-1, along with RAE-1, in activated mouse oligodendrocytes led to their susceptibility to killing by activated $CD4^+$ T cells enriched for the expression of NKG2D. Finally, the authors highlight that these important considerations led to the development of strategies aiming to block NKG2D-mediated activation to effectively reduce pathogenesis, as tested in animal models of RA, EAE, and IBD and currently in phase 2 clinical trials in patients with active Crohn's disease.

Similar to the gut, the liver is a tolerogenic organ that harbors a large amount of innate lymphoid cells and under constant exposure to pathogens. One particular feature of the liver is the elevated presence of invariant Natural Killer T cells (iNKT), which constitute the main immune cell type residing in this tissue in mice. On this topic, Dulaimi et al. demonstrated the contribution of NKG2D on iNKT cells in liver inflammation. They employ a model of Con-A-induced hepatitis in NKG2D wild-type (WT) vs. deficient (knockout, KO) mice to assess the relevance of the NKG2D-NKG2DL pathway on iNKT cells. The authors showed an upregulation of RAE-1 on hepatocytes upon Con-A treatment associated with an increased production of IFNγ, TNFα, and IL-4 in liver iNKT cells of NKG2D-WT mice compared to the KO group. One key finding is that antibody-mediated NKG2D blockade ameliorates disease by reducing liver injury and increasing mouse survival, which correlated with reduced cytokine production and FASL expression by iNKT cells. Increased survival due to NKG2D blockade was not to the level comparable to CD1d-deficient mice, however, suggesting additional pathway(s) by which iNKT cells drive liver damage in this model. An added value of this work is the characterization of thymic and peripheral iNKT cells in NKG2D-deficient mice showing that they are phenotypically and functionally similar to wildtype mice. The literature on NKG2D in iNKT cells is scarce (2), these valuable studies clearly demonstrate the impact of NKG2D in NKT cell activation upon liver inflammation. Yet, the actual contribution of NKG2D on iNKT cells as opposed to NK cells and other T cell subsets in this model remains to be determined. Notably, these data further probe the deleterious effect driven by NKG2D in inflammation, feeding the model that NKG2D-mediated liver damage could contribute to tumor development as discuss by Sheppard et al. in the next section.

## NKG2D IN CANCER IMMUNITY

While the NKG2D-NKG2DL axis is recognized as an attractive target for immunotherapy against cancer and inflammatory disorders—more information is needed to optimally design and tailor strategies that either boost or block this peculiar pathway. Hosomi et al. review the mechanisms involved in the upregulation of NKG2DL expression in cells upon genotoxic stress. These include activation of the DNA damage pathway through the ATM or ATR protein kinases and through the stimulator of interferon genes (STING)-dependent pathway upon accumulation of cytosolic DNA. Less studied is the role of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) in tumor immunity. The authors discuss their recent findings along other groups' studies on the mechanisms that suppress NKG2D-NKG2DL-mediated killing of cancer cells. On tumor cells, CEACAM1 was shown to prevent NKG2D ligand cell surface expression via intracellular retention as an incompletely glycosylated protein. On NK cells, co-engagement of NKG2D and CEACAM1 suppresses NKG2D-mediated signaling in a SHP1-dependent manner, hence reducing NK cell cytotoxicity against CEACAM1-expressing tumor cells. These data evidence the impact of CEACAM1 as a negative regulator of NKG2D ligands on cancer cells.

The normal liver contains a large population of resident lymphocytes largely enriched in NK cells, yet it is the site of a number of ineffectively controlled chronic infections, of primary hepatocellular carcinoma (HCC), and metastasis from colorectal cancer (CRC). Easom et al. studied NK cells in HCC and secondary liver tumors arising from CRC metastasis and evidenced surprisingly high frequencies of NK cells infiltrating tumors—a large majority of which displaying a liver resident phenotype ($CXCR6^+CD69^+$). Intratumor NK cells displayed fairly high levels of cell surface NKG2D, albeit lower compared to NK cells located in healthy adjacent tissues. It would be very interesting to assess whether the presence and amount of NK cell infiltrating liver tumors correlates with disease prognosis in HCC and metastatic CRC patients. Further, the authors compared the level of granzyme B (GzB) expression between healthy peripheral NK, intrahepatic NK cells [including liver resident (lr) NK and non-lrNK cells] and tumor-infiltrating NK cells. They show a heterogeneous expression of $GzB^{hi}$ and $GzB^{low}$

expressing cells in intrahepatic and tumor-infiltrating NK cells which contrasted with the homogeneous $GzB^{hi}$ level detected in peripheral NK cells. The percentage of IFN-γ-producing NK cells was not significantly different in tumor vs. healthy liver, yet significantly lower than in circulating NK cells. Further studies will be needed to directly link the reduced NK cell functionality with NKG2D and NKG2DL expression in HCC patients. The authors also performed overnight coculture experiments of healthy intrahepatic NK cells with a MICA+ HCC-derived cell line and showed the ability of the HCC cell line to downregulate NKG2D and reduce NK cell activation. This effect was cell-cell contact dependent and NK cell activation could be restored by exposure to IL-15. Future work employing advanced cytometry to assess intertumoral NK cell functional heterogeneity (3) and autologous tumor cells in functional assays will be key, along with developing ways to use IL-15 to potentiate NKG2D-mediated response *in situ* with limited toxicity toward healthy hepatocytes.

The persistence of chronic inflammation is well-established as a contributing factor for tumor development, especially in the liver. The role of NKG2D-NKG2DL in this process is currently understudied, relying on a few clinical and experimental evidence and conflicting with its canonical function in tumor suppression. Sheppard et al. discuss the literature regarding the prognostic value for NKG2D ligands in various cancer types as well as experimental evidence supporting a tumor-promoting effect of NKG2D and $CD8^+$T cells in models of liver injury and cancer. A role for NK cells in HCC development is supported by recent evidence that the accumulation of $CD49a^+$ NK cells in liver tumors associates with tumor progression and poor clinical outcome (4). Sheppard et al. discussion stems from their recent finding that NKG2D promotes rather than delays tumor progression in a long-term model of chemically-induced liver cancer (5). These studies showed that HCC progressed more rapidly in NKG2D-WT than NKG2D-KO mice leading to increased tumor burden and tumor grade in a tissue that expresses elevated levels of membrane-bound NKG2DL. The authors propose that at early stages of tumor development, the expression of NKG2D ligands on precancerous lesions facilitate tumor clearance, however, over time, chronic activation of $NKG2D^+$ effector cells residing in the healthy liver combined with a partially or fully impaired NKG2D-dependent tumor rejection within the tumor bed could favor tumor progression. They postulate that in settings of chronic inflammation, NKG2D sustains a feedback loop of tissue injury and repair in the healthy tissue adjacent to the tumor which exacerbates the formation of inflammatory niches favorable for neotumor development. This hypothesis awaits to be supported by further demonstration in different models of cancer and by the identification of the cell type(s) contributing to the dual function of NKG2D. These findings stimulate further discussion and encourage careful considerations regarding the need to stratify patients that may benefit from NKG2D-based therapies.

Beyond NK cells and $CD8^+$T cells, the NKG2D-NKG2DL system is highly relevant to γδT cell-based therapy. Bhat et al. examined the effect of six pharmacological inhibitors of the epigenetic modifier histone deacetylases on the expression and shedding of several NKG2D ligands in a pancreatic and a prostate carcinoma cell line. Exposure to Valproic acid (VPA) significantly increased the cell surface expression of MICA, MICB, and ULBP2, but not ULBP1, and their release as soluble forms, highlighting discrepancy with data obtained in other cancer types. Decreased NKG2D expression at the protein and RNA levels were observed in activated Vδ2 T cells, but not PBMC when treated with VPA and co-cultured with tumor cell lines, albeit this did not affect their capacity to degranulate. The authors also established a flow cytometry-based method to distinguish high vs. low levels of histone acetylation (H3K9c) associated with changes in the relative distribution of Vδ2 T cell subpopulations when treated with VPA. While shedding further light on the epigenetic regulation of NKG2D and its ligands in γδ T cells and tumor cells, respectively, the biological significance of the findings remains to be demonstrated using fresh tumor tissues from prostate and pancreatic cancer patients to appreciate the implications in forecasting VPA treatment in combination with γδ T cell-based therapy.

With immunotherapy rapidly expanding as a novel option in the treatment landscape of several cancer types, two comprehensive reviews discuss the rationale for the development of immune-based therapies exploiting the NKG2D-NKG2DL axis. Frazao et al. review clinical studies that describe the role of the NKG2D-NKG2DL axis in cancer immunosurveillance indicating how NKG2D and NKG2D ligand polymorphisms affect cancer development and the patient response to chemotherapy. The authors review clinical studies reporting that low NK cell numbers within tumors associate with bad prognosis and that the expression profile of NK cell receptors strongly influence prognosis and disease outcome. Interestingly, they point out the exception of patients with chronic myeloid leukemia (CML) for whom NK cell counts are a significant predictive parameter for relapse after imatinib discontinuation. They further discuss the potential of Chimeric Antigen Receptor (CAR)-NK cells in complement or alternative to CAR-t cells, focusing on the benefits of employing NKG2D-CAR, an approach currently under trial.

Schmiedel and Mandelboim review focuses on the various mechanisms of NKG2D ligand regulation and how these can impact tumor evasion. The authors also discuss current approaches that target NKG2D-NKG2DL expression for cancer immunotherapy including approaches that directly or indirectly interfere with the release of soluble ligands in advanced cancer and those that aim to boost anti-tumor responses to overcome the immunosuppressive tumor milieu.

## CONCLUSIONS

Overall, these articles illustrate key findings on the expression and function of the NKG2D-NKG2DL axis and highlight current challenges in the field. Outstanding fundamental questions remain regarding the role of NKG2D in ILCs, how chronic NKG2D signaling may affect NK and T cell metabolic status potentially leading to a stage of "exhaustion," and how NKG2DL might play a role in cell recruitment to inflamed tissues. Further

work is also needed to generate a holistic view on the evolution of NKG2DL expression with disease stepwise progression over time and their prognostic and/or predictive value in cancer patients.

Several approaches are underway to enhance NKG2D-mediated tumor recognition *in situ* and upon adoptive transfer of NK cells or T cells to treat cancer patients. Concomitant with blocking inhibitory checkpoints and increasing NK cell longevity, these approaches hold great promise for an effective targeting of solid tumors with no tumor-associated antigens identified. The three main challenges yet to be overcome are how to deal with low level of ligands on advanced cancer cells and cancer stem cells (6), and how to favor migration of adoptively transferred cells to the tumor microenvironment rather than residing in the healthy tissues, hence limiting toxicity and autoimmunity.

## AUTHOR CONTRIBUTIONS

NG wrote the editorial. LL edited the editorial.

## FUNDING

LL was funded in part by the Parker Institute for Cancer Immunotherapy. NG receives funds from the AstraZeneca Innovation funds (RSRO_P71752).

## ACKNOWLEDGMENTS

We express our gratitude to all the authors who have contributed to this Research Topic and to the reviewers for their valuable work.

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**Conflict of Interest:** LL and the University of California, San Francisco have licensed intellectual property rights regarding NKG2D for commercial applications. NG received funds from AstraZeneca. The funder was not involved in the writing of this editorial or the decision to submit it for publication.

frontiers in Immunology

REVIEW
published: 12 February 2018
doi: 10.3389/fimmu.2018.00231

# More than Decoration: Roles for Natural Killer Group 2 Member D Ligand Expression by Immune Cells

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The activating immune receptor natural killer group 2 member D (NKG2D), which is expressed by natural killer cells and T cell subsets, recognizes a number of ligands expressed by "stressed" or damaged cells. NKG2D has been extensively studied for its role in tumor immunosurveillance and antiviral immunity. To date, the majority of studies have focused on NKG2D-mediated killing of target cells expressing NKG2D ligands. However, with a number of reports describing expression of NKG2D ligands by cells that are not generally considered stressed, it is becoming clear that some healthy cells also express NKG2D ligands. Expression of these ligands by cells within the skin, intestinal epithelium, and the immune system suggests other immune functions for NKG2D ligand expression in addition to its canonical role as a "kill me" signal. How NKG2D ligands function in this capacity is just now starting to be unraveled. In this review, we examine the expression of NKG2D ligands by immune cells and discuss current literature describing the effects of this expression on immunity and immune regulation.

**Keywords: natural killer group 2 member D ligands, natural killer group 2 member D, immune cells, immune regulation, natural killer cells, T cells**

OPEN ACCESS

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

***Received:*** *15 November 2017*
***Accepted:*** *26 January 2018*
***Published:*** *12 February 2018*

***Citation:***
*Trembath AP and Markiewicz MA (2018) More than Decoration: Roles for Natural Killer Group 2 Member D Ligand Expression by Immune Cells. Front. Immunol. 9:231. doi: 10.3389/fimmu.2018.00231*

## INTRODUCTION

The demands placed on the immune system are immense and highly complex. It is tasked with protecting the body against untold external threats while maintaining a balance between immune defense and autoimmune damage, the stakes are literally life and death. Fortunately, millions of years of evolution have resulted in immunological systems which are equally complex and necessarily efficient. Increasingly, we are coming to appreciate that few immune mechanisms are "single use," with many systems having distinct functions dependent upon setting and context. While this immunological multipurposing leads to a capable and nuanced immune response, it puts the onus on us to tease out the different roles played by many immune system components. A prime example is presented in the activating immune receptor natural killer group 2 member D (NKG2D) and its ligands.

**Abbreviations:** ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia mutated- and Rad3-related protein; CAR, chimeric antigen receptor; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; DAP10, DNAX-activating protein of 10 kDa; DAP12, DNAX-activating protein of 12 kDa; IFN-γ, interferon gamma; *Klrk1*, killer cell lectin-like receptor K1; LPS, lipopolysaccharide; MCMV, murine cytomegalovirus; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MICA, MHC class I polypeptide-related sequence A; MICB, MHC class I polypeptide-related sequence B; MULT1, murine ULBP-like transcript 1; NKG2D, natural killer group 2 member D; NOD, non-obese diabetic; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; RAE-1, retinoic acid early inducible 1; RAET1, retinoic acid early transcript 1; TACE, TNF-α-converting enzyme; TNF-α, tumor necrosis factor alpha; ULBP, UL16-binding proteins.

Natural killer group 2 member D, which is encoded by the gene *killer cell lectin-like receptor K1* (*Klrk1*) and designated CD314, is one of the best-studied activating immune receptors. NKG2D is expressed by all human and mouse natural killer (NK) cells, all human $CD8^+$ T cells, activated mouse $CD8^+$ T cells, NKT cells, subsets of γδ T cells, and rare $CD4^+$ T cells in both human and mouse (1–4). NKG2D binds to a number of endogenous ligands that are induced by cellular stress and originally believed to be effectively absent from healthy cells (5, 6). There are eight known human NKG2D ligands. These are major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA) and B (MICB), and the retinoic acid early transcript 1 (RAET1) family of proteins, which are better known as the UL16-binding proteins (ULBP1-6). There are nine known ligands for NKG2D in mouse. They are RAE-1α-ε, H60a-c, and murine ULBP-like transcript 1 (MULT1), which are all orthologs of human RAET1. NKG2D ligands are all distantly related to MHC class I molecules, but do not associate with β2 microglobulin or bind peptide, and are tethered to the cell membrane *via* a GPI anchor or transmembrane domain (7). Specifically, MICA, MICB, ULBP4, H60a, H60b, and MULT1 have transmembrane domains, while ULBP1, ULBP3, and ULBP6, RAE-1α-ε, and H60c are attached to the cell surface *via* GPI anchors. Interestingly, ULBP2, ULBP5, and certain alleles of MICA can be associated with the membrane *via* a transmembrane domain or by GPI anchor (7, 8). Ligands can be shed from the cell surface *via* proteolytic cleavage, alternative splicing, phosphoinositide phospholipase C, or exosome release (9). While the ligands do have different binding affinities with NKG2D, all NKG2D ligands are believed to signal similarly through NKG2D (5, 6). NKG2D ligands have generally been considered markers of "altered self," being induced by stress, such as cellular transformation or infection, and acting as a distress signal to target affected cells for immune killing.

Natural killer group 2 member D has been extensively studied for its involvement in antitumor surveillance and viral immunity, where it directs NK cell and $CD8^+$ T cell recognition of NKG2D ligand-expressing cancerous or virally infected cells. NKG2D functions as a homodimer, with a short cytoplasmic tail that does not contain any signaling motifs. To signal, NKG2D associates with one of two adapter proteins, DNAX-activating protein of 10 kDa (DAP10) or DNAX-activating protein of 12 kDa (DAP12). In human and mouse T cells and NK cells, NKG2D associates with DAP10, which has a YINM motif that induces PI3 kinase and Grb2-Vav signaling (7, 10). In mouse NK cells, NKG2D also associates with DAP12, which is an immunotyrosine-based activation motif-bearing signaling molecule that signals through Syk and Zap70 (11–13). On NK cells, NKG2D is a primary activating receptor, triggering NK cell cytotoxicity and cytokine production in response to ligand-expressing cells. The function of NKG2D on $CD8^+$ T cells is less well-defined with both co-stimulatory and T cell receptor-independent functions being described (1, 14–17). In addition to this well-studied role directing immune killing of ligand-expressing cells, a growing body of evidence suggests that NKG2D–NKG2D ligand interactions play other important roles in shaping the immune response. This idea came about after the appreciation of the importance of NKG2D ligand expression by otherwise healthy tissues (18). Numerous reports show expression of NKG2D ligands by healthy tissues, but until relatively recently, the effects of this NKG2D ligand expression were not explored in-depth. The expression of NKG2D ligands by healthy cells is the focus of a review by Eagle et al., wherein the authors address the potential significance of NKG2D ligand expression by both healthy hematopoietic and non-hematopoietic cells and discuss the need for more systematic study of the role of NKG2D–NKG2D ligand signaling in apparently healthy cells (18). In the years since this review, further evidence has accumulated that NKG2D ligand expression by healthy cells has distinct functions beyond targeting cells for immune killing. One major type of healthy cells, which evidence suggests routinely express NKG2D ligands, is cells of the hematopoietic lineage, specifically leukocytes. The role of NKG2D ligands in host defense as well as the mechanisms regulating ligand expression are discussed in detail elsewhere (6, 7). In this review, we focus on the expression of NKG2D ligands by immune cells and discuss what role this expression plays in the modulation of immune responses.

## FUNCTION OF NKG2D LIGAND EXPRESSION BY T CELLS

In their 1998 paper first describing the human NKG2D ligand MICA, Zwirner and colleagues showed that MICA was weakly expressed by freshly isolated $CD4^+$ and $CD8^+$ T cells, but that expression could be strongly induced in culture by addition of the polyclonal T cell activator phytohemagglutinin (19). Further investigation showed that MICA was induced on human T cells upon activation with anti-CD3 and anti-CD28 or PMA stimulation, and this induction could be inhibited in a dose-dependent manner by the NF-κB inhibitor sulfasalazine (20). In these studies, the authors suggest that MICA expression by T cells could participate in the maintenance of immune homeostasis through NKG2D-mediated NK cell killing of activated T cells (21). Indeed, a number of studies in both human and mouse have since observed expression of NKG2D ligands by activated T cells and found that this expression makes them susceptible to NKG2D-mediated killing. In mice, a study by Rabinovich et al. showed that upon activation, T cells from either C57BL/6 or Balb/c mice became susceptible to syngeneic killing by NK cells or lymphokine-activated killer cells (22). In Balb/c mice, this killing was mediated by NKG2D and was due to upregulation of an NKG2D ligand, most likely H60 (22). Curiously, however, no NKG2D ligands were detected on activated C57BL/6 T cells, suggesting that recognition and killing of activated syngeneic C57BL/6 T cells are mediated through a different receptor (22). In a model of graft-versus-host disease, Noval Rivas and colleagues found that transferred host-specific $CD4^+$ T cells were limited by NKG2D-dependent killing by host NK cells (23). They found that upon antigen stimulation, monoclonal antigen-specific $CD4^+$ T cells upregulated mRNA encoding the NKG2D ligands: MULT1 and H60. However, it should be noted that surface expression of MULT1 was not observed by flow cytometry, and surface expression of H60 proteins was not investigated (23). In humans, a similar finding was reported by Cerboni et al., who

found that primarily MICA, but also ULBP1-3, was expressed by activated human $CD4^+$ and $CD8^+$ T cells upon antigen stimulation in an ataxia telangiectasia mutated/ataxia telangiectasia mutated- and Rad3-related protein (ATM)-dependent manner. In addition, expression of these ligands by activated T cells resulted in NKG2D-mediated NK cell lysis, again suggesting a potential mechanism for limiting T cell responses (24). Nielsen et al. also found that activated $CD4^+$ T cells expressed MICA, MICB, and ULBP1-3 and were susceptible to NK cell lysis (25). Further evidence supporting this role comes from a recent study that showed expression of MICA and MICB by liver-infiltrating T cells in patients with chronic hepatitis B correlated with enhanced NK cell activation and NKG2D-dependent depletion of $CD4^+$ T cells upon short-term *ex vivo* culture (26). However, it appears that NKG2D-mediated T cell killing does not always result in a reduced immune response. For instance, during *Mycobacterium tuberculosis* infection, NK cells were shown to control regulatory T cell (Treg) numbers through NKG2D-mediated lysis of NKG2D ligand-expressing Tregs (27).

As discussed earlier, multiple studies demonstrate that NKG2D ligand expression by human and murine T cells has an important function in regulating T cell responses by directing the elimination of activated T cells. However, there is also evidence of additional functions for NKG2D ligands expressed on apparently healthy T cells. Li et al. found that NKG2D ligands were expressed by double positive Balb/c thymocytes prior to fate determination, suggesting a role for NKG2D ligand expression in thymocyte development (28). It was also shown that human peripheral blood mononuclear cells (PBMCs), and in particular $CD4^+$ T cells, can release soluble NKG2D ligands in response to superantigen stimulation. These soluble ligands were found to downregulate NKG2D expression by $CD8^+$ T cells, which showed impaired proliferation, cytokine production, and cytotoxic activity (29). Finally, emerging evidence suggests that expression of NKG2D ligands by T cells directly affects the production of cytokines. A recent article reported that expression of ULBP proteins by $CD4^+$ T cells from inflamed Crohn's disease intestine positively correlated with release of IL-10, while the frequency of $\gamma\delta^+$ and $CD56^+$ cells expressing NKG2D negatively correlated with inflammation and pro-inflammatory cytokine release (30). Additionally, our laboratory recently demonstrated that T cells from non-obese diabetic (NOD) mice express H60a upon activation and that NKG2D–H60a interaction during cytotoxic T lymphocyte (CTL) differentiation reduces NOD CTL effector cytokine production (31).

## FUNCTION OF NKG2D LIGAND EXPRESSION BY B CELLS

Information regarding expression of NKG2D ligands by B cells is limited, and functional evidence is even scarcer. In humans, peripheral B cells have been shown to express ULBP1-3 (32). This study did not assess functional effects of ligand expression by these cells, but the authors did show that acute myeloid leukemia cells show very low NKG2D ligand expression compared to B cells from healthy patients, which may be a result of malignant transformation (32). Similarly, data from our laboratory demonstrate NKG2D ligand expression by B cells in aging mice (33). However, this expression was much greater in mice lacking NKG2D expression and correlated with increased B cell lymphoma development. This suggests that these NKG2D ligand-expressing B cells in older mice are premalignant, rather than healthy cells. A recent report hints that NKG2D ligand expression on B cells may play a role in cytokine production in Crohn's disease. B cells in inflamed intestine were found to express ULPB1-6 and MICA/B, and a significant correlation between MICA/B expression by B cells and IL-1β and tumor necrosis factor alpha (TNF-α) production was demonstrated. In addition, a significant correlation was observed between ULBP expression by B cells and TNF-α release (30).

## FUNCTION OF NKG2D LIGAND EXPRESSION BY NK CELLS

With NKG2D being one of its major activating receptors, a large amount of NKG2D research has focused on NK cells. Despite this, relatively few studies have assessed the expression and function of NKG2D ligands on NK cells. In a 2003 study examining impaired NK cell function in NOD mice, Ogasawara and colleagues found that activated NK cells from NOD and NK1.1 NOD, but not C57BL/6 mice, express NKG2D ligands. RT-PCR analysis showed that these cells were positive for RAE-1α, β, and γ, and H60 mRNA transcripts. Only retinoic acid early inducible 1 (RAE-1) protein expression was confirmed by flow cytometry, as an anti-H60 antibody did not yet exist (34). The authors posit that expression of NKG2D ligands by NK cells themselves may contribute to the observed down modulation of NKG2D and NK cell dysfunction in NOD mice (34).

More recent studies suggest that expression of NKG2D ligands by NK cells may also direct NKG2D-mediated killing of NK cells, acting to regulate the NK cell response in a similar manner to that observed with T cells. The first of these studies describes the unique acquisition of tumor-derived RAE-1 by murine NK cells through the transfer of pieces of tumor membrane to NK cells in a process termed "trogocytosis." These RAE-1-expressing NK cells were susceptible to NKG2D-dependent, perforin-mediated killing by other NK cells (35). Lopez-Cobo et al. also observed that human NK cells could acquire ULBP1-3 from target cells, which not only made them targets of autologous NK cell killing but also allowed for propagation of further NKG2D ligand transfer during this NK cell–NK cell interaction (36). In another study, type I interferons were shown to preserve NK cell expansion during murine cytomegalovirus infection by reducing NK cell expression of NKG2D ligands and reducing NKG2D-mediated fratricide (37).

Expression of NKG2D ligands by NK cells appears to have other functions besides targeting NK cells for killing. Brennan et al. observed expression of ULBP2 by stimulated human NK cells and found that expression was highest by recently activated and proliferating NK cells. This expression did not target NK cells for fratricide and led the authors to suggest that ULBP2 is a marker of newly activated "mature" NK cells (38). Recently, it was demonstrated in our laboratory that expression of ULBP proteins by activated NK cells plays an important role

in tuning NK cells through regulation of TNF-α-converting enzyme (TACE) activity. We found that ULBP family members are upregulated on NK cells following activation with IL-12, IL-15, and IL-18. The interaction of NKG2D and NKG2D ligand, both expressed by NK cells, enhanced TACE activity, resulting in increased TNF-α and ULBP release from the cell surface (39).

## FUNCTION OF NKG2D LIGAND EXPRESSION BY MONOCYTES AND MACROPHAGES

During the original characterization of MICA, Zwirner et al. found that MICA protein was expressed by monocytes from multiple donors using Western blot (19). Since, the expression of NKG2D ligands by monocytes and macrophages has been investigated by a number of groups, with results suggesting two primary functions. One of these functions was suggested by Hamerman et al., who showed that toll-like receptor (TLR) signaling through MyD88 in murine macrophages induced RAE-1 and that NK cells cocultured with these RAE-1-expressing macrophages internalized NKG2D from the surface both *in vitro* and *in vivo* (40). This suggests that ligand expression by macrophages is involved in communication between macrophages and NK cells. This idea is supported by another study showing that expression of RAE-1 by murine macrophages downregulates NKG2D surface expression by NK cells and inhibits the NK cell response against B16 tumors (41). MICA expression by human monocytes was also shown to enhance NK cell interferon gamma (IFN-γ) production and antitumor function *via* an NKG2D-dependent mechanism (42, 43).

In addition to communication with, and regulation of, NK cells, it appears that expression of NKG2D ligands also makes macrophages susceptible to regulation by direct NKG2D-mediated killing. Autologous killing of macrophages and monocytes by NK cells or NKG2D-expressing $CD4^+$ T cells was shown after induction of NKG2D ligand expression on monocytes by lipopolysaccharide (LPS) stimulation, *in vitro* culture with IL-10, or on monocytes from patients with systemic lupus erythematosus (44–46). Other observations include upregulation of NKG2D ligands by human monocytes, as well as murine macrophages and microglial cells, in response to GM-CSF and other myeloid growth factors, including FLT-3 ligand and stem cell factor (32, 47). MICB and ULBP1 were also shown to be upregulated on healthy monocytes from glioblastoma patients in response to tumor-derived lactate dehydrogenase (48). Additionally, MICA and MICB were observed on foam cells from atherosclerotic lesions as well as human monocyte-derived macrophages treated with acetylated low-density lipoprotein, mimicking atherosclerotic conditions (49). A study by Ge et al. showed that while ULBP1 and MICA were expressed at similar levels by PBMC from children with Kawasaki disease and healthy controls, NKG2D expression by NK cells and $CD8^+$ T cells was decreased in diseased patients, which correlated with increased cytokine production by monocytes (50). This observation suggests that NKG2D ligand expressed by monocytes (and possibly other PBMCs) interacting with NKG2D has functional effects in modulating monocyte function.

## FUNCTION OF NKG2D LIGAND EXPRESSION BY DENDRITIC CELLS (DCs)

Dendritic cells are cornerstones in initiating and directing an immune response. Given the evidence presented thus far of NKG2D ligand expression as a means of communication between immune cells, it is little surprise that NKG2D ligands are induced on DCs and appear to play a role in regulating DC, NK cell, and T cell function. In humans, ULBPs are not only upregulated after infection of DCs with influenza, measles, and respiratory syncytial virus but also after treatment with poly(I:C) (51, 52). Stimulation with LPS also induces ULPB2, MICA, and MICB, demonstrating danger signaling *via* TLRs can drive NKG2D ligand expression in DCs (42, 52).

Jinushi and colleagues described a pathway whereby IL-15 signaling drives expression of IFN-α, which induces MICA/B expression by monocyte-derived DCs (53, 54). They found that these DCs activated NK cells in a MICA/B-NKG2D interaction-dependent manner (54). The involvement of IL-15, a critical cytokine in NK cell development and activation, in inducing NKG2D ligand on DCs suggests the potentially important role of this ligand expression in the regulation of an NK cell-mediated response. It was later found that human DCs release exosomes bearing ULBP1 and IL-15 receptor and that these exosomes are capable of cross-presenting exogenous IL-15 to NK cells (55). Together, the presence of ULBP1 and IL-15 receptor by DC-derived exosomes promoted NK cell activation and proliferation (55). NKG2D ligands on DCs may be important regulators of T cell function as well. ULBP1 expression was observed by DCs in areas of T cell interaction in lymph nodes, suggesting a role for ULBP1 on DCs in the induction or reactivation of T cell responses (56). Zloza et al. found that transgenic expression of RAE-1ε on DCs at the time of priming rescued memory recall by $CD8^+$ T cells in the absence of $CD4^+$ T cells. They found that RAE-1 expression by DCs did not affect effector T cell responses, but conferred a high rate of survival of $CD4^+$ T cell-deficient animals in a model of influenza in which viral elimination is ordinarily $CD4^+$ T cell-dependent. Additionally, they showed that RAE-1 stimulation rescues HIV-specific $CD8^+$ T cell responses in $CD4^+$ T cell-deficient HIV-positive donors (57).

Evidence suggests that NKG2D ligand expression by DCs may not always be activating, but can also negatively regulate immune function. Transgenic expression of RAE-1ε by DCs causes downregulation of NKG2D on NK cells and impaired NKG2D-dependent NK cell functions, including tumor rejection (58). Correlative evidence comes from a study by Fabritius and colleagues who found expression of RAE-1γ on DCs in the spleen and lymph nodes of C57BL/6 mice and demonstrated that deletion of NKG2D accelerates rejection of cardiac allografts (59). Additionally, NKG2D ligand expression on DCs infected with an ULBP-expressing cytomegalovirus resulted in decreased MHC class I expression by the DCs (60). This effect is consistent with

earlier studies from our laboratory that demonstrate that high NKG2D ligand expression decreases MHC class I expression by both tumor cells and normal cells (61). While this decrease in MHC class I increases NK cell responses, it likely decreases the response of $CD8^+$ T cells (60, 61).

## OTHER IMMUNE CELLS

Many studies report expression of mRNAs encoding NKG2D ligands in both primary and secondary immune tissues (62–64). Much of this expression, especially in the thymus and spleen, is likely attributed to immune cell types already discussed. However, other immune cells have been found to express NKG2D ligands in both humans and mice, although the function of this expression is not entirely clear. RAE-1 and H60 are expressed by freshly isolated bone marrow cells from Balb/c, but not C57BL/6 mice, and this expression is responsible for the rejection of Balb/c bone marrow by C57BL/6 mice in an NKG2D-dependent manner (65). GR-$1^+$$CD11b^+$$F4/80^+$ myeloid-derived suppressor cells (MDSCs) from RMA-S tumor-bearing mice were also found to express RAE-1. This expression enhanced the production of IFN-γ by NK cells and made the MDSCs susceptible to NK cell killing both *in vitro* and *in vivo* (66). Similarly, tumor-infiltrating myeloid cells in glioblastoma patients were shown to express MICB and ULBP1 (48). It has yet to be determined if expression of NKG2D ligands in these instances is an incidental effect of rapid cellular division in bone marrow or immune dysregulation in the tumor environment, or if they play a distinct role in immune cell development and regulation. However, given the cellular energy involved in protein expression, and the potential immune triggering consequences, it seems unlikely that NKG2D ligands would be induced without a biologically important function.

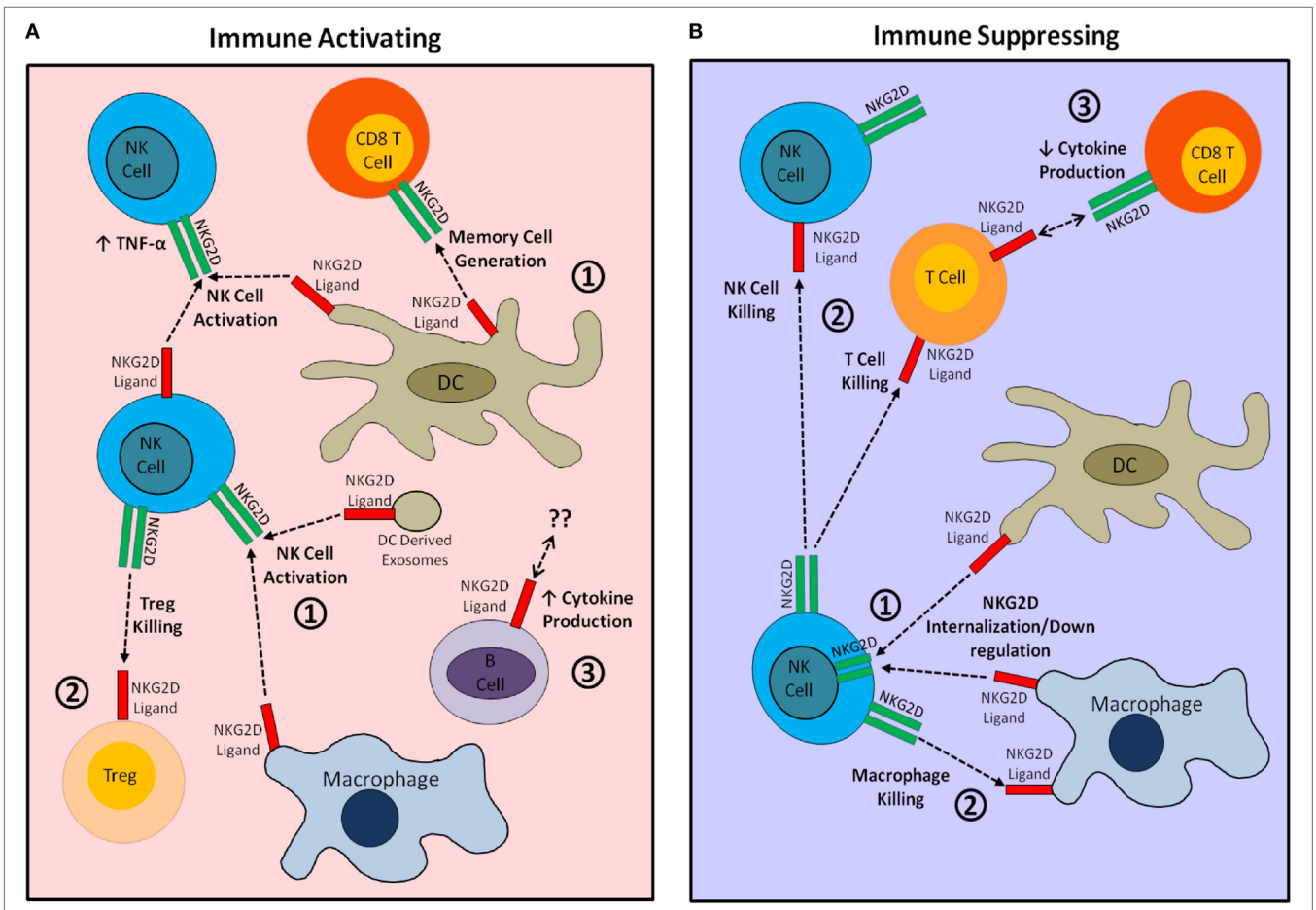


**FIGURE 1** | Visual summary of immunostimulatory and immunosuppressive effects of natural killer group 2 member D (NKG2D) ligand expression by cells of the immune system. **(A)** The immunostimulatory effects of NKG2D ligand expression by immune cells. (1) NKG2D ligand expression by dendritic cells (DCs) and macrophages provides activating and differentiation signals to NKG2D-bearing natural killer (NK) cells and $CD8^+$ T cells. (2) Expression of NKG2D ligand by regulatory T cells (Tregs) targets these cells for killing by NK cells, thereby increasing the overall immune response. (3) NKG2D ligand expression may affect B cell cytokine production. **(B)** The immunosuppressive effects of NKG2D ligand expression by immune cells. (1) Widespread expression of NKG2D ligands by DCs or macrophages causes internalization of NKG2D, impairing NKG2D-mediated immune activation. (2) NKG2D ligand-bearing immune cells are directly targeted for killing by autologous NKG2D-bearing NK and T cells. (3) NKG2D–ligand interaction during CTL generation may decrease CTL cytokine production.

**TABLE 1** | Summary of natural killer group 2 member D (NKG2D) ligands expressed by human immune cells and their observed immunological effects.

| Immune cell type | Observed NKG2D ligands | Immune activating effects | Immune suppressing effects | Reference |
|---|---|---|---|---|
| T cells | • Major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA)<br>• MHC class I polypeptide-related sequence B (MICB)<br>• ULBP1-3 | • Enhanced natural killer (NK) cell activation<br>• NK cell killing of regulatory T cells | • NK cell killing of T cells<br>• Downregulation of NKG2D through release of soluble ligands | (19–21, 24–27, 29) |
| B cells | • ULBP1-6<br>• MICA<br>• MICB | • Correlation with increased IL-1β and tumor necrosis factor alpha (TNF-α) production | – | (30, 32) |
| NK cells | • ULBP1-6 | • Increased TNF-α release | • NK cell–NK cell killing | (36, 38, 39) |
| Monocytes/ macrophages | • MICA<br>• MICB<br>• ULBP1-3 | • Increased NK cell interferon gamma production | • Macrophage/monocyte killing | (19, 42–46) |
| Dendritic cells | • MICA<br>• MICB<br>• ULBP1-3,5 | • Activation of NK cells<br>• NKG2D ligand bearing exosomes promote NK cell activation and proliferation | – | (51, 52, 54–56) |

**TABLE 2** | Summary of natural killer group 2 member D (NKG2D) ligands expressed by immune cells in mice and their observed immunological effects.

| Immune cell type | Observed NKG2D ligands | Immune activating effects | Immune suppressing effects | Reference |
|---|---|---|---|---|
| T cells | • H60a<br>• H60b*<br>• murine ULBP-like transcript 1 (MULT1)* | – | • Natural killer (NK) cell killing of T cells<br>• Decreased $CD8^+$ T cell cytokine production | (22, 23, 31) |
| B cells | • Retinoic acid early inducible 1 (RAE-1)<br>• MULT1 | • Protection from B cell lymphoma | | (33) |
| NK cells | • RAE-1α-γ<br>• H60* | – | • NK cell–NK cell killing | (34, 35, 37) |
| Monocytes/ macrophages | • RAE-1 | • Correlation with microglial proliferation | • Downregulation of NKG2D on NK cells | (40, 41, 47) |
| Dendritic cells | • RAE-1 | • Enhanced $CD8^+$ T cell memory generation in the absence of $CD4^+$ T cells | • Downregulation of NKG2D on NK cells and impaired NK cell function<br>• Decreased major histocompatibility complex-I expression | (57, 58, 60, 61) |
| Myeloid-derived suppressor cells | • RAE-1 | • Increased NK cell IFNγ production and killing of MDSCs | – | (66) |

**Observed at the mRNA level only.*

## CONCLUSION

The effects of NKG2D ligand expression by immune cells are summarized in **Figure 1** and **Tables 1** and **2**. A major theme is that expression of NKG2D ligands by immune cells serves to downregulate immune responses. This is done either by modulating the activity of other NKG2D-bearing cells or making the ligand-bearing cells susceptible to NKG2D-mediated killing. A unique aspect of NKG2D ligand expression is the paradoxical ability of these ligands to not only stimulate a robust immune killing response in some instances but also exhibit expression on a diverse array of healthy tissues. This can be explained in large part by the expression of inhibitory "self" molecules by healthy cells, which limit or prevent their killing (67–70). Interestingly, animals engineered with ubiquitous transgenic expression of NKG2D ligands show surprisingly understated effects on immune development and response. With the exception of an early report, which found weight loss, hyperkeratosis, and increased white blood cell numbers in mice ubiquitously expressing MICB (71), studies involving transgenic expression of MICA, RAE-1, or H60a have shown effects limited to downregulation of NKG2D expression, impaired NKG2D-dependent NK cell and $CD8^+$ T cell functions, and decreased MHC class I expression (15, 61, 65, 72–76), with limited effects on immune responses. Such a finding does not preclude the critical role NKG2D may play in immune function and regulation. As we have seen, NKG2D ligand expression by immune cells appears to play opposing roles in regulating immune responses. In some instances, ligand expression regulates immune cell responses by targeting the ligand-bearing cell for NKG2D-mediated killing. NKG2D ligand expressing immune

cells can also reduce NKG2D-dependent immunity by directly causing internalization of NKG2D itself. In other cases, NKG2D ligands expressed by immune cells stimulate the activation and proliferation of NKG2D-positive cells without necessarily inducing killing against the ligand-bearing cell. In addition, NKG2D ligands on non-immune cells can recruit immune cells to the site of expression (17, 30). It is likely that NKG2D ligand expression by immune cells similarly acts to recruit immune cells and enhance a local immune response. Which of these immune modulatory effects of NKG2D ligand expression prevails is likely situational, depending upon the combination of many factors present, and could be a key component in maintaining an effective yet measured immune response. Yet another compelling reason to better understand the function of NKG2D ligand expression by immune cells comes from the wide interest in targeting NKG2D ligand expression in both cancer and autoimmunity. Such an understanding will help avoid side effects and improve the efficacy of therapies such as NKG2D chimeric antigen receptor T cells, which are being developed to target tumors, and anti-NKG2D mAbs, which are being evaluated for the treatment of Crohn's disease and type I diabetes (77–81).

Finally, evidence is accumulating that NKG2D–NKG2D ligand interaction between immune cells has functions beyond a stimulatory capacity, including T cell development, differentiation, and memory generation. So far, we only have a glimpse of how NKG2D signaling affects immune cells in these ways. Another major unresolved question is whether the NKG2D ligands are functionally different. There are hints that this may be the case, though evidence suggests that differences may be driven more by ligand tethering, GPI-anchor versus transmembrane domain, which affects distribution on the cell surface, and physical size, than by the affinity for or interaction with individual ligands for the NKG2D receptor itself (82, 83). Further research in these areas may shed light on the complex and sometimes conflicting roles for NKG2D reported in disease and tumor immunity. In summary, it is apparent that NKG2D ligand expression by immune cells themselves warrants more rigorous investigation as a multifaceted mechanism regulating immune system function.

## AUTHOR CONTRIBUTIONS

AT wrote the majority of the manuscript. MM contributed to writing and editing the manuscript.

## FUNDING

This work was supported by the National Institutes of Health Centers of Biomedical Research Excellence Grant P20GM104936 (to MM) and the V Foundation for Cancer Research D2017-020 (to MM).

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**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: 08 March 2018
doi: 10.3389/fimmu.2018.00441

# NKG2D: A Master Regulator of Immune Cell Responsiveness

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NKG2D is an activating receptor that is mostly expressed on cells of the cytotoxic arm of the immune system. Ligands of NKG2D are normally of low abundance, but can be induced in virtually any cell in response to stressors, such as infection and oncogenic transformation. Engagement of NKG2D stimulates the production of cytokines and cytotoxic molecules and traditionally this receptor is, therefore, viewed as a molecule that mediates direct responses against cellular threats. However, accumulating evidence indicates that this classical view is too narrow. During NK cell development, engagement of NKG2D has a long-term impact on the expression of NK cell receptors and their responsiveness to extracellular cues, suggesting a role in NK cell education. Upon chronic NKG2D engagement, both NK and T cells show reduced responsiveness of a number of activating receptors, demonstrating a role of NKG2D in induction of peripheral tolerance. The image that emerges is that NKG2D can mediate both inhibitory and activating signals, which depends on the intensity and duration of ligand engagement. In this review, we provide an overview of the impact of NKG2D stimulation during hematopoietic development and during acute and chronic stimulation in the periphery on responsiveness of other receptors than NKG2D. We propose that NKG2D interprets the context of the immunological environment through detection of cellular cues and in response sets the appropriate activation threshold for a large number of immune receptors. This perspective is of particular importance for future therapies that aim to exploit NKG2D signaling to fight tumors or infection.

**Keywords: NKG2D, education, peripheral tolerance, activation, NK cells, T cells**

OPEN ACCESS

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

***Received:*** *21 November 2017*
***Accepted:*** *19 February 2018*
***Published:*** *08 March 2018*

***Citation:***
*Wensveen FM, Jelenčić V and Polić B (2018) NKG2D: A Master Regulator of Immune Cell Responsiveness. Front. Immunol. 9:441. doi: 10.3389/fimmu.2018.00441*

## INTRODUCTION

NKG2D, encoded by *Klrk1*, is an activating cell surface receptor that is predominantly expressed on cytotoxic immune cells. NKG2D is abundantly present on all NK cells, NKT cells, and subsets of γδ T cells. While naïve human $CD8^+$ T cells express NKG2D, in mice they upregulate its expression only after activation (1). $CD4^+$ T cells generally do not express NKG2D even after activation, but in humans its expression can be induced under certain pathological conditions, such as Crohn's disease juvenile-onset lupus and cytomegalovirus infection (2–4). In mice, CD4 T cells were shown to induce NKG2D expression in models for inflammation, such as colitis and chronic inflammatory arthritis (5, 6). The molecular structure of NKG2D allows it to bind a number of structurally different MHC-I-like ligands. NKG2D ligands have in common that under homeostatic conditions their expression is generally low. In contrast, upon cellular stress, such as infection or oncogenic transformation, their expression can be highly induced (7). In humans, the NKG2D ligands are MICA, MICB, and six members of the ULBP family. In mice, ligands can be divided into three subgroups: five different

isoforms of the Rae1-family (α-ε), MULT1, and three different isoforms of H60 (a, b, and c) (7).

The NKG2D receptor consists of a homodimer of two disulfide-linked transmembrane proteins, with very short intracellular domains that do not have signaling properties. In mice, NKG2D therefore uses the adaptor molecules DAP10 and DAP12 to relay its signaling, whereas in humans NKG2D associates exclusively with DAP10 (8). Two NKG2D isoforms have been identified in mice, a short (NKG2D-S) and a long (NKG2D-L) form, which differ 13 amino acids in length as result of alternative splicing of the *Klrk1* transcript (9). Due to this difference in length, NKG2D-L can only associate with DAP10, whereas NKG2D-S can form a complex with both DAP10 and DAP12. In humans, only the NKG2D-L isoform is expressed explaining why this receptor exclusively signals through DAP10 (10, 11). DAP10 and DAP12 initiate different signaling cascades. DAP10 possesses a YINM motif which allows binding p85 of phosphatidylinositol-3 kinase (PI3K) (12). In addition, DAP10 binds Grb2, which associates with Vav1. All three of these molecules are required to mediate the full signaling potential of NKG2D over DAP10 (13). DAP12 contains an immune receptor tyrosine-based activation motif, which is phosphorylated by Src-kinases upon NKG2D triggering (14). This event allows binding and activation of the tyrosine kinases, Syk and Zap70 (12). T cells and naïve NK cells predominantly express the NKG2D-L isoform, which is therefore thought to promote cellular processes downstream of the PI3K signaling cascade, such as co-stimulation, cytotoxicity, and cell survival (15–17). In mice, NKG2D-S is induced in activated NK cells, in which it promotes signaling through Syk/Zap70, resulting in enhanced cytotoxicity and cytokine production (17).

NKG2D plays an important role in the recognition and elimination of potentially dangerous cells (1, 18). It has been shown to mediate immune responses against tumors (18), virally infected cells (8, 19), and organ transplants (20). For this reason, NKG2D was originally thought to predominantly mediate direct cytotoxicity in response to the encounter of ligand on stressed target cells (1). However, in most cases, NKG2D is only able to mediate immune cell activation if it occurs within an inflammatory context. Both NK and T cells generally require a secondary signal before NKG2D is able to mediate a measurable effect (21–23). The primary function of NKG2D therefore appears to be regulation of signaling through other receptors. Its unique feature is that it is able to both inhibit and potentiate signaling of a large number of receptors in multiple ontologically distinct immune cell subsets and during different stages of the life cycle of immune cells, such as hematopoietic development, priming, and effector responses (8). In this review, we will give a brief overview of the literature regarding the role of NKG2D in various immunological settings. The model that emerges from accumulated evidence is that NKG2D is a master regulator of activation thresholds for a large number of receptors, both when NKG2D is directly engaged, and long after its signaling has ceased.

## NKG2D AND NK CELLS

As part of innate immunity, NK cells play an important role in the early cytolytic defense against infections and tumors. NK cells are members of the type 1 family of innate lymphoid cells (24, 25). On their cell surface they express a large number of structurally distinct, germline-encoded receptors that can transfer both activating and inhibitory signals into the cell (26). These receptors respond to external cues from peripheral cells that communicate either inhibitory homeostatic signals, or activating signals in case of cellular stress (7). Under normal conditions inhibitory signals prevail, which keeps NK cells inactive. When cells become stressed, for example, upon infection or oncogenic transformation, activating signals dominate causing loss of equilibrium and NK cell activation (27). To prevent autoimmunity or anergy, an extensive set of regulatory mechanisms is in place that determines the activation threshold values beyond which the balance between inhibitory and stimulating cues shifts in favor of activation. Already during their development, in a process known as "education," "licensing," or "arming," NK cell activation thresholds are set, mostly in response to inhibitory receptors. NK cell education ensures proper reactivity, as well as tolerance toward self in response to locally expressed ligands. This process functionally mimics positive and negative selection of T cells in the thymus (28–30). Outside of the bone marrow, the responsiveness of NK cells is further fine-tuned by engagement of self-ligands by their receptor array, which mediates peripheral tolerance.

NKG2D is expressed from the earliest NK cell precursor stages onward (31). Initially, its expression is relatively low, but increases over time and stays high in mature cells (32). In mice, NK cells express both NKG2D isoforms, even though the long form predominates in a resting state (9). Expression of the NKG2D-S isoform strongly increases after NK cell activation, whereas levels of the L-form abate (10). Nevertheless, both DAP10 and DAP12-mediated signaling is engaged upon NKG2D stimulation in activated murine NK cells (15). NKG2D has been implied in NK cell education, effector cell function, and peripheral tolerance through modification of the activation threshold of NK cell receptors. During development, NKG2D regulates both expression levels and responsiveness of a plethora of receptors (**Figure 1**). NK cells of mice with a germline deficiency for NKG2D show reduced levels of c-kit (CD117), the activating receptor DNAM-1, as well as the inhibitory receptors Ly49A, Ly49G2, and Ly49F (33, 34). In addition, whereas *Klrk1*$^{-/-}$ NK cells fail to respond to target cells expressing NKG2D ligands (33, 35), mice lacking either NKG2D, or its ligands Rae1δ and Rae1ε produce higher levels of IFNγ following stimulation with cellular targets (33, 34, 36). As a result, NKG2D-deficient mice display better NK cell-mediated control of murine cytomegalovirus infection (33). This effect seems specific for NKG2D, since deletion of CD16, another activating receptor expressed on all NK cells, does not affect CD16 independent NK cell function both in humans and in mice (37–39).

Humans do not express Ly49 molecules, nor does human NKG2D engage DAP12. Unfortunately, the impact of NKG2D on NK cell development in humans is difficult to determine. To date, no deficiency for this receptor has been documented and in the periphery most NK cells express NKG2D, precluding comparison of NK cell subpopulations with and without this receptor. This makes it difficult to translate many observations made in NKG2D-deficient mice directly to the human situation. However,

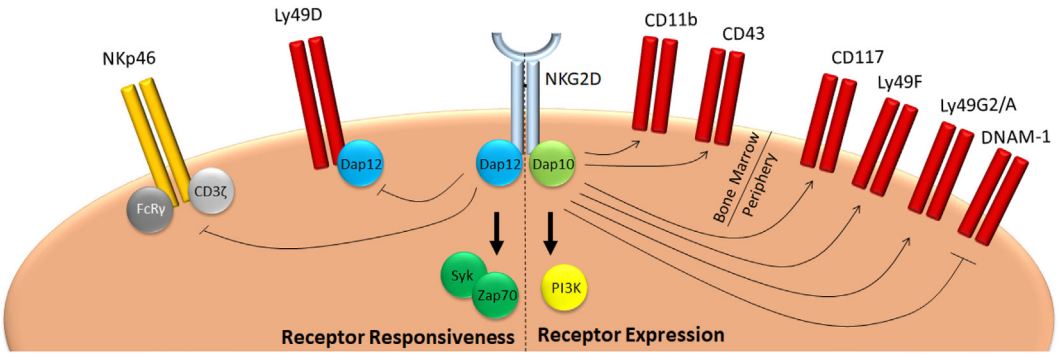


**FIGURE 1** | NKG2D mediates education of NK cells. NKG2D signaling during development negatively affects responsiveness of NK cells in two ways. On one hand, it promotes expression of inhibitory Ly49 molecules, such as Ly49F, G2, or A, whereas it inhibits expression of activating receptors, such as DNAM-1. On the other, it reduces responsiveness of activating receptors, such as NKp46 and Ly49D. As a consequence, mice deficient for NKG2D have a hyper-responsive phenotype and are better protected against infection with pathogens which are controlled by NK cells, such as cytomegalovirus (33, 34).

the maternal decidua contains a large population of NKG2D$^-$ NK cells, which is replaced by NKG2D$^+$ cells during the first trimester of pregnancy (40). Notably, this is associated with strong changes in the KIR repertoire and reduced pro-inflammatory NK cell function (40, 41). These observations suggest that also in humans NKG2D may affect NK cell development and education.

The impact of NKG2D on NK cell development is likely to depend on its interaction with the IL-15 receptor (IL-15R). IL-15R signaling is known to be important for the development, homeostasis, and survival of NK cells (42). Both NKG2D and the IL-15R were shown to be able to bind DAP10 in murine cells (16). Jak3, as part of the canonical IL-15R signaling pathway, phosphorylates the YINM sequence on DAP10 and was shown to be able to potentiate NKG2D signaling (16). *Klrk1*$^{-/-}$ mice show a higher proliferation rate as well as faster differentiation and transition to more mature stages resulting in perturbation in size of immature NK cell subpopulations (33). Importantly, NKG2D-deficient NK cells are prone to apoptosis, which could not be corrected by addition of IL-15 (33), suggesting a common signaling pathway. Both NKG2D and the IL-15R can activate PI3 kinase, a molecule important for the regulation of proliferation and survival (43). It therefore seems likely that the PI3K pathway is engaged by these receptors to mediate common regulatory effects. Whether NKG2D prevents a general hyper-reactivity of NK cells or if it sets the activation threshold of a specific activating NK cell receptor still needs to be investigated.

NKG2D plays a key role in effector responses of NK cells in the periphery (**Figure 2**). NKG2D itself is an important mediator of tumor immuno-surveillance, since animals deficient for NKG2D demonstrate a reduced ability to fight prostate carcinoma and B cell lymphoma, but not chemically induced fibrosarcoma (35). In addition, NKG2D ligation was shown to reduce activation thresholds for several NK receptors, both in humans and in mice. In human NK cells, NKG2D promotes CD16 signaling and ADCC, since blocking of NKG2D receptors results in a reduced ability of NK cells to mediate anti-HIV-1 antibody-dependent cellular cytotoxicity (44). Conversely, NKG2D was shown to be able to synergistically activate human NK cells when they were simultaneously stimulated through CD16, NKp46, or 2B4 (23, 45). For the 2B4-NKG2D pair, it was shown that synergy is achieved through conversion on the signaling adaptor Vav1, which overcomes c-Cbl-mediated inhibition (46). In mice, NKG2D was shown to augment Ly49H-dependent proliferation (47). Wild type Ly49H$^+$ NK cells proliferate faster than their *Klrk1*$^{-/-}$ counterparts following infection with a virus that drives overexpression of the NKG2D ligand Rae1γ (47). Both NKG2D and Ly49H use DAP12 for their signal transduction providing a possible overlap in signaling cascades. It should be noted, however, that the Rae1γ transgenic virus mediates important NKG2D-independent effects (48) and additional evidence is required before NKG2D and Ly49H signaling can be directly linked.

NKG2D has also been implied in the generation of peripheral tolerance of NK cells, an effect that was first identified in cancer patients. Whereas some tumors downregulate NKG2D ligands to prevent recognition, others paradoxically induce expression NKG2D ligands (49). Rather than activating NK cells, high NKG2D ligand expression mediates downregulation of the NKG2D receptor on tumor infiltrating lymphocytes (TILs) and reduced responsiveness to NKG2D engagement (50). Under physiological conditions, this process is thought to prevent NK hyper-responsiveness against peripheral ligands for which education in the bone marrow is incomplete. In addition, it allows induction of NK cell hypo-responsiveness at times when this is required, for example during pregnancy (41). The human placenta was shown to produce both soluble and exosome-bound NKG2D ligands. During pregnancy, the placenta accumulates a large number of NK cells, which are inhibited from recognizing the fetus as non-self. Both supernatants of umbilical cord cells and placental exosomes were shown to downmodulate NKG2D and inhibit NK cell cytotoxicity (51–53). Loss of NK cell functionality after exosome treatment could be prevented using blocking antibodies directed against NKG2D. NKG2D-induced hypo-responsiveness was therefore proposed as a mechanism to mediate fetal tolerance. Indeed, several polymorphisms in NKG2D and MICA are associated with recurrent miscarriage (54).

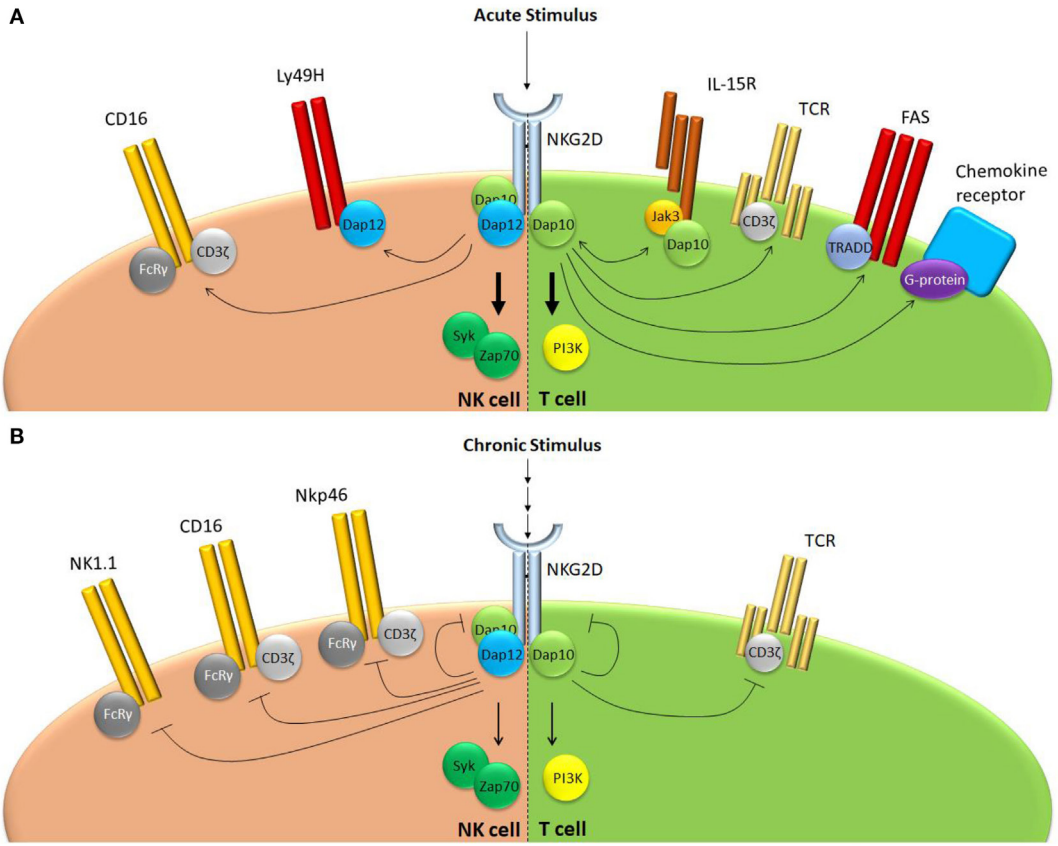


**FIGURE 2** | NKG2D regulates receptor responsiveness differently following acute or chronic stimulation. **(A)** Following acute stimulation, NKG2D promotes responsiveness of a range of structurally unrelated receptors that use largely distinct intracellular signaling modalities. **(B)** Chronic NKG2D engagement mediates its own downregulation and subsequent hypo-responsiveness to stimulation. In addition, chronic NKG2D stimulation impairs T cell receptor responsiveness in T cells. In NK cells, chronic NKG2D stimulation reduces missing self-signaling (not shown) as well as responsiveness of a number of receptors that share the FcεRIγ signal adaptor.

Chronic NKG2D engagement by NK cells results in reduced responsiveness of multiple receptors other than NKG2D. An impaired ability to kill RMA-S cells was observed in mice that transgenically overexpress Rae1ε or MICA and upon chronic exposure of NK cells to NKG2D ligands *in vitro*. This indicates a role for NKG2D in regulation of missing self-recognition (55–57). When murine NK cells were long-term exposed to cells overexpressing the NKG2D ligand H60, they showed reduced responsiveness to CD16, NK1.1, and NKp46 stimulation (55). NKG2D-induced peripheral tolerance demonstrated receptor specificity, since Ly49H and Ly49D function were reduced, but still partially preserved (55). Similarly, when human NK cells were chronically exposed to MICA, downregulation of CD3ζ was observed, rendering them hypo-responsive to CD16, NKp46, and NKp30, but not 2B4 stimulation (58). In mice, NK cell hypo-responsiveness following chronic NKG2D engagement requires signaling through both DAP10 and DAP12 adaptor molecules (55). Interestingly, most receptors affected by chronic NKG2D exposure, such as NK1.1, NKp46, or CD16, do not make complexes with either DAP10 or DAP12, but rather use the adaptor molecule FcεRIγ (59). This implies that prolonged NKG2D stimulation influences signaling components downstream of FcεRIγ. Again, it seems that this level of regulation is specific for NKG2D since sustained exposure of murine NK cells to CD16, another broadly expressed NK cell activating receptor, did not affect any other activation pathway besides CD16-mediated NK cell activation (55). NKG2D-mediated peripheral tolerance appears to depend on the level of stimulation. Chronic exposure of murine NK cells to Rae1ε or Rae1δ, NKG2D ligands of relatively low affinity, induced general hypo-responsiveness of NK cells to activating stimuli ("desensitization"). Tolerance could be broken through administration of soluble MULT1, an NKG2D ligand that binds this receptor with high affinity (36). How this effect is mediated molecularly, or why a difference in the affinity of NKG2D ligands has such opposing effects on NK cell activity is still unknown.

In summary, NKG2D affects all stages of the life cycle of NK cells through modification of NK cell receptor activation thresholds. In addition to its important role in recognition and elimination of potentially dangerous cells, NKG2D mediates NK cell education in the bone marrow and peripheral tolerance upon chronic ligand exposure. Further investigation is needed to

determine how NKG2D affects activity of receptors that do not share its downstream signaling components.

## NKG2D AS A MODULATOR OF RECEPTOR RESPONSIVENESS IN T CELLS

Inappropriate activation of T cells, for example by self antigens, may lead to devastating tissue destruction. Therefore, priming of naïve T cells requires not only T cell receptor (TCR) engagement, but also co-stimulation via membrane-bound receptors and/or cytokines (60). Co-stimulatory molecules ensure survival of T cells and control responsiveness of TCR engagement of effector cells in the periphery. The most well-documented co-stimulatory molecule is CD28. Upon engagement of B7 molecules, this protein activates several signaling cascades, including the NF-kB and PI3 kinase pathways (61). Deficiency of CD28 causes a reduction in proliferation and cytokine production by T cells upon infection (61). T cells expressing a mutant form of CD28 that lacks the PI3K binding YMNM motif have a specific impairment in producing cytokines (62). This indicates that co-stimulatory functions are associated with specific signaling cascades. Indeed, deficiency of CD28 does not abrogate the cytotoxic response against LCMV infection (63), demonstrating that co-stimulation does not necessarily induce a general state of increased responsiveness, but is able to potentiate specific receptors.

NKG2D has been well-documented as a co-stimulatory molecule for T cells. Upon MICA engagement, activated human CD8 T cells produce more IFNγ, TNF, and IL-2 in response to TCR stimulation (22). Moreover, the cytolytic response of antigen-specific CD8 T cells against peptide-pulsed target cells is enhanced if these cells overexpress ligands for NKG2D (22, 64). For priming of naïve CD8 T cells, NKG2D is insufficient as a co-stimulatory molecule. *In vitro* stimulation of human peripheral blood CD8 T cells showed that CD28-mediated co-stimulation is required for NKG2D to be able to promote cytokine production and cytotoxicity following TCR stimulation (65). The *in vivo* role of NKG2D on T cells long remained elusive, because NKG2D deficiency on NK cells results in their hyper-responsiveness, thus affecting viral titers and the subsequent CD8 T cell responses (33, 66). Specific elimination of NKG2D on CD8 T cells demonstrated that this receptor has a non-redundant role in promoting cytokine production following viral infection in mice (66).In contrast, deficiency of NKG2D on these cells did not impact their ability to expand or mediate cytotoxicity (66). Conversely, when mice are infected with a cytomegalovirus strain that transgenically expresses the NKG2D ligand Rae1γ, no major differences in effector CD8 T cell numbers are observed, but these cells do display increased cytokine production and cytotoxic potential (48). This indicates that NKG2D is able to promote cytotoxicity in antiviral CD8 T cells, but is not required by these cells to obtain their normal cytolytic potential.

In contrast to αβ T cells, γδ T cells do not require priming and are directly able to mediate cytokine production and cytolytic killing upon TCR engagement (67). γδ T cells constitutively express NKG2D. However, most γδ T cells are not able to mediate cytotoxicity upon NKG2D triggering alone (68, 69). Indeed, also in γδ T cells does NKG2D function to potentiate TCR responsiveness with regards to cytokine production and target cell lysis (68, 70).

The TCR functionally defines the T cell and, therefore, has been given most attention regarding its interaction with NKG2D. However, NKG2D mediates sensitization of receptors in T cells beyond the TCR (**Figure 2**). Most notably, NKG2D has been associated with IL-15 receptor signaling and memory CD8 T cell formation. Upon stimulation, the IL-15 receptor activates the adaptor molecules Jak1 and Jak3, which mediate phosphorylation of Stat5, but also DAP10, thus potentiating NKG2D signaling through DAP10 (16). Indeed, both DAP10 expression and IL-15 stimulation mediate NKG2D expression on the surface of murine effector CD8 T cells (15, 71). Conversely, NKG2D potentiates IL-15R signaling in memory CD8 T cell precursors. Upon LCMV infection, murine CD8 T cells deficient for NKG2D have a reduced capacity to form central memory cells, which is DAP10 and IL-15 dependent (72). In absence of NKG2D, memory precursor CD8 T cells show reduced phosphorylation of Akt, a downstream target of PI3 kinase, following IL-15 stimulation. As a result, these cells have a reduced ability to sustain levels of the pro-survival protein Mcl-1, leading to lower number of central memory cells (72). Thus, both in NK and CD8 T cells, NKG2D and the IL-15R potentiate each other's signaling through DAP10 even though the functional outcome differs between these cell subsets. In murine memory T cell precursors, NKG2D also mediates down-regulation of the transcription factor T-bet by limiting JNK2 signaling (73). Whether this effect is downstream of the IL-15R or of another ligand was not shown (73).

FAS, also known as CD95, is a member of the TNF receptor superfamily. Dependent on the intracellular adaptor molecules that are associated to this receptor, FAS ligation may induce apoptosis, growth arrest, or proliferation (74). In a large number of human tumors, infiltrating CD4 T cells were shown to express high levels of NKG2D (75). Rather than inducing a cytolytic response, MICA expression by these tumors induced expression of FASL by NKG2D-expressing CD4 T cells. Whereas, tumor cells themselves were protected against FASL-induced apoptosis, it did mediate growth arrest in $NKG2D^-$, but not in $NKG2D^+$ cells (75). In glioblastoma cells, FAS was shown to recruit the p85 subunit of PI3 kinase, which enhanced invasive growth (76). Since NKG2D/DAP10 signals through PI3K in T cells, it seems likely that this mechanism is adopted in tumor infiltrating T cells to prevent FASL-mediated growth arrest.

Finally, NKG2D stimulation affects responsiveness of T cells to chemokines. Deficiency of NKG2D does not result in changes in migration *per se*, as naïve or effector T cells of NKG2D-deficient mice do not show differences in tissue homing (66). However, in a mouse that transgenically overexpressed the NKG2D ligand Rae1ε in pancreatic β-islet cells, CD8 T cells rapidly accumulated in the pancreas, which was associated with strong changes in the local chemokine milieu (77). This effect was dependent on DAP10, but did not require antigen-recognition by T cells (77). Surprisingly, accumulation of CD8 T cells in the pancreas of these animals did not lead to massive destruction of β-cells or diabetes. The molecular mechanism how NKG2D impacts chemotaxis is not known, but the involvement of DAP10 again implies a role for

PI3 kinase. Many chemokine receptors signal through PI3 kinase, either directly, or via G-protein linked signaling (78). Thus, NKG2D may indirectly impact the sensitivity of PI3K-linked chemokine receptors to change the migration behavior of T cells.

Similar to NK cells, chronic exposure of T cells to NKG2D ligands impairs their responsiveness to stimulation. Characterization of T cells infiltrating human tumors overexpressing NKG2D ligands showed that they downregulate the NKG2D receptor (50). As a result, activated T cells become less responsive to NKG2D (co-) stimulation (50). Surprisingly, chronic NKG2D ligand encounter also results in reduced responsiveness of the TCR. Re-stimulation of TILs from a MICA overexpressing human melanoma through their TCR showed that they have a reduced capacity to produce IFNγ compared to TILs derived from melanoma that did not express NKG2D ligands (50). To recapitulate this observation, various experimental models have been generated in which mice overexpress NKG2D ligands, either ubiquitously or in specific organs. These confirmed that chronic NKG2D stimulation impairs responsiveness to tumor antigens in particular (57, 79). The molecular mechanism underlying impaired responsiveness of the TCR following chronic NKG2D stimulation is still a matter of debate. Co-cultivation of human peripheral blood CD8 T cells for several days with fibroblasts overexpressing MICA showed that these cells downregulated CD3ζ, a key intracellular signaling component of the TCR (58). This effect was shown to depend on activation of caspase 3/7, which mediated proteolytic degradation of CD3ζ (58). How chronic NKG2D drives caspase activation and how this is achieved without inducing apoptosis remains unclear. Interestingly, when animals which ubiquitously express Rae1ε were infected with murine cytomegalovirus, there was no difference observed in the number of antigen-specific CD8 T cells, nor in their ability to produce cytokines (80). This implies that anergy induced by chronic NKG2D stimulation can be overcome in a sufficiently inflammatory environment (80).

## NKG2D AND B CELLS

A surprising role for NKG2D was recently identified in regulation of signaling thresholds in B cells. Mice deficient for NKG2D show a specific reduction in the number of B cells in spleen (33). In addition, *Klrk1*$^{-/-}$ mice have a twofold reduction in B1a cells in the peritoneal cavities (81). This phenotype partially depended on DAP10-mediated signaling, indicating that this may also be of relevance for the human situation. NKG2D is not expressed on mature B cells or committed B cell precursors. Nevertheless, NKG2D is expressed very early during NK cell development. Since B1a cells represent an innate B cell population, it was postulated that in mice NKG2D is expressed on a common precursor for B1a and NK cells (81). Importantly, in a model for B1a cell controlled bacterial infection, *Klrk1*$^{-/-}$ mice behaved similarly to B cell-deficient animals (81). Indeed, NKG2D deficiency resulted in reduced B cell receptor signaling in B1a cells. Thus, also in the B cell lineage, NKG2D appears to have a long-term impact on receptor sensitivity.

## CONCLUDING REMARKS

NKG2D has great potential as a therapeutic target, since it has the potency to enhance cytolytic immune responses against important diseases, such as cancer. Indeed, chimeric receptors using NKG2D signaling domains have successfully been used *in vitro* to potentiate antitumor T cells (82, 83). However, the complexity of NKG2D biology should be acknowledged before the power of this receptor can be harnessed therapeutically. NKG2D ligands may be used to communicate cellular stress, in which case NKG2D should induce immune cell activation. However, when NKG2D ligands are expressed chronically or at low intensity, for example during pregnancy in the placenta, they communicate a need for reduced receptor responsiveness and inhibit immune cell function. Similarly, when NKG2D ligands are encountered by NK cell precursors they set the proper bandwidth for mature NK cell responsiveness. Thus, NKG2D appears to be a sensor of the immunological context in which an immune cell operates and it adjusts responsiveness of its other receptors accordingly. The signaling pathways via which NKG2D mediates its effects on other receptors are only beginning to be unraveled, especially in NK cells. Future studies must reveal how we will be able to exploit NKG2D-based therapies, without introducing unforeseen effects.

## AUTHOR CONTRIBUTIONS

VJ wrote the section on NK cells. FW wrote the section on T cells. BP wrote the other sections and edited the article.

## FUNDING

This work was supported by the University of Rijeka (865.10.2101 to FW and 803.10.1103 to BP) and the Croatian Science Foundation (IP-2016-06-8027 to FW and IP-2016-06-9306 to BP).

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**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: 12 March 2018
doi: 10.3389/fimmu.2018.00476

# NKG2D and Its Ligands: "One for All, All for One"

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The activating receptor NKG2D is peculiar in its capability to bind to numerous and highly diversified MHC class I-like self-molecules. These ligands are poorly expressed on normal cells but can be induced on damaged, transformed or infected cells, with the final NKG2D ligand expression resulting from multiple levels of regulation. Although redundant molecular mechanisms can converge in the regulation of all NKG2D ligands, different stimuli can induce specific cellular responses, leading to the expression of one or few ligands. A large body of evidence demonstrates that NK cell activation can be triggered by different NKG2D ligands, often expressed on the same cell, suggesting a functional redundancy of these molecules. However, since a number of evasion mechanisms can reduce membrane expression of these molecules both on virus-infected and tumor cells, the co-expression of different ligands and/or the presence of allelic forms of the same ligand guarantee NKG2D activation in various stressful conditions and cell contexts. Noteworthy, NKG2D ligands can differ in their ability to down-modulate NKG2D membrane expression in human NK cells supporting the idea that NKG2D transduces different signals upon binding various ligands. Moreover, whether proteolytically shed and exosome-associated soluble NKG2D ligands share with their membrane-bound counterparts the same ability to induce NKG2D-mediated signaling is still a matter of debate. Here, we will review recent studies on the NKG2D/NKG2D ligand biology to summarize and discuss the redundancy and/or diversity in ligand expression, regulation, and receptor specificity.

**Keywords: NKG2D ligands, NKG2D receptor, NK cells, virus infection, cancer**

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***Edited by:***
*Nadia Guerra, Imperial College London, United Kingdom*

***Reviewed by:***
*Nicolas Dulphy, Paris Diderot University, France*
*Carsten Watzl, Leibniz Research Centre for Working Environment and Human Factors (LG), Germany*

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

***Received:*** *31 January 2018*
***Accepted:*** *22 February 2018*
***Published:*** *12 March 2018*

***Citation:***
*Zingoni A, Molfetta R, Fionda C, Soriani A, Paolini R, Cippitelli M, Cerboni C and Santoni A (2018) NKG2D and Its Ligands: "One for All, All for One". Front. Immunol. 9:476. doi: 10.3389/fimmu.2018.00476*

## INTRODUCTION

NK cells and T cells respond to pathogens and tumors by the integration of signals deriving from numerous cell surface receptors that can initiate, enhance or suppress lymphocyte effector functions. While the antigen-specific T cell receptor—generated by somatic genetic recombination—dictates T cell recognition and activation, NK cells use a vast repertoire of germ-line encoded receptors. Many of them are also expressed by T cells, with NKG2D being one of the best characterized receptors shared by both cell types (1).

NKG2D is a C-type lectin-like receptor expressed on NK cells, γδ T cells, $CD8^+$ T cells, and some autoreactive or immunosuppressive $CD4^+$ T cells and represents a major recognition receptor for the detection and elimination of damaged, transformed, and pathogen-infected cells. Its ligands belong to the H60 (a–c), RAE (α–ε), and MULT1 families in mice, and to the MIC (MICA and MICB) and ULBP (ULBP1–ULBP6) families in humans, where their repertoire is more complex than in other species (1, 2). In fact, MIC molecules are encoded by the most highly

polymorphic human genes after the classical HLA molecules, while murine ligands have a low allelic diversity (2).

But which is the reason and biological significance of having more than 100 different alleles for MICA, 40 for MICB, and less than 20 for the ULBPs, or, in other words, why do we have such a diversity of NKG2D ligand genes and alleles? The answer lies in the million years of co-evolution between host and pathogens. "Cat and mouse" evolution of the host immune system and pathogen immune evasion mechanisms are a dominant view of immunogenetics, and among the different classes of pathogens, there is ample evidence that viruses, in particular herpesviruses, could be the major driving force for the evolution of NKG2D ligand diversity. Moreover, gene duplications and further mutations within the alleles result in such a ligand variety that there is not a single viral protein or RNA described so far able to target all of them (2, 3). Thus, possessing a high number of NKG2D ligands together with genetic polymorphisms is clearly advantageous to the host, allowing it to counteract viral immune evasion strategies. In fact, in the paradigmatic example of cytomegalovirus, although viral immune evasion genes are strongly diversified, they are not entirely successful, since NK cells are functionally active, with NKG2D playing a role in the elimination of infected cells (4–8). On the other hand, there is no convincing evidence to date that non-viral infectious pathogens are a significant drive for the evolution for NKG2D ligand diversity, and for the "cat and mouse" competition, thus further supporting a role for the viral-mediated selective pressure.

Although the origin and evolution of NKG2D ligand variety dates back to host–pathogen competition, infections are far to be the only examples in which NKG2D ligands are induced. In fact, they can be expressed at different levels on some normal cells (9–11), but more typically they are upregulated on tumor cells (12). The pathways underlying the regulation of their expression are generally activated by different forms of stress and cellular abnormalities, often associated with tumor transformation and progression, and they can act at different levels: regulation of transcription and protein synthesis, posttranslational modifications, and release of ligands in the microenvironment have been all described as important mechanisms controlling NKG2D ligand expression (13). Thus, primary tumors frequently express NKG2D ligands but, as in the case of viral infections, several mechanisms have been identified that elude the detection and elimination of tumor cells by the immune system, suggesting an NKG2D-mediated immune editing of the tumor (14). As with virus-infected cells, alerting the immune system will be then the final result of a balance between expression of ligands and tumor immunoevasion strategies (15).

Yet, although we have many information today on both the NKG2D receptor and its numerous ligands, some key questions still await a full comprehension and exhaustive answer. Are the ligands regulated in different manner depending on the cell type, stimulus, microenvironment, etc? And if so, are there general rules for which the different ligands are induced in various cell types by different stimuli? Do different ligands and/or alleles bind to NKG2D with different affinities? Are the ligands redundant in their functions? Or are they specific?

In this review, we will try to discuss these aspects, illustrating the diversity of ligand expression, regulation, and receptor specificity, in the context of viral infections and tumor transformation.

## NKG2D LIGAND REGULATION: REDUNDANCY AND SPECIFICITY

NKG2D ligand expression at the cell surface results from multiple levels of regulation, and as a consequence, it is often due to the contribution of distinct pathways acting collectively. We will discuss the specialization of some regulatory processes intrinsic to one or few ligands, as well as the redundancy of other molecular mechanisms able to simultaneously control the expression of several NKG2D ligands in normal conditions and in response to different stimuli. Moreover, most of the studies reported that modulation of NKG2D ligand expression on "target" cells affected NK cell recognition and killing (**Table 1**).

## TRANSCRIPTIONAL REGULATION

NKG2D ligands can be regulated at transcriptional level by a plethora of molecular pathways; both the multiplicity of transcription factors (TFs) and the diversity in the regulatory sequences in NKG2D ligand gene promoters can significantly contribute to generate the extensive heterogeneous expression of these proteins in different cell types. Indeed, distinct TFs are able to regulate the transcription of a number of NKG2D ligands in different systems; on the other hand, some cell lineage specific transcriptional regulators of selected ligands have also been described in cancer cells. Finally, epigenetic mechanisms have a robust impact on the transcriptional regulation of diverse NKG2D ligands.

NKG2D ligand-inducing cell stresses, including proliferative signals, malignant transformation, infection, or oxidative stress, share the ability to activate a DNA damage response involved in maintaining the integrity of the genome (52, 53). In this context, the sensor kinases ATM and ATR can trigger a signaling cascade in which different downstream checkpoint kinases, such as Chk1 and Chk2, are activated together with the key tumor suppressor p53 (54). Interestingly, although the activity of these kinases is needed for the induction of MIC, ULBPs or Raet1 genes (16, 17), p53 can differently regulate several NKG2D ligands, e.g., ULBP1–2, with no evident effects on the expression of MICA/B (22–24). On the other hand, the same pathway connected with proliferative signals (19, 20) or triggered by oxidative stressors (18) can enhance the activity of either NF-κB or E2F1, thus inducing the transcription of MICA/B in humans, or RAE-1 in the mouse. In addition, genomic damage or cytosolic DNA can lead to the activation of the DNA sensor pathway regulated by STING/TBK1 and IRF3, identified as regulators of RAE-1 ligands (21). These observations suggest that triggering of DDR (16), together with the induction of a senescence program (17, 18, 55) and/or the involvement of cytosolic DNA/RNA sensors (21), represents a major signal of activation/alarm for NKG2D-expressing cells (e.g., NK cells), likely establishing a primary checkpoint for aberrant cell proliferation or infection. Of note, the expression and/or function of effector proteins of these pathways can be often altered/defective in cancer cells, suggesting that their contribution in

**TABLE 1** | Mechanisms regulating NKG2D ligand expression in steady state conditions and in response to stimuli.

| Regulatory level | Condition/stimuli | Pathway/molecule | Ligand modulation | Cell type | NK cell functions | Reference |
|---|---|---|---|---|---|---|
| **TRANSCRIPTIONAL** | | | | | | |
| | Ionizing radiation, cisplatin, and 5-FU | DDR | ↑ MICA, ULBP1–3<br>↑ RAE-1, MULT1 | HFF; C1, C2 cell lines | ↑ Cytotoxicity | (16) |
| | Doxorubicin and melphalan | DDR/E2F1 | ↑ MICA | Multiple myeloma | ↑ Cytotoxicity<br>↑ IFNγ | (17, 18) |
| | Activation/proliferation | NF-κB<br>NF-κB<br>E2F1 | ↑ MICA<br>↑ MICA<br>↑ RAE-1ε | T cells<br>T cells<br>Primary fibroblasts, embrionic brain cells | n.d.<br>↑ Cytotoxicity<br>↑ Cytotoxicity | (9)<br>(19)<br>(20) |
| | Ara-C | STING/TBK/IRF3 | ↑ RAE-1 | B cell lymphoma | ↑ Cytotoxicity | (21) |
| | RITA | p53 | ↑ ULBP1, ULBP2 | Cancer cell lines | ↑ Cytotoxicity<br>↑ IFNγ | (22) |
| | | p53 | ↑ ULBP2 | Cancer cell lines | ↑ Cytotoxicity | (23) |
| | Vincristine | p53 | ↑ ULBP1 | Multiple myeloma | ↑ Cytotoxicity | (24) |
| | Heat shock response | HSF1 | ↑ MICA, MICB | Cancer cell lines<br>Multiple myeloma | n.d.<br>↑ Cytotoxicity | (25)<br>(26) |
| | ER-induced stress | CHOP | ↑ ULBP1<br>↑ MULT1 | Intestinal epithelial cells | ↑ Cytotoxicity | (27) |
| | Steady state | STAT3 | ↑ MICA | Colonrectal cancer<br>Multiple myeloma | ↑ Cytotoxicity<br>↑ IFNγ<br>↑ Cytotoxicity | (28)<br>(29) |
| | Steady state | IKZE1/3, IRF4 | ↑ MICA | Multiple myeloma | ↑ Cytotoxicity | (30, 31) |
| **RNA SPLICING** | | | | | | |
| | Steady state | RBM4 | ↓ ULBP1 | HAP1 cell line | n.d. | (32) |
| **POSTTRANSCRIPTIONAL** | | | | | | |
| | Steady state | AUF1 | ↓ ULBP2, MICB | Epithelial cells | n.d. | (33) |
| | Steady state | miR34a, c<br>miR-519a-3p<br>miR-93<br>miR-20a | ↓ ULBP2<br>↓ MICA, ULBP2<br>↓ MICA, MICB, ULBP3<br>↓ MICA, MICB, ULBP2 | Melanoma cell lines<br>Mammary epithelial cell line<br>Glioma cell lines<br>Cancer cell lines | ↓ Cytotoxicity<br>↓ Cytotoxicity<br>↓ Cytotoxicity<br>↓ Cytotoxicity | (34)<br>(35)<br>(36)<br>(37, 38) |
| | IFNγ | miR-520b | ↓ MICA | Cancer cell lines | n.d. | (39) |
| | HCMV, KSHV, and EBV | miR-UL112; miRK12-7; and miR-BART2-5 | ↓ MICB | Infected HFF cells; cancer cell lines | ↓ Cytotoxicity | (40, 41) |
| | JCV | miR-J1-3p | ↓ ULBP3 | Infected cancer cell lines | ↓ Cytotoxicity | (42) |
| **Posttranslational** | | | | | | |
| | Steady state | Ubiquitination | ↓ MULT1 | Cancer cell lines | n.d. | (43) |
| | KSHV | K5 ubiquitin ligase | ↓ MICA | Cancer cell lines | ↓ Cytotoxicity | (44) |
| | Histamine | Ubiquitination | ↓ MICA | Monocytic leukemia | ↓ Cytotoxicity | (45) |
| | Steady state | ADAMs and MMPs (protease-mediated shedding) | ↓ MICA, MICB, ULBP2 | Cancer cell lines | ↓ Cytotoxicity<br>n.d. | (46–48)<br>(49–51) |

*↓ Decrease; ↑ increase; HFF, human foreskin fibroblasts; HCMV, human cytomegalovirus; EBV, Epstein–Barr virus; KSHV, Kaposi's sarcoma-associated herpes virus; JCV, human polyoma virus JC; ADAM, a disintegrin and metalloprotease; MMP, matrix metalloprotease; n.d., not done; CHOP, C/EBP homology protein.*
*Ref. (48) provides an ample overview on protease-mediated shedding of NKG2D ligands.*

the regulation of one or more specific ligands guarantees the expression of these molecules in different types of tumors or at different disease stages.

Mechanisms of chromatin remodeling are also widely implicated in the transcriptional regulation of almost all NKG2D ligands. Both hypomethylating agents and histone deacetylase inhibitors have been shown to upregulate MICA/B and ULBPs surface levels in different tumors and infected cells (56–61), thus indicating that transcriptional silencing of these genes is largely dependent on events of DNA methylation and deacetylation. Accordingly, epigenetic dysregulation of NKG2D ligand promoters is an important immune evasion mechanism

helping cancer or infected cells to acquire resistance to NK cell surveillance.

An example of a molecular pathway selectively targeting MICA and MICB molecules is the heat-shock response that regulates their expression in different models. In this regard, ChIP experiments indicated that the MICA/B promoters are occupied by the HSF1 in heat-shocked cells, or in cells where the basal repression of this factor by the chaperone HSP90 is abrogated by specific small molecule inhibitors or proteotoxic stress (25, 26). Although promoter sequence analyses indicate that potential canonical heat-shock elements also exist in some of the ULBP genes (62, 63), no evidence regarding heat shock-induced ULBP expression has been reported to date.

In a different context, accumulation of improperly folded proteins or alterated UPR, as shown in dysregulated intestinal epithelial cells and human cell lines, can induce the selective expression of diverse ULBPs *via* C/EBP homology protein-mediated transactivation, and increase NKG2D-mediated epithelial cytolysis (27). In addition, a critical role for the TF ATF4 has been also described for UPR-induced upregulation of ULBP1 in human cell lines (32).

Different mechanisms of transcriptional repression have been described only for MICA; they are caused by cell type-specific proteins, the expression and/or function of which is often critical for the survival and proliferation of cancer cells. A cogent example is the TF STAT3 shown to directly interact with MICA promoter and repress its transcription in colon cancer cells (28); the same mechanism was found to occur also in multiple myeloma cells where the serine–threonine kinase GSK3 was identified as an important upstream regulator of STAT3 contributing to the inhibition of MICA expression (29). Furthermore, three TFs highly expressed in multiple myeloma and pivotal regulators of malignancy-specific gene expression, the Ikaros family zinc finger protein-1 and -3 (IKZF1 and IKZF3) and IRF4, are potent repressors of MICA expression in this hematological cancer (30, 31).

Connected with these mechanisms able to negatively regulate the expression of NKG2D ligands at transcriptional level is the polymorphism of their promoters. It is well known that promoters of MICA and MICB are polymorphic (12 MICA/B promoter haplotypes) with some polymorphisms associated with reduced expression (64, 65) or increased susceptibility to specific diseases (66). Accordingly, binding sites for TFs in MICA/B promoters have been demonstrated to be interrupted by polymorphisms within these regions, resulting in allele-specific regulation (65). These findings indicate that individuals with the same alleles might show variation in the expression of MICA and MICB because of polymorphism in their promoters. Altogether, these modifications may lead to selective transcriptional regulation of distinct NKG2D ligands.

In summary, despite most of the transcriptional regulatory mechanisms are common to distinct ligands, assuring the recognition and elimination of stressed cells, a number of studies indicate a certain level of specificity in the type of "stressor" that allows the expression of a particular ligand. Heat-shock response acts essentially on MICA/B but not on ULBPs (25); ligand induction in proliferating cells is characteristic of some NKG2D ligands like RAE-1 but not MULT1 or H60 (20) and in proliferating T cells MIC molecules are induced with a faster kinetic when compared with ULBPs (19, 67); ER-induced stress results in selective upregulation of ULBP1 in humans and MULT1 in mice (27). Thus, different stimuli induce specific cellular responses leading to the expression of one or few ligands, originating a sophisticated mechanism to alert the immune response.

## RNA SPLICING

Regulation of RNA splicing represents another mechanism to control NKG2D ligand expression. In particular, Gowen et al. have shown that the RNA-binding protein (RBP) RBM4 supports ULBP1 expression by suppressing a novel alternatively spliced isoform of ULBP1 mRNA and appears to be specific for the differential splicing of ULBP1 but not of other NKG2D ligands (32). Although alternative splicing isoforms have been described for MICA (68), ULBP4 (69) and ULBP5 (70), the molecular mechanisms involved in their regulation is still unknown.

## POSTTRANSCRIPTIONAL REGULATION

Stabilization of NKG2D ligand mRNA is considered an important mode to strictly control ligand expression mainly under normal conditions. In this context, a new pathway by which NKG2D ligand mRNAs (i.e., MICA, MICB, and ULBP2) are constitutively targeted by AUF1 proteins that mediate RNA degradation has been identified (71). In response to EGFR activation, either by its ligand or by some type of stress, AUF1 molecules are excluded from the nucleus allowing NKG2D ligand mRNA to be stabilized. In addition, the oncogenic RBP IMP3, which selectively binds to ULBP2 but not ULBP1 and ULBP3 mRNA, leads to ULBP2 transcript destabilization and reduced ULBP2 surface expression in several human cell lines (33). Similarly, Nachmani et al. identified other RBPs able to bind to MICB RNA and regulate its expression (72).

A number of studies have shown that distinct NKG2D ligands are regulated by microRNAs (miRNAs), which are short, non-coding RNAs that exert their regulation of gene expression posttranscriptionally by targeting 3′-untranslated region (3′UTR) of the target mRNAs and leading to degradation or translation inhibition (73). Different sites for cellular miRNAs within the 3′UTRs and/or the 5′UTR of MICA, MICB, and ULBP1 have been identified (39, 74–77). Interestingly, several viruses use miRNAs to hinder NKG2D ligand expression and evade the NKG2D-dependent immunosurveillance. Indeed, Stern-Ginossar and colleagues identified a group of endogenous cellular miRNAs regulating MICB and MICA expression by targeting a specific site also used by the human cytomegalovirus (HCMV) miRNA miR-UL112 (40, 74). Despite MICA and MICB display almost identical putative binding sequence for miR-UL112, the cooperation between miR-UL112 and cellular miRNA was reported to suppress only MICB expression during HCMV infection, suggesting that additional factors are involved in determining a functional binding site (40). Similarly, results of functional approaches and basic bioinformatic tools

demonstrated that other herpesvirus miRNAs (i.e., Kaposi's sarcoma-associated herpesvirus, KSHV, and Epstein–Barr virus) downregulate preferentially MICB but not MICA expression (41). The human polyoma viruses BKV and JCV use an identical miRNA to evade NK cell control by downregulating the stress-induced ligand ULBP3 (42).

Moreover, miRNA-mediated NKG2D ligand regulation occurs also in cancer cells. Indeed, the tumor-suppressive miR34a and miR34c strongly downregulated ULBP2 in human melanoma (34). The downregulation of ULBP2 and MICA expression by miR-519a-3p has been implicated in the inhibition of NK cell-mediated cytotoxicity of breast cancer cells (35), whereas miR-93 mimics decreased cell surface expression of MICA, MICB, and ULBP3 by translational repression, thus contributing to the immune evasion of glioma cells (36). Recently, miR-17-92 cluster was also reported to downregulate MICA/B protein expression in ovarian tumors (37), and breast cancers (38). In the latter, the authors provided the evidence that miR-17-92 members affected ULBP2 expression by inhibiting the MAPK/ERK signaling pathway (38). Finally, also some cytokines regulate NKG2D ligand expression by miRNAs. In particular, IFN-γ increased expression of miR-520b able to inhibit MICA transcript levels in different types of cancer cell lines (39).

All together these studies highlight the fact that cellular miRNAs and RBPs represent an important way to keep a low NKG2D ligand expression in steady state conditions, and they emerge as a general mechanism to regulate both ULBPs and MIC molecules.

## POSTTRANSLATIONAL REGULATION

The surface expression levels of a determined NKG2D ligand can be finely controlled by mechanisms implicated in the regulation of its release as soluble form by various processes including protease-mediated cleavage, exosome secretion, and alternative splicing. The choice of one of these processes is mainly dependent on the ligand type as well as its allelic variant.

In general, both MIC and ULBP molecules are cleaved by proteases belonging to two distinct families, the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteases (ADAMs) (46–51) that undergo modulation of their activity and expression (78–81). Different susceptibility to the protease-mediated cleavage has been described for several NKG2D ligands. As such, ULBP1, ULBP2, and ULBP3 are released from cells with different kinetics and by distinct mechanisms, being ULBP1 and ULBP3 more resistant to cleavage and preferentially secreted into exosome-like vesicles (82). Similarly, the short-allelic variant MICA*008, the prototype of a group of MICA alleles named MICA*A5.1, is resistant to proteolytic cleavage and mostly released from cells in association with exosomes (83). Also the MICA-129 dimorphism, causing a valine to methionine exchange at position 129, has been described to affect MICA shedding, but the mechanism behind is largely unknown (84). Thus, the existence of NKG2D ligands and/or allelic variants with distinct sensitivity to proteases might have relevant functional consequences. In this regard, genotoxic agents have been reported to selectively stimulate the shedding of MICB or of the allelic variant MICA*019 in a ADAM10-dependent manner, whereas the release of the short MICA*008 allele was not perturbed. Therefore, during the course of chemotherapy, MICA*008 appears to be more stable on the tumor cell surface thus favoring the recognition and killing by NK cells (85). In another study, expression of the metalloprotease tissue inhibitor of metalloproteinase 3 (TIMP3), induced by specific miRNAs in HMCV-infected cells, resulted in an enhanced activity of ADAM17 and MMP14 and increased MICA shedding (86). Moreover, an increased protease-mediated shedding of MICA, MICB, and ULBP2 was described in HIV-infected $CD4^+$ T cells (87).

On the other side, the soluble form of the high-affinity mouse NKG2D ligand, MULT1, promotes NK cell activation and tumor rejection (88). Indeed, in an *in vivo* mouse model, Deng and coworkers reported that in the presence of this soluble ligand, NK cells were not desensitized because soluble MULT1 prevented the chronic interactions between NKG2D and its ligands on cells of the tumor microenvironment (88). However, these effects appear to be restricted to the mouse and could depend on the capacity of MULT1 to bind to NKG2D with an elevated affinity respect to other mouse NKG2D ligands.

In regard to the exosome secretion, both MIC and ULBP ligand family members can be released by this class of nanovesicles (89–91), and some of them such as ULBP3 or ULBP1 (82) or the allelic variant MICA*008 (83) are secreted exclusively by exosomes. Interestingly, increased exosome secretion has been observed in response to different types of stress (90, 92, 93); however, unlike the protease-mediated shedding, it is still unclear whether the release of NKG2D ligands *via* exosomes could result in the reduction of their surface expression.

In addition to shedding and exosomal secretion, alternative splicing represents another mode to generate soluble forms of some ligands as demonstrated for ULBP4 and ULBP5 (69, 70).

Several reports have provided also evidence that other post-translational mechanisms concur to regulate NKG2D ligand expression at protein level, including protein turnover and ubiquitination. For instance, stability of ULBP1 and MICB at the plasma membrane is lower than for other ligands, and in part occurs because of a rapid internalization (94, 95). The intracellular sequestration of immature forms of MICA in the endoplasmic reticulum was observed in melanoma cancer cells and proposed as an immune escape strategy (96).

Reduction of MICA expression by ubiquitination has been described in different models (44, 45). It is used as evasion strategy by KSHV because it encodes the K5 ubiquitin ligase that ubiquitinates MICA cytoplasmic tail, thus causing a profound downregulation of this ligand on the surface of infected cells (44). Of note, the truncated allelic variant MICA*008, lacking lysine residues in its cytoplasmic tail, was resistant to KSHV-induced downregulation suggesting a selective advantage for individuals carrying such allele. Moreover, it has been shown that the murine ligand MULT1 is ubiquitinated and degraded in normal cells, and this process is reduced in response to heat shock or ultraviolet irradiation (43). Thus, targeting the ubiquitination machinery in cancer or virus-infected cells might increase their susceptibility to NK cell-mediated killing.

In conclusion, structural characteristics inherent to a specific ligand/allelic variant affecting the different susceptibility to

the protease-mediated cleavage, the ubiquitination-mediated degradation and its stability on the cell surface, represent all fundamental elements to successfully complete the long route that finally allows the ligand to be expressed on the cell surface.

## DISTINCT LIGANDS OR ALLELIC VARIANTS DIFFER IN THEIR ABILITY TO MODULATE NKG2D-MEDIATED SIGNALING

Given that NKG2D ligands are characterized by variable domain structure, distinct mode of membrane anchor and diverse affinity for their receptor, it is likely that they are not equally able to evoke activating signals. This paragraph summarizes recent findings that support the capability of NKG2D ligands to differently regulate NKG2D signaling events in both NK and T cells.

Engagement of human NKG2D elicits cytolytic responses overcoming inhibitory signals on NK cells and enhancing TCR-dependent activation in $CD8^+$ T cells, Vγ2Vδ2, and gut intraepithelial Vγ1δ1 T cells (97–100). In $CD8^+$ T cells, both co-stimulatory and T cell receptor independent functions have been described (101–104), whereas Vγ9Vδ2 T cells can be directly activated by NKG2D in the absence of TCR-dependent antigen recognition (105). Of note, NKG2D engagement alone can elicit effector functions only in NK cells preactivated by cytokines including IL-15 (106) and IL-2 (107), whereas the synergistic engagement of at least another activating receptor is required on freshly isolated NK cells (108). Thus, NKG2D can provide either a co-stimulatory signal or a direct activating signal depending on the cell context and/or the initial influence of cytokine environment.

In humans, NKG2D homodimer forms a hexameric complex with two homodimers of the transmembrane adaptor DNAX-activating protein 10 (DAP10), which is involved in intracellular signal propagation (109). Indeed, the cytoplasmic tail of DAP10 contains a tyrosine based signaling motif (YINM), which is tyrosine phosphorylated by Src-family kinases upon antibody-mediated NKG2D engagement (110). DAP10 phosphorylation promotes the recruitment of the p85 regulatory subunit of PI3K and of the Grb2/Vav1 complex, that, in turn, is required for the phosphorylation of Src homology 2 domain-containing leukocyte protein of 76 kD (SLP-76) and of phospholipase C gamma (PLCγ2) (109–111).

Even though the interaction with target cells exposing one or more NKG2D ligands triggers a functional response, the relative contribution of distinct ligands to specific signaling pathways remains elusive.

Of note, persistent stimulation with membrane-bound or soluble NKG2D ligands down-modulates receptor expression and ultimately impairs NKG2D-dependent functions on both NK and $CD8^+$ T cells (46, 91, 112–115). This functional impairment is achieved by a rapid NKG2D internalization from plasma membrane and sorting along the endocytic compartments till lysosomes, where internalized receptor complexes are degraded (113, 116–118).

In addition to reduce surface receptor expression, NKG2D endocytosis also plays an indispensable role in NKG2D-mediated signaling. Indeed, recent findings demonstrate that receptor endocytosis is required for cytotoxic granule secretion and IFNγ production. In particular, the activation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) was found to occur in signaling-competent endocytic compartments where the internalized complexes are also transported, demonstrating that NKG2D continues to signal before reaching lysosomes for degradation (119). Whether distinct ligands differ in their ability to promote signaling from endosomes is currently unknown.

Regarding the extent of receptor internalization and the rate of NKG2D lysosomal degradation, the nature of the ligand appears to play a pivotal role, as demonstrated by comparing the ability of membrane-bound MICA and ULBP2 to regulate NKG2D expression (118). Indeed, MICA promotes a more rapid NKG2D down-modulation if compared with ULBP2, leading to a stronger lysosomal degradation (**Figure 1A**). Although MICA and ULBP2 resulted equally able to elicit NKG2D-mediated NK cell cytotoxic function, the ability to further perform cytotoxicity resulted dramatically impaired only upon MICA-induced NKG2D down-modulation.

All together these results suggest that distinct ligands have the potential to activate selective signaling pathways resulting in different routes of receptor endocytosis. To this regard, we have reported that phosphorylation of the ubiquitin ligase c-Cbl, a negative regulator of NKG2D signaling (120), and the activation of the ubiquitin pathway is indispensable for MICA- but not ULBP2-induced NKG2D internalization and degradation (**Figure 1A**) (118). This selective behavior can be either attributable to MICA and ULBP2 distinct mode of membrane anchor (transmembrane and GPI-linked, respectively) and/or to differences in their affinity/avidity for NKG2D. To this regard, recent evidences demonstrate that allelic variants of the same ligand that differ in their avidity for NKG2D can also vary in their ability to tune the threshold of NKG2D signaling (121, 122).

Regarding MICA, the dimorphism in the position 129 strongly affects ligand ability to promote NK cell effector functions and to co-stimulate $CD8^+$ T cell activation (121). MICA-129Met allele binds to NKG2D with higher avidity than MICA-129Val variant (123) and appears to be more efficient in the induction of proximal signaling events such as Src phosphorylation and in triggering NK cell degranulation and IFNγ release (121). In $CD8^+$ T cells, MICA-129Met co-stimulates IL-2 production and proliferation with a more rapid kinetic than the MICA-129Val variant. On the other hand, NKG2D engagement by the MICA-129Met isoform results in a stronger receptor down-modulation in both NK and T cells leading to a severe impairment of NKG2D-mediated functions and avoiding excessive cell activity (**Figure 1B**).

By comparing the allelic variants of ULBP6, the most polymorphic ULPB ligand (124, 125), the amino acid substitution (from Arg to Leu) in position 106 reported in the ULBP0602 variant was found to be responsible for the great enhancement in affinity and stability of NKG2D interaction compared with the ULBP0601 allelic variant (122). Unexpectedly, the higher affinity variant resulted less able to elicit both NKG2D down-modulation and functional responses in NK cells as well as in

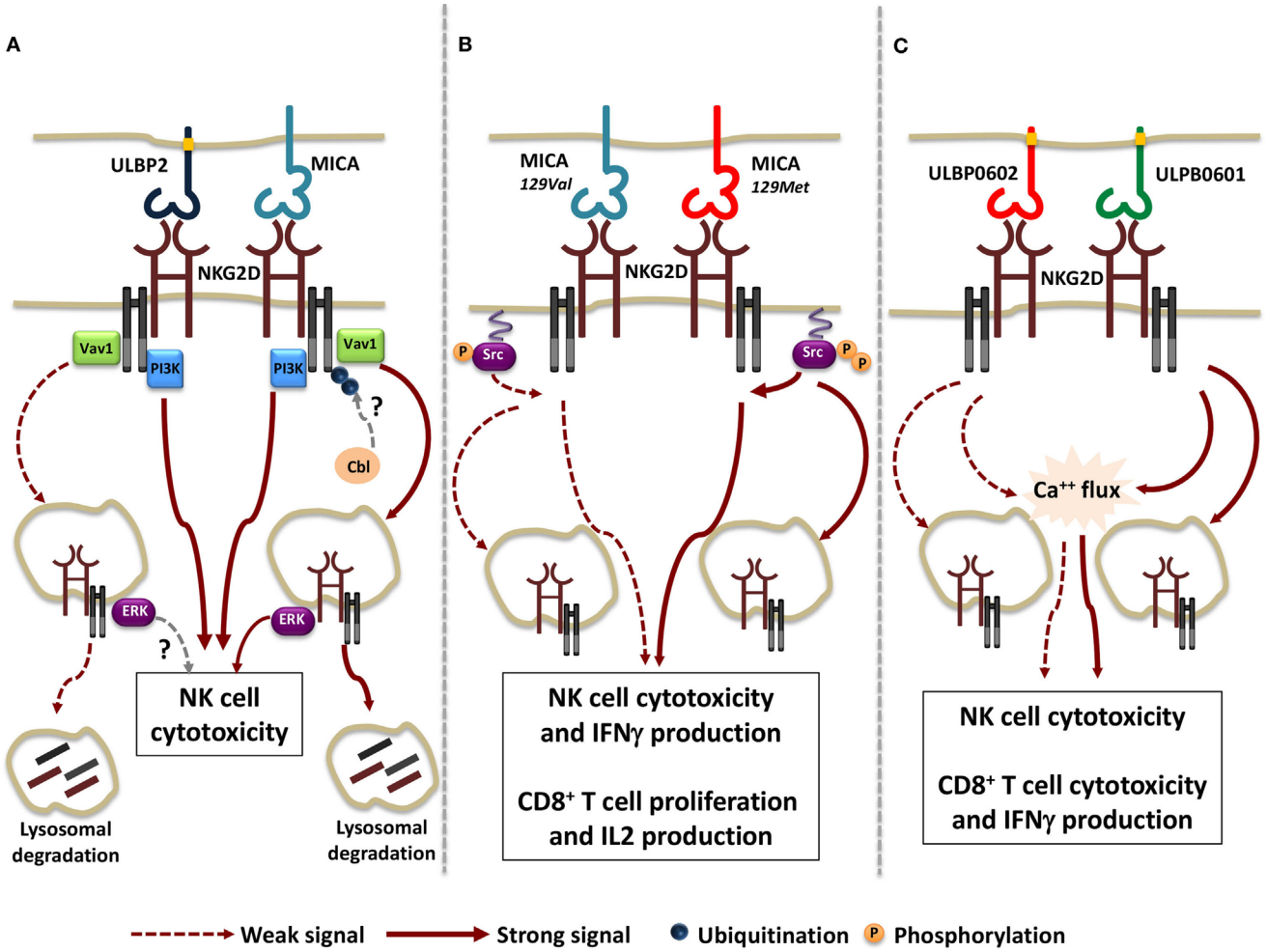


**FIGURE 1** | Functional consequences of the interaction of NKG2D with different ligand/allelic variants. **(A)** Transmembrane MICA and GPI-linked ULBP2 ligands result equally able to trigger Vav1 and PI3K activation and to induce NK cell cytotoxic function. However, a stronger receptor internalization and lysosomal degradation due to the activation of the ubiquitin pathway was observed upon MICA engagement. Whether Cbl is the ubiquitin ligase regulating NKG2D/DNAX-activating protein 10 ubiquitination and whether ULBP2 ligand is able to activate NKG2D-mediated signals from endosomal compartment is not clear (dashed arrows). **(B)** MICA-129Met, which binds to NKG2D with higher avidity compared with MICA-129Val allele, induces stronger Src phosphorylation, thus triggering both NK cell and $CD8^+$ T cell effector functions with higher efficiency. Concomitantly, a higher extent of NKG2D down-modulation is also induced upon MICA-129Met allele engagement. **(C)** The rigid and stable binding to NKG2D of the high-affinity ULBP0602 variant impairs its ability to induce $Ca^{++}$ flux and effector functions in NK cell and $CD8^+$ T cells as well as NKG2D down-modulation. As consequence, ULBP0602 engagement results less efficient compared with the low affinity variant ULBP0601.

$CD8^+$ T cells and γδT cells (**Figure 1C**). To explain these findings, the authors speculated that a rigid and stable interaction with the higher affinity ligand ULBP0602 limits the ability of cytotoxic lymphocytes to serially kill their targets.

Collectively, these results outline a hierarchy of cellular responses to different allelic variants of NKG2D ligands, suggesting that they elicit heterogeneous functional outcomes (**Figures 1B,C**). In addition, they support the notion that the strength of NKG2D-mediated signaling positively correlates with the rapidity and degree of receptor down-modulation. The interconnection between signaling and endocytosis guarantees a rapid and tight regulation of NKG2D activation preventing strong intracellular signals that could drive autoimmune responses.

A further level of complexity is given by the potential ability of soluble NKG2D ligands to modulate NKG2D signal propagation by regulating receptor surface expression. Several lines of evidence have demonstrated that the presence of soluble ligands in the sera of neoplastic patients correlates with a reduced NKG2D surface expression (113, 114, 126), suggesting that soluble NKG2D ligands share with their respective membrane-bound forms the ability to regulate receptor expression. However, a direct comparison between ligands shed after proteolytic cleavage and ligands released in exosomes, demonstrate a higher ability of the latter to induce receptor down-modulation. Regarding MICA, the GPI-linked allele MICA*008 that is released in association with exosome membranes, is a more potent NKG2D down-modulator compared with metalloproteinase-shed

MICA variants (83). Accordingly, exosome-released ULBP3 molecules reduce NKG2D surface expression and compromise NKG2D-mediated NK cell cytotoxic function with higher efficiency than the metalloproteinase-shed ULBP2 ligands (82). These results may be explained by the presence of ligands on exosomal membranes that can multimerize and bind to NKG2D with higher avidity than the soluble counterpart. Whether the ability of exosomal multimeric ligands to efficiently down-modulate receptor expression reflects their ability to induce intracellular signals and elicit selective functional responses is currently unknown.

## CONCLUSION

Diversified modalities of NKG2D ligand regulation can be applied to all NKG2D ligands while others are specific just for one or a few of them. In steady state conditions, NKG2D ligand expression is tightly repressed to maintain immune homeostasis. In response to external "danger" signals (i.e., stress and pathogens) or during neoplastic transformation, increased transcriptional activity of NKG2D ligand genes together with a perturbation of their regulatory mechanisms at mRNA and protein levels leads to the ultimate ligand cell surface expression. The sum of critical factors (the type of stressor, the structural characteristics of the ligand/allelic variant and the cellular context) determines the expression of one or more but not all ligands on the cell surface. Thus, despite some common regulatory mechanisms, NKG2D ligands are not equal or redundant in terms of final outcome, and the "specificity" of these cellular responses triggered by a multiplicity of ligands assures a sophisticated mechanism to alert the immune response. The "one for all" aspect is another compelling and still unsolved issue of the NKG2D-dependent immunosurveillance, where the large variety of ligands appears functionally non-redundant, even though they all engage the same receptor. In this regard, the expression of a certain ligand or a particular allelic variant is essential to drive a proper immune response. To date, many questions still remain open, and it is unclear if a hierarchy exists and if one of them can dominate in the triggering ability when distinct ligands have reached the cell surface; alternatively it is plausible that the ligands may act "all for one," contributing in concert to the NKG2D-mediated functional response. Thus, a detailed characterization of the cell biology of single NKG2D ligand will be indispensable to warrant targeted modulation of this system in the course of a viral infection or neoplastic transformation.

## AUTHOR CONTRIBUTIONS

AZ, RM, CF, ASoriani, RP, MC, CC, and ASantoni equally contributed to the manuscript writing.

## FUNDING

This work was supported by Italian Association for Cancer Research (AIRC Investigator Grant cod. 16014 and AIRC 5x1000 cod. 9962), grants by "Sapienza" University of Rome (RM116154C8F24748) and the Italian Ministry of University and Research (PRIN 2015-W729WH to CC).

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**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: 29 March 2018
doi: 10.3389/fimmu.2018.00620

# Cutting an NKG2D Ligand Short: Cellular Processing of the Peculiar Human NKG2D Ligand ULBP4

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Stress-induced cell surface expression of MHC class I-related glycoproteins of the MIC and ULBP families allows for immune recognition of dangerous "self cells" by human cytotoxic lymphocytes *via* the NKG2D receptor. With two MIC molecules (MICA and MICB) and six ULBP molecules (ULBP1–6), there are a total of eight human NKG2D ligands (NKG2DL). Since the discovery of the NKG2D–NKG2DL system, the cause for both redundancy and diversity of NKG2DL has been a major and ongoing matter of debate. NKG2DL diversity has been attributed, among others, to the selective pressure by viral immunoevasins, to diverse regulation of expression, to differential tissue expression as well as to variations in receptor interactions. Here, we critically review the current state of knowledge on the poorly studied human NKG2DL ULBP4. Summarizing available facts and previous studies, we picture ULBP4 as a peculiar ULBP family member distinct from other ULBP family members by various aspects. In addition, we provide novel experimental evidence suggesting that cellular processing gives rise to mature ULBP4 glycoproteins different to previous reports. Finally, we report on the proteolytic release of soluble ULBP4 and discuss these results in the light of known mechanisms for generation of soluble NKG2DL.

**Keywords: NKG2D, ULBP4, NK cells, shedding, antibodies**

OPEN ACCESS

***Edited by:***
*Nadia Guerra, Imperial College London, United Kingdom*

***Reviewed by:***
*Alessandro Poggi, Ospedale Policlinico San Martino, Italy*
*Mar Vales-Gomez, Consejo Superior de Investigaciones Científicas (CSIC), Spain*

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

***Received:*** *31 January 2018*
***Accepted:*** *12 March 2018*
***Published:*** *29 March 2018*

***Citation:***
*Zöller T, Wittenbrink M, Hoffmeister M and Steinle A (2018) Cutting an NKG2D Ligand Short: Cellular Processing of the Peculiar Human NKG2D Ligand ULBP4. Front. Immunol. 9:620. doi: 10.3389/fimmu.2018.00620*

## INTRODUCTION

NKG2D is an activating, homodimeric C-type lectin-like immunoreceptor almost exclusively, but broadly, expressed on human cytotoxic lymphocytes endowing such killer cells with the capacity to detect and destroy dangerous "self cells" by means of the "induced-self" recognition mode (1–4). Upon NKG2D ligation, activating signals are intracellularly transduced *via* the NKG2D-associated adaptor protein DAP10 with subsequent activation of the phosphatidylinositol-3-kinase and the Grb2-Vav signaling pathways (5, 6). These signaling pathways stimulate cellular cytotoxicity, but also promote cytokine secretion by NK cells, CD8 αβ T cells, and γδ T cells (3, 7–9). NKG2D-mediated "induced-self" recognition is facilitated by various MHC class I-related cell surface glycoproteins, which usually are not or barely expressed on "healthy" cells but are strongly upregulated at the cell surface upon cellular stress, exposure to PAMPs, viral infection, or malignant transformation, thereby promoting cytolysis of "harmful" cells through engagement of NKG2D (1, 4, 10).

In humans, there are eight known ligands for NKG2D including the two MHC-encoded and MHC class I chain-related glycoproteins A and B (MICA and MICB) as well as the six non-MHC-encoded, UL16-binding proteins (ULBP1–6) (1, 4, 11, 12). MICA/B molecules are comprised of an MHC class-I-like α1α2 superdomain followed by an Ig-like α3 domain, a transmembrane domain, and a cytoplasmic domain (1, 4, 12, 13). By contrast, ULBP ectodomains comprise only the MHC class I-like α1α2 superdomain, which serves as NKG2D binding platform, and which is directly attached to the

cellular membrane *via* a glycosylphosphatidylinositol (GPI) anchor (ULBP1, -2, -3, and -6) or followed by a transmembrane domain and a short (ULBP4) or long (ULBP5) cytoplasmic tail, respectively (11, 12, 14, 15). More recently, it has been shown that the common truncated MICA allelic variant MICA*08 can also be membrane attached *via* a GPI anchor (16).

ULBP4 is encoded at the centromeric end of the ULBP gene cluster on the long arm of human chromosome 6 by the *RAET1E* locus (11, 17). ULBP4 glycoproteins have first been described in 2003 by Cosman and colleagues (18) as well as by Coukos and colleagues (19). Both groups identified ULBP4 based on *in silico* screens of human genomic sequences searching for relatives of the ULBP family members ULBP1, ULBP2, and ULBP3, which had previously been discovered during a search for binding partners of the HCMV glycoprotein UL16 and been named accordingly (14). Of note, ULBP4, like ULBP3, is not bound by the HCMV glycoprotein UL16 (18, 20, 21) and therefore can be considered a misnomer. Both original studies (18, 19) described ULBP4 cDNA encoding for a polypeptide of 263 amino acids (including the signal peptide) and giving rise to a mature cell surface-bound protein of 235 amino acids (~27 kDa). This ULBP4 polypeptide is encoded by four exons with exon 1 encoding for the signal peptide, exon 2 for the α1 domain, exon 3 for the α2 domain, and exon 4 for the short serine-rich stalk, the transmembrane region, and a short cytoplasmic domain (**Figure 1A**). This originally reported ULBP4 variant has meanwhile been termed isoform 1 by the Uniprot database[1] (22). Five additional ULBP4 isoforms (isoforms 2–6) are referenced in the Uniprot database originating from alternative splicing (23, 24) and will be discussed later. Sequence analyses and phylogenetic trees constructed from the comparison of ULBP α1α2 superdomains strongly suggest that ULBP4 has diverged from other primate ULBP molecules earliest and before the separation of Old and New World monkeys (25). In addition, ULBP4 is the most polymorphic member of the ULBP family of proteins (11, 26, 27), although there is no functional rationale for this polymorphism. There are some reports of ULBP4 being expressed by various tumors, EBV-infected B cells, and cytokine-activated NK cells that may be relevant for the NKG2D-mediated immunosurveillance and immunoregulation in these settings (28–33). In our approach to this peculiar NKG2DL, we realized that ULBP4 glycoproteins are poorly characterized and that there exist substantial inconsistencies when comparing the literature and publicly available databases on rather basic issues such as on biochemical properties and expression by cell lines and tissues. Hence, we set out to study expression and biochemical properties of ULBP4 molecules.

# MATERIALS AND METHODS

## Cells

C1R and HepG2 cells were cultivated in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA), HCT116 and HaCaT cells in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich), and Hela cells in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA, USA). All media were supplemented with 10% FCS (Biochrome, Berlin, Germany), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (both from Sigma-Aldrich), and RPMI 1640 in addition with 1 mM sodium pyruvate (Thermo Fisher). 293F cells were cultured in FreeStyle™ F17 Expression Medium (Thermo Fisher) with 0.1% Pluronic F-68 (Thermo Fisher), 100 U/ml penicillin, 100 mg/ml streptomycin, and 8 mM L-glutamine. C1R stably transfected with RSV.5neo containing the cDNA MICA*07, MICA*08 (21), ULBP4 (isoform 0), and ULBP4 (isoform 1) were cultivated in presence of 1.8 mg/ml G418.

## Antibodies

Anti-ULBP4 mAb clone 709116 and ULBP4-specific goat polyclonal antibodies (pAb) were from R&D (Minneapolis, MN, USA), anti-ULBP4 mAb clone 6E6 from Santa Cruz (Dallas, TX, USA) and the anti-hexahistidine-tag mAb His.H8 from Thermo Fisher. Secondary staining reagents allophycocyanin-conjugated goat anti-mouse Ig (GAM-APC), Alexa-Fluor-488-conjugated donkey anti-goat Ig (DAG-488), and phycoerythrin-conjugated streptavidin (SA-PE) were from Jackson ImmunoResearch (West Grove, PA, USA). MICA-specific mAb AMO1 and MICA/B-specific mAb BAMO3 were previously described (34). Anti-ULBP4 mAb DUMO1 was generated by immunizing BALB/c mice with P815-ULBP4 transfectants and soluble ULBP4 (sULBP4) by standard hybridoma technology as previously described (35) and isotyped as IgG1. Supernatants of hybridoma were screened for binding to 293-ULBP4 transfectants by flow cytometry. DUMO1 binds to P815, 293, and COS-7 cells transfected with ULBP4, but not to the respective mock transfectants, and DUMO1 does not cross-react with ULBP1, ULBP2, and ULBP3, respectively (data not shown).

## Flow Cytometry

Cells were harvested, washed twice with fluorescence-activated cell sorter (FACS) buffer (PBS, 2% FCS, 2 mM EDTA, and 0.01% sodium azide) and stained with 10 µg/ml of the primary antibody for 20 min at 4°C. Then, cells were washed again with FACS buffer and stained with DAG-488 (10 µg/ml) or GAM-APC (1.25 µg/ml) for 20 min at 4°C. After additional washing, flow cytometry analyses were performed using a FACS Canto II (BD Biosciences, Heidelberg, Germany) and data analyzed using FlowJo (Tree Star, Ashland, OH, USA). Biotinylated soluble truncated standard ULBP4 (ststULBP4) was immobilized on streptavidin-coated microspheres (Bangs Laboratories, Fishers, IN, USA) by incubating 5 µg microspheres with 5 µg/ml biotinylated ststULBP4 for 15 min at 4°C. Subsequently, ststULBP4-loaded microspheres were washed twice with FACS buffer, stained with antibodies, and analyzed by flow cytometry as described earlier. The specific fluorescence intensity was calculated by subtracting the median fluorescence intensity (MFI) of the isotype control from the MFI of the antibody of interest.

## Production of sULBP4

A cDNA encoding for the first 222 amino acids of ULBP4 (Met 1 to Ser 222) was cloned into the pFUSE vector (InvivoGen, San Diego, CA, USA) in front of an AviTag and a hexahistidine tag

[1] http://www.uniprot.org/uniprot/Q8TD07 (Accessed: January 31, 2018).

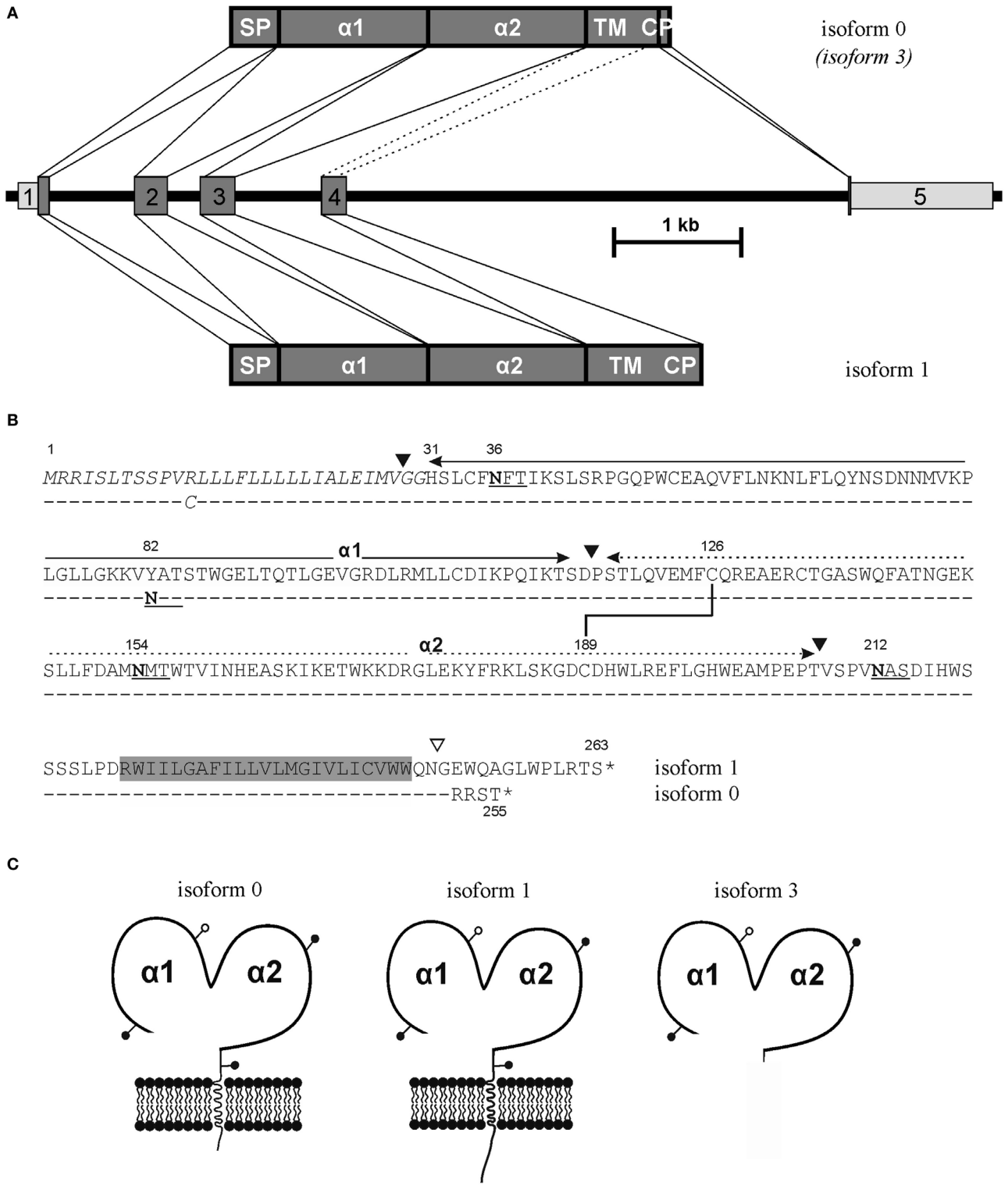


**FIGURE 1** | ULBP4 isoforms generated by differential splicing. **(A)** Differential usage of an internal splice site in exon 4 gives rise to the major full-length ULBP4 isoforms 0 and 1, respectively, while exclusion of exon 4 by alternative splicing (stippled lines) gives rise to the putatively secreted soluble ULBP4 isoform 3. Depicted is the exon–intron structure of the ULBP4/RAET1E gene and the alternatively spliced gene products representing isoforms 0, 3, and 1. **(B)** Amino acid sequence alignment of ULBP4 isoform 1 [upper; GenBank accession no. AY252119 (18)] and isoform 0 (lower; GenBank accession no. MH020173). Exon boundaries are indicated by black triangles except for the exon 4/5 boundary of isoform 0 (open triangle). N-linked glycosylation sites are underlined with asparagines in bold. Signal peptide sequence (Met 1 to Gly 30) is italicized, transmembrane domain highlighted in gray. Disulfide bond between Cys 126 and Cys 189 highly conserved in the MHC class I fold is indicated. **(C)** Scheme of ULBP4 isoforms 0, 1, and 3 with N-glycosylation sites indicated by pins.

(**Figure 2A**) replacing the IL-2 signal sequence and the Fc-encoding region of the vector. The resulting plasmid encoding for soluble carboxyterminally truncated and tagged ULBP4 (stULBP4) was used for transient transfection of 293F cells. Supernatants were harvested 4 days later and stULBP4 affinity-purified using HisPurTM Ni-NTA spin columns (Thermo Scientific) according to the manufacturer's protocol. Eluted stULBP4 was concentrated using Amicon® Ultra Centrifugal Filter units (Merck, Darmstadt, Germany) and further purified by size exclusion chromatography using a Hiload™ 16/60 Superdex™ 200 column (GE Healthcare, Chicago, IL, USA) on an ÄKTA purifier (GE Healthcare). stULBP4 was collected and analyzed by SDS-PAGE (**Figure 2B**). For determining concentrations of sULBP4 and sMICA in culture supernatants, ststULBP4 (His 31 to Ser 222 followed by an AviTag and a hexahistidine tag) or soluble truncated standard MICA07 (ststMICA) (Glu 24 to Gln 304 followed by an AviTag and a hexahistidine tag) was produced as described above for stULBP4 in 293F cells and purified by Ni-NTA spin columns except that the vector-encoded IL-2 signal sequence was placed in front of the coding regions of mature ULBP4 and MICA glycoproteins. Biotinylation of ststULBP4 was done using BirA Biotin-protein ligase (Avidity, Aurora, CO, USA) according to the manufacturer's protocol.

## Quantitative Real-Time PCR

Cellular RNA was isolated using pegGOLD TriFast™ (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. RNA from human esophagus and skin was purchased from Thermo Fisher. RNA was treated with RNAse-free DNAse (Promega, Madison, WI, USA) and subsequently converted into cDNA using M-MLV RT RNAse (H−) Point Mutant (Promega) according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using SYBR Green technology (Roche, Basel, Switzerland) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For amplification of ULBP4, oligonucleotides Ex3fw (5′-CTGGCTCAGGGAATTCTTAGG-3′) and Ex4rv (5′-CTAGAAGAAGACCAGTGGATATC-3′) were used. To selectively amplify ULBP4 isoforms 0 or 1, the forward primer ULBP4_fw (5′-TACCAGATAGATGGATCATCCTG-3′) was combined with the reverse primers ULBP4_Iso0_rv (5′-CTAGGTGGATCTTCTGCCATT-3′) or ULBP4_Iso1_rv (5′-CTAAGACGTCCTCAAGGGCC-3′), respectively (all from Sigma-Aldrich). Copy numbers were normalized with the ΔΔCt method using 18S rRNA as previously described (35).

## Immunoblotting

HeLa cells were harvested, washed twice with PBS and resuspended with ice-cold NP40-Lysis-Puffer [1% NP40, 50 mM Tris, 150 mM NaCl, Complete Protease Inhibitor (Roche, Mannheim, Germany)]. Lysates were incubated for 20 min on ice and then centrifuged for 15 min at 17,000 *g*. ULBP4 was immunoprecipitated with biotinylated DUMO1 coupled to streptavidin magnetic beads (Thermo Fisher Scientific) for 3 h at 4°C. Immunoprecipitates were washed several times and eluted using denaturation buffer [0.5% SDS, 40 mM dithiothreitol (DTT)] for 5 min at 95°C. Deglycosylation was performed using endoglycosidase H or peptide-N-glycosidase F (both from New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocols. Immunoprecipitates were separated using SDS-PAGE and blotted onto a PVDF membrane (Carl Roth, Karlsruhe, Germany). Membrane was blocked using 5% non-fat dried milk powder (AppliChem) in TBST (150 mM NaCl, 10 mM Tris, 0.1% Tween 20) and then probed with 2 µg/ml ULBP4-specific pAb, and subsequently with 0.16 µg/ml HRP-conjugated donkey anti-goat-Ig (Santa Cruz). Immunoblots were developed with HRP-Juice Plus (PJK, Kleinbittersdorf, Germany) using a Fusion SL imaging system (Vilber Lourmat).

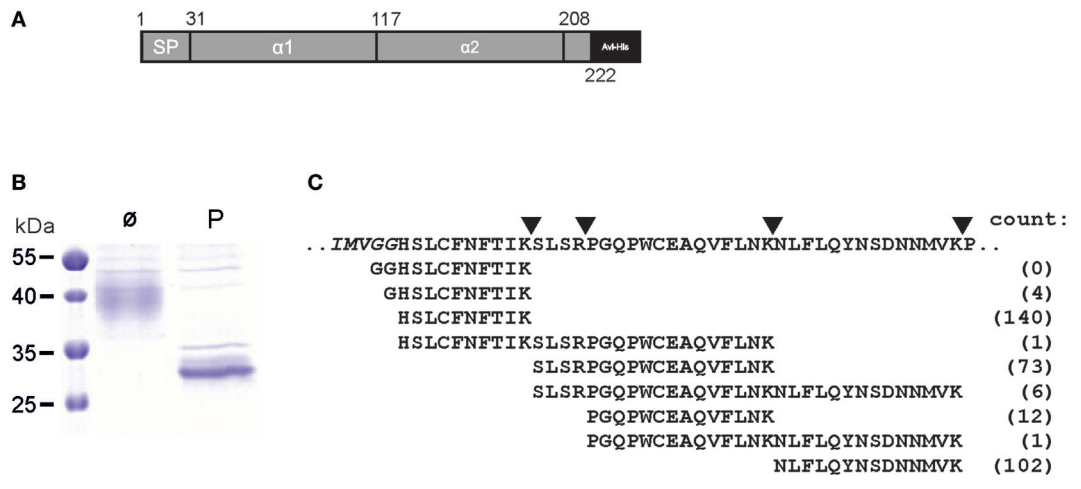


**FIGURE 2** | Aminoterminus of mature ULBP4 glycoproteins. **(A)** Scheme of soluble carboxyterminally truncated and tagged ULBP4 (stULBP4) used for mass spectrometric analyses. **(B)** Affinity-purified stULBP4 from supernatants of transfected 293F cells was treated either with PNGaseF (P) or left untreated (ø), separated by SDS-PAGE, and visualized by Coomassie staining. **(C)** Abundancy of aminoterminal stULBP4 fragments as determined by mass spectrometry. stULBP4 was digested with trypsin and LysC, and resulting fragments were analyzed and counted by mass spectrometry.

## Detection of sULBP4 and sMICA

For detection of sULBP4 and sMICA, anti-ULBP4 pAb (R&D Systems) or mAb AMO1 were immobilized on MagPlex-C microspheres (Luminex, Austin, TX, USA), respectively, according to the manufacturer's instructions. Cell culture supernatants were diluted 1:1 in PBS with 1% BSA (dilution buffer) and added together with SA-PE and either biotinylated DUMO1 (for sULBP4 detection) or biotinylated BAMO3 (for sMICA detection) to 500 microspheres. Standard curves were generated by titrating ststULBP4 or ststMICA. Samples were incubated overnight at room temperature, washed twice in dilution buffer, and measured on a Luminex 100 system (Luminex). All samples were measured in triplicates.

## In-Solution Digestion

Purified stULBP4 protein samples were precipitated with acetone, and subsequently in-solution digestion was performed as described (36). In brief, protein pellets were washed and resuspended in denaturation buffer containing 6 M urea and 2 M thiourea. Proteins were reduced with 1 mM DTT, alkylated with 5.5 mM iodoacetamide and digested with the endopeptidase LysC (Wako) for 3 h and sequencing-grade trypsin (Promega) overnight. Peptide mixtures were concentrated and desalted using the Stop and Go Extraction technique (37).

## Liquid Chromatography and Mass Spectrometry

A binary buffer system consisting of buffer A (0.1% formic acid) and buffer B (80% acetonitrile, 0.1% formic acid) was used for peptide separation on an Easy-nLC 1200 (Thermo Fisher Scientific). This system was coupled *via* a nano electrospray ionization source to the quadrupole-based Q Exactive HF benchtop mass spectrometer (38). Peptide elution from the in-house packed 18 cm (1.9 µm C18 Beads, Dr. Maisch Germany) column was achieved by increasing the relative amount of B from 10 to 38% in a linear gradient within 23 min at a column temperature of 40°C. Followed by an increase to 100% B within 7 min and gradients were completed by a re-equilibration to 5% B.Q Exactive HF settings: MS spectra were acquired using 3E6 as an AGC target, a maximal injection time of 20 ms and a 60,000 resolution at 300 *m*/*z*. The mass spectrometer operated in a data-dependent Top15 mode with subsequent acquisition of higher-energy collisional dissociation fragmentation MS/MS spectra of the top 15 most intense peaks. Resolution for MS/MS spectra was set to 15,000 at 200 *m*/*z*, AGC target to 1E5, maximal injection time to 25 ms and the isolation window to 1.6 Th.

## Mass Spectrometry Data Processing and Analysis

All acquired raw files were processed using MaxQuant (1.5.3.30) (39) and the implemented Andromeda search engine (40). For protein assignment, electrospray ionization-tandem mass spectrometry (ESI-MS/MS) fragmentation spectra were correlated with the Uniprot human database (v. 2017) with manually added peptide sequences of ULBP4 starting with all possible N-termini of the mature protein. Searches were performed with tryptic specifications and default settings for mass tolerances for MS and MS/MS spectra. Carbamidomethyl at cysteine residues was set as a fixed modification, while oxidation at methionine, acetylation at the N-terminus, and conversion from Asn to Asp were defined as variable modifications. The minimal peptide length was set to seven amino acids, and the false discovery rate for proteins and peptide spectrum matches to 1%. The match-between-run feature was used with a time window of 0.7 min.

## Statistics

Statistical analyses as detailed in the figure legends were performed using Prism 7 (GraphPad, San Diego, CA, USA).

# RESULTS

## Aminoterminus of Mature ULBP4 Glycoproteins Is Recessed

We noted conflicting predictions for the aminoterminus of mature ULBP4 glycoproteins. In the original reports, the aminoterminus of mature ULBP4 glycoproteins, as generated by cleavage of the putative aminoterminal signal peptide, has been assigned in the absence of experimental evidence to glycine 29 along the exon 1/exon 2 boundaries (18, 19) presumably based on sequence alignments with other ULBP and MHC class I-related molecules (**Figures 1A,B**). However, we noted that the database Uniprot (22), in contrast to the existing literature, had assigned the aminoterminus of mature ULBP4 molecules to histidine 31 based on "manual assertion according to rules" (see text footnote 1). To clarify these conflicting predictions, we addressed this issue experimentally: soluble, carboxyterminally truncated and tagged ULBP4 ectodomains encompassing the α1, the α2 domain, and most of the serine-rich region, preceded by the "natural" ULBP4 signal peptide (stULBP4; Met 1 through Ser 222) were ectopically expressed in 293F cells, purified from the supernatants by Nickel-NTA spin columns (**Figures 2A,B**), fragmented by digestion with trypsin and LysC, and resulting stULBP4 fragments were subjected to liquid chromatography–mass spectrometry (LC–MS). Mass spectrometry identified all expected tryptic fragments almost completely covering the mature stULBP4 polypeptide (data not shown). The aminoterminus of mature ULBP4 molecules, however, is generated by cleavage of the signal peptide during cotranslational translocation into the ER, and, accordingly, potentially resulting in the peptides GGHSLCFNFTIK, GHSLCFNFTIK, or HSLCFNFTIK, respectively, depending on the actual cleavage site. While the fragment HSLCFNFTIK was detected at high abundance, the peptides GGHSLCFNFTIK and GHSLCFNFTIK were not or only very rarely detected (**Figure 2C**). These data strongly suggest that mature ULBP4 glycoproteins start with histidine 31 in line with the prediction by Uniprot and not as previously assumed with glycine 29. It remains to be determined how such a recessed β1 strand of the ULBP4 α1 domain may impact ULBP4 folding and function differently as compared with other ULBP molecules. Our mass spectrometric analyses also showed that the three putative N-glycosylation sites predicted by Uniprot at Asn 36 (α1 domain), Asn 154 (α2 domain),

and Asn 212 (serine-rich region) all were glycosylated. In addition, we also detected N-linked glycosylation at Asn 82 (**Figures 1B,C**). Position 82 is polymorphic and occupied either by asparagine (stULBP4) or tyrosine (Uniprot reference sequence at http://www.uniprot.org/uniprot/Q8TD07), and therefore ULBP4 glycosylation can be expected to be variable within the human population with potential consequences for expression and detection. While Asn 82, Asn 154, and Asn 212 were glycosylated in almost all (>99%) tryptic fragments analyzed, glycosylation efficiency at Asn 36 appeared slightly reduced with ~90% of the fragments glycosylated (data not shown).

## Carboxyterminus of ULBP4 Is Variable due to Alternative Splicing

Original studies of ULBP4 reported that exon 4, in addition to the serine-rich stalk region, entirely encodes for both transmembrane and cytoplasmic ULBP4 residues (18, 19) (**Figure 1**). In the course of our studies on ULBP4, we characterized a commercially available EST clone (clone 601078687F1) derived from a cervical carcinoma cell line encoding for a ULBP4 variant with a divergent carboxyterminus (GenBank accession no. MH020173). This ULBP4 variant differs from the originally published sequence by an alternate and shorter cytoplasmic tail which is created by alternative splicing due to an alternative splice donor site within exon 4 (**Figure 1**). Accordingly, the 5′ portion of exon 4 is merged in frame with an additional exon located ~4 kb downstream (exon 5) which encodes the four carboxyterminal amino acids followed by a long 3′ UTR (**Figure 1**). Consequently, such ULBP4 isoforms have a distinct cytoplasmic domain shortened by eight amino acids as compared with the originally reported ULBP4 isoform 1 (**Figure 1**) (18, 19). In addition, Uniprot lists five other ULBP4 isoforms (isoforms 2–6) based on reports of alternatively spliced transcripts for which experimental evidence of protein expression is lacking or scarce (see text footnote 1). Among these, isoform 3 is the only isoform that also includes exon 5 as reported here for the new ULBP4 isoform. Isoform 3 was identified in the course of a broad screen by the secreted protein discovery initiative (24) that used computational and experimental approaches to identify human cDNA clones encoding for putatively secreted proteins preceded by a signal peptide. Of note, the cDNA of isoform 3 is composed of exons 1, 2, 3, and 5, but lacks exon 4 which encodes for the transmembrane region. Hence, isoform 3 corresponds to an alternatively spliced and potentially soluble isoform of the new full-length ULBP4 isoform reported here to which we refer as isoform 0 in the following (**Figure 1**). The original studies have shown that ULBP4 isoform 1 can be expressed at the cell surface of transfected cell lines and is functionally recognized by NK cells (18, 19). To assess whether the newly reported isoform 0 likewise gives rise to a ULBP4 glycoprotein that can be functionally expressed at the cell surface and consequently be recognized *via* NKG2D by NK cells and T cells, we generated a ULBP4-specific mAb.

### ULBP4 Antibodies

The ULBP4-specific mAb DUMO1 was generated by immunizing mice with mouse mastocytoma cells P815 stably transfected with ULBP4. DUMO1 binds to ststULBP4 immobilized on microspheres (ststULBP4_im), but not to control microspheres (**Figure 3A**). DUMO1 also bound to 293F cells and C1R cells transfected with either ULBP4 isoform 0 or isoform 1, but not to the corresponding mock transfectants (**Figures 3C,D**). Apart from corroborating specificity of DUMO1 for cellular ULBP4, these data also demonstrate that ULBP4 isoform 0 can be broadly expressed at the cell surface. While our data also indicate that ULBP4 isoform 0 may allow for a brighter surface expression than ULBP4 isoform 1, this possibility needs to be validated by further experiments. For control, we included the few commercially available antibodies said to be ULBP4-specific. While ULBP4-specific pAb and mAb 709116 bound ststULBP4_im and ULBP4 transfectants similarly to DUMO1, we did not detect binding of mAb 6E6 neither to ststULBP_im nor to the ULBP4 transfectants by flow cytometry (**Figures 3A,B**), although mAb 6E6 brightly detected denatured ststULBP4 in immunoblotting (data not shown). mAb 6E6 has previously been used to demonstrate ULBP4 surface expression on EBV-infected cells and on placental exosomes (31, 41) as well as on ULBP4 transfectants[2] which is puzzling in the light of our results.

### ULBP4 Expression

Among human tissues, we found ULBP4 transcripts by qPCR most abundantly in tissues which are of ectodermal origin such as skin, esophagus, and cervix (data not shown). A preferential ULBP4 expression in human skin has already been reported by Cosman and colleagues (18) and a strong expression bias toward tissues of ectodermal origin is also documented by publicly available databases.[3] We wondered whether isoforms 0 and 1 may be differentially expressed in such tissues but detected both at a comparable abundance in both skin and esophagus (**Figure 4A**). Assessing abundance of ULBP4 transcripts in a broad variety of human tumor cell lines, we detected ULBP4 transcripts most abundantly in the cervix carcinoma cell line HeLa, whereas ULBP4 transcripts were undetectable in liver cancer cells HepG2 or in the erythroleukemia line K562 (**Figure 4B** and data not shown) well in line with publicly available data sets.[4] Of note, pronounced ULBP4 surface expression has been claimed based on binding of mAb 709116 for HepG2 cells by the supplier,[5] for the erythroleukemia line K562 (42) and for cytokine-activated NK cells (32). By using DUMO1 and ULBP4-specific pAb, we were unable to detect ULBP4 surface expression on HeLa, HepG2, HCT116, and HaCat cells, respectively. By contrast, mAb 709116 bound to a substantial portion of HCT116 cells and brightly stained HepG2 cells (**Figure 4D**) as reported by the supplier and as previously reported for another not publicly available mAb (29), although we were unable to detect ULBP4 transcripts in HepG2 cells. Collectively, our data on mAb 6E6 and 709116 advise substantial caution when interpreting results obtained with these commercially available ULBP4 antibodies

[2]https://www.scbt.com/scbt/product/ulbp4-antibody-6e6 (Accessed: January 31, 2018).
[3]https://www.proteinatlas.org/ENSG00000164520-RAET1E/tissue(Accessed: January 31, 2018).
[4]https://www.proteinatlas.org/ENSG00000164520-RAET1E/cell (Accessed: January 31, 2018).
[5]https://www.rndsystems.com/products/human-ulbp-4-raet1e-apc-conjugated-antibody-709116_fab6285a (Accessed: January 31, 2018).

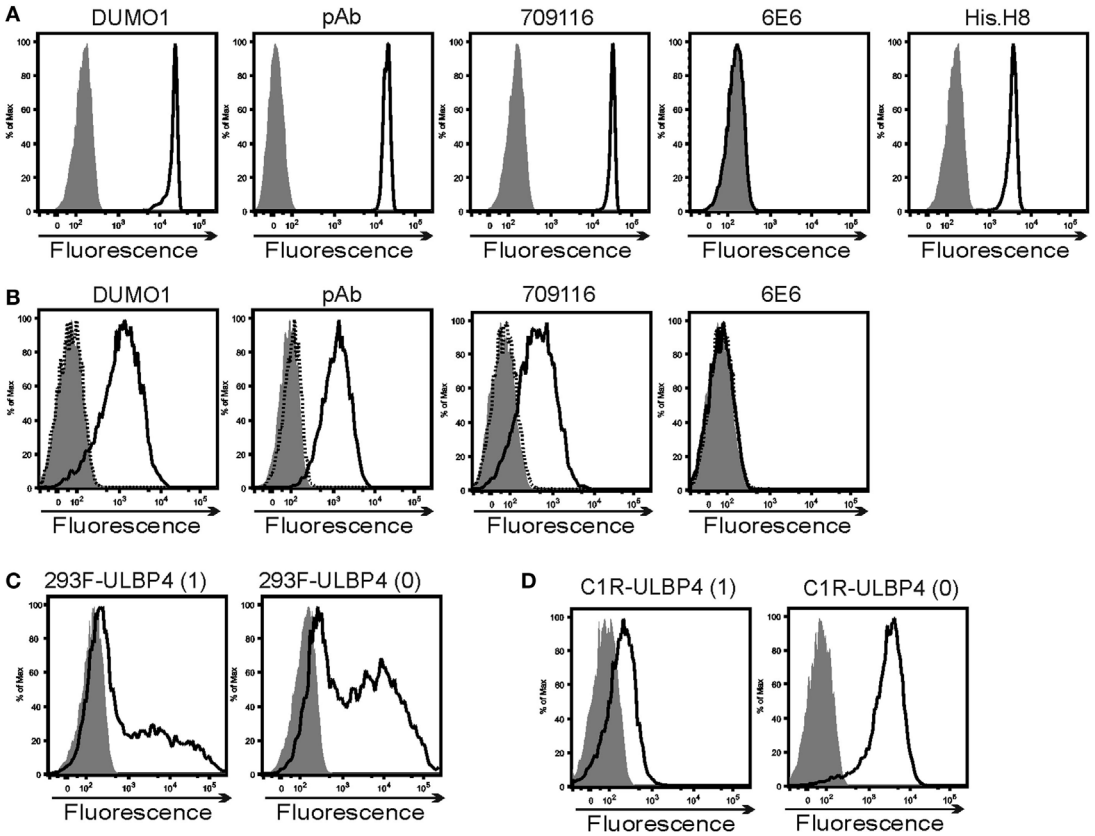


**FIGURE 3** | Characterization of ULBP4-specific antibodies. **(A)** Binding of various ULBP4 antibodies or anti-His-tag mAb (solid lines) to biotinylated ststULBP4 immobilized on streptavidin-coated microspheres. Control stainings are shaded. **(B)** Binding of various ULBP4 antibodies to C1R-ULBP4 isoform 0 cells (solid lines). Control stainings are shaded. No binding of ULBP4 antibodies to C1R-mock transfectants was detected (dotted). **(C,D)** Binding of DUMO1 to **(C)** 293F or **(D)** C1R cells transfected with ULBP4 isoform 1 or ULBP4 isoform 0. Control stainings are shaded.

and strongly suggest to validate such data by independent methodological approaches such as by qPCR and/or immunoblotting. Our data also indicate that in contrast to other NKG2DL such as MICA, MICB, and ULBP1–3, which are frequently expressed on a broad variety of human tumor cell lines (43), ULBP4 may be not or only sparsely expressed on the surface of human tumor cell lines. In fact, immunoblotting of lysates of HeLa cells revealed that the vast majority of detectable ULBP4 glycoproteins is sensitive for digestion with endoglycosidase H, and therefore retained intracellularly in the ER or Golgi complex well in accordance with the lack of detectable cell surface expression (**Figures 4C,D**). Assessing expression of ULBP4 isoform 1 versus isoform 0 in more than 30 human cell lines, we found that three cell lines including HeLa cells (HeLa, LNCaP, and MG-63) and primary keratinocytes expressed both isoforms, 8 cell lines expressed only isoform 0, but none expressed only isoform 1 (data not shown). Based on these findings, one may consider the possibility that the previously reported isoform 1 rather is a byproduct of inefficient splicing of ULBP4 primary transcripts, whereas isoform 0 may represent the physiologically more relevant isoform. Future research on ULBP4 should take such considerations into account.

## Soluble ULBP4

Isoform 3 may represent a soluble variant of ULBP4 generated by alternative splicing of the full-length isoform 0 excluding exon 4 (**Figure 1**). However, evidence for isoform 3 thus far relies only on analyses of transcripts/cDNA, but not on protein data (24). Therefore, existence and secretion of sULBP4 based on isoform 3 transcripts has to await validation by experimental evidence. In addition, there are reports claiming the existence of other sULBP4 isoforms generated by alternative splicing (23, 44). However, for the originally reported soluble RAET1E2 isoform (Uniprot isoform 4) (44), no corresponding transcripts could be detected in a subsequent study by the same group (23). This latter study reported three other rarely occurring ULBP4 splice variants all comprising the entire exon 4 encoding for the transmembrane domain. Accordingly, these variants (Uniprot isoforms 2, 5, and 6) reportedly are cell membrane-bound variants (23), which obviously cannot be secreted without further processing. Considering physiologic expression of ULBP4 in skin, we assessed freshly isolated human keratinocytes for ULBP4 transcripts. By pairing a primer located in exon 1 with a reverse primer in the 3′ end of exon 4 (isoform 1) or in the 5′ end of exon 5 (isoform 0), respectively, we detected predominant expression

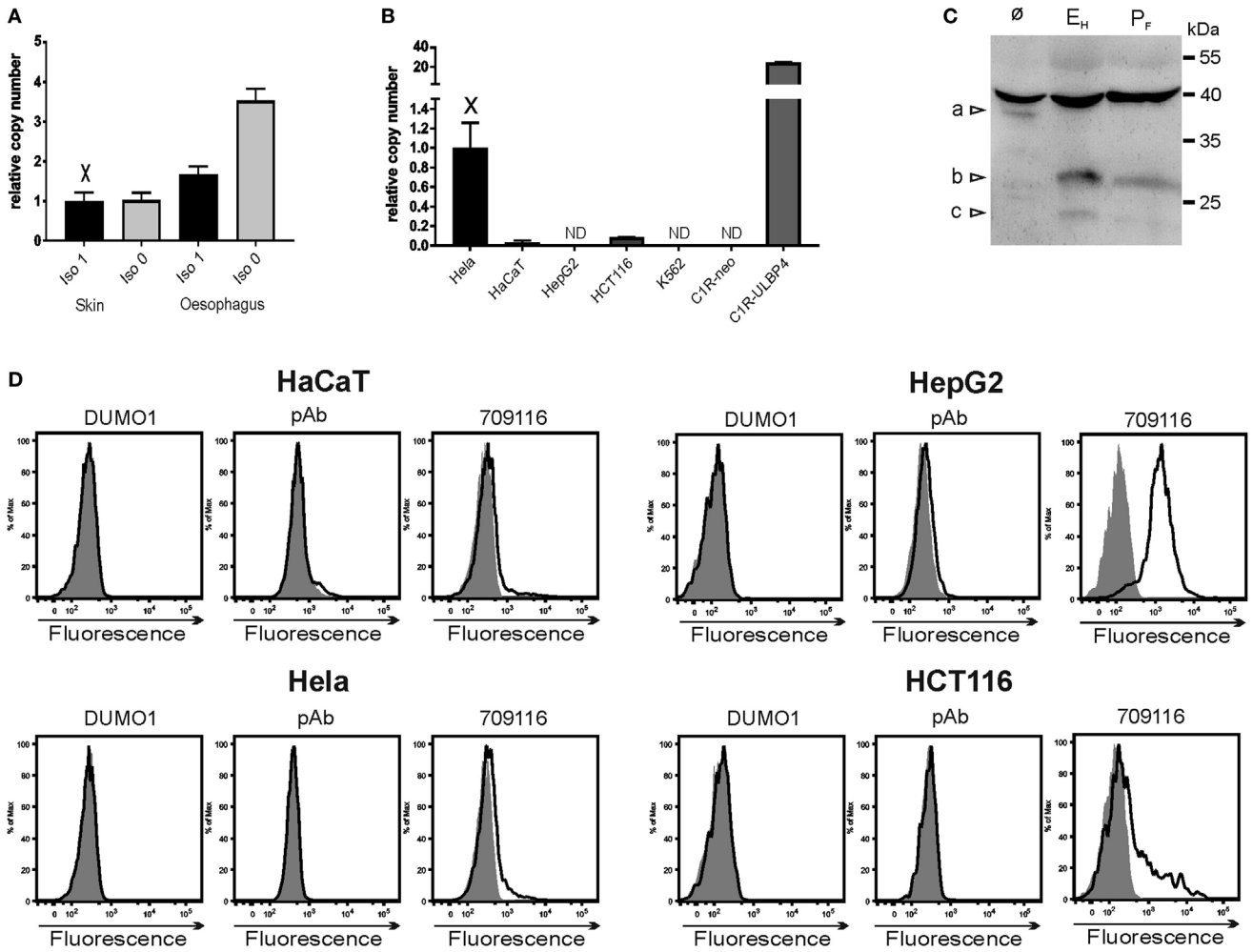


**FIGURE 4** | Detection of ULBP4 expression. **(A)** Abundance of ULBP4 isoforms 0 and 1 in human skin or esophagus as determined by quantitative PCR (qPCR). **(B)** Abundance of ULBP4 transcripts in various human cell lines as determined by qPCR. Abbreviation: ND, not detectable. **(C)** ULBP4 glycoproteins in lysates of HeLa cells were detected after immunoprecipitation with mAb DUMO1 with ULBP4-specific pAb in immunoblotting. Lysates were treated with endoglycosidase H ($E_H$) or PNGase F ($P_F$) before SDS-PAGE where indicated. Triangles indicate positions of glycosylated ULBP4 [a] in untreated lysates, and putative deglycosylated isoforms 0/1 [b] or 3 [c] in lysates treated with endoglycosidase H and PNGase F. **(D)** Binding of ULBP4 antibodies (solid lines) to various human cell lines. Control stainings are shaded.

of transcripts corresponding to both full-length isoforms 0 and 1. At considerably lower frequency, we also detected transcripts corresponding to isoforms 2 and 3, but not to isoforms 4–6 (data not shown). Hence, our data indicate, that sULBP4 may be generated physiologically by alternative splicing under exclusion of exon 4 (i.e., ULBP4 isoform 3) although existence of such sULBP4 glycoproteins remains to be shown experimentally. We and others had previously reported that most human NKG2DL can be released from cells either by proteolytic shedding or by exosomal secretion (12, 34, 45–47). MICA (but not MICA*08), MICB, and ULBP2 have been shown to be shed by metalloproteases such as ADAM10, ADAM17, and MMP14 (48–51) while MICA*08 and ULBP3 were shown to be preferentially released in exosomes (45, 52). However, neither proteolytic shedding nor exosomal release has been reported for ULBP4 possibly due to the scarcity of *bona fide* ULBP4-specific antibodies.

To address release of sULBP4 from ULBP4-expressing cells, we established a sandwich assay specifically detecting sULBP4 using immobilized anti-ULBP4 pAb and biotinylated mAb DUMO1. Using this assay, sULBP4 was clearly detectable in supernatants of C1R cells stably transfected with ULBP4 isoforms 0 and 1, respectively (**Figure 5B**). To assess whether metalloproteases are involved in the generation of sULBP4, we added batimastat (BB-94), a potent, broad spectrum metalloprotease inhibitor, to cultures of C1R transfectants. As expected and in line with the previous reports, addition of BB-94 to C1R-MICA*07 and C1R-MICA*08 cells inhibited release of sMICA*07, but not of sMICA*08, in a dose-dependent manner (**Figure 5A**). Of note, release of sULBP4 from both C1R-ULBP4 transfectants was also inhibited in a BB-94 dose-dependent manner demonstrating that both ULBP4 isoforms 0 and 1 can give rise to sULBP4 due to the action of metalloproteases (**Figures 5B,C**). Finally,

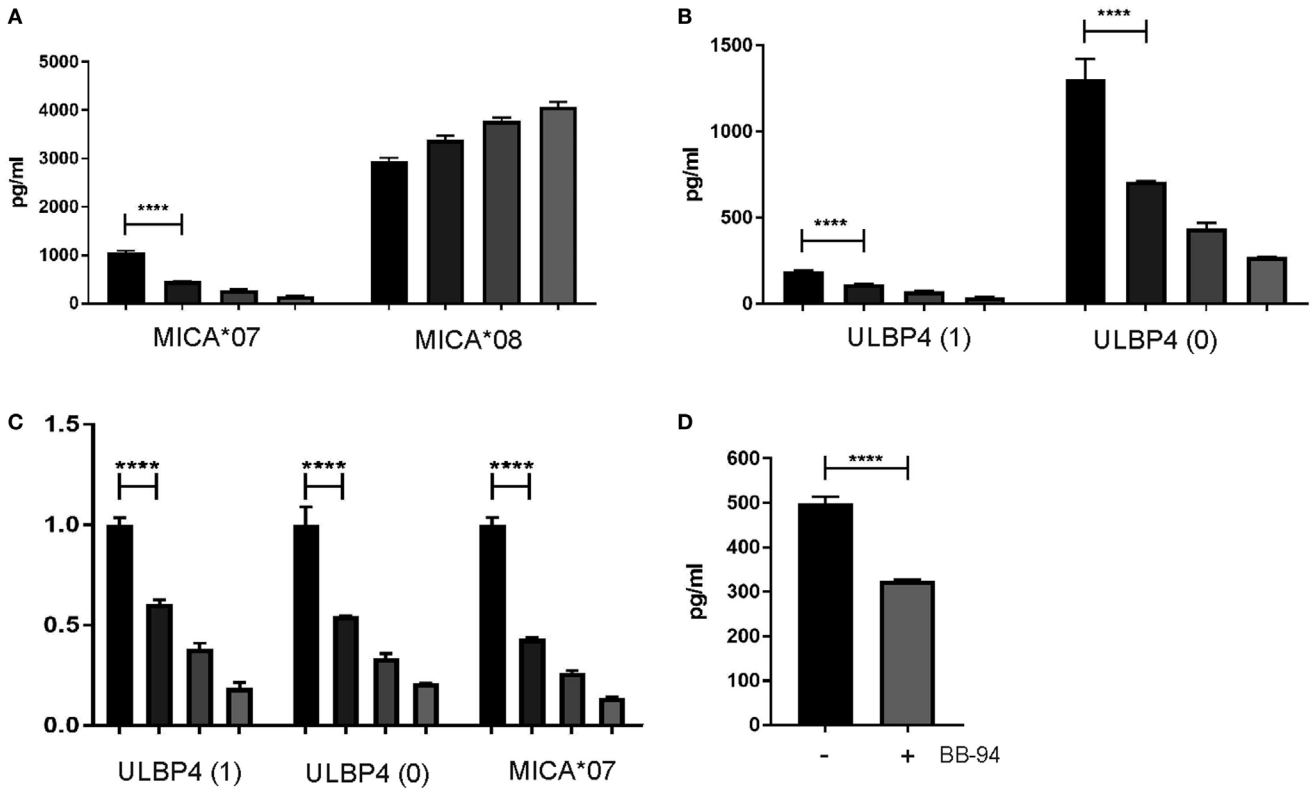


**FIGURE 5** | Proteolytic release of soluble ULBP4 (sULBP4). **(A–C)** C1R cells ($1 \times 10^6$ cells/ml) stably transfected with ULBP4 isoforms 0, ULBP4 isoform 1, MICA*07, or MICA*08, respectively, were cultivated in the presence of various concentrations of batimastat for 42 h (from left to right: 0, 0.2, 1, and 5 μM batimastat). Subsequently, supernatants were analyzed for concentrations of **(A)** sMICA or **(B)** sULBP4. **(C)** Relative changes of concentrations (mean ± SD) as depicted in panels **(A,B)** with values for control-treated samples (0 μM batimastat) set as 1. One representative experiment out of three is shown. Statistical significance was only assessed for differences between samples treated with 0 versus 0.2 μM batimastat using a one-way ANOVA with Tukey's multiple comparisons test (****$p < 0.0001$). **(D)** Detection of sULBP4 in supernatants of HeLa cells grown for 65 h in absence or presence of 10 μM batimastat (BB-94). Statistical significance was calculated using unpaired *t*-test (****$p < 0.0001$).

we assessed sULBP4 in the culture supernatants of various cell lines expressing ULBP4 transcripts (**Figure 4B**). In line with the highest abundance of ULBP4 transcripts in HeLa cells, we only detected low amounts sULBP4 in the supernatants of HeLa cells (**Figure 5D**), but not of HCT116, HepG2, and HaCat cells (data not shown). Release of sULBP4 by HeLa cells could only partially be blocked by addition of BB-94 (**Figure 5D**) indicating that HeLa cells generate sULBP4 not only by shedding of full-length isoforms 0 and/or 1 through metalloproteases. Since we detected in HeLa cells both transcripts of ULBP4 isoform 3 (data not shown) and ULBP4 proteins of a molecular mass corresponding to isoform 3 (**Figure 4C**), secretion of isoform 3 may explain the BB-94 independent release of sULBP4 by HeLa cells.

## CONCLUDING REMARKS

ULBP4 glycoproteins are among the least characterized human NKG2D ligands. Our present study suggests that this may be explained by a highly restricted expression in tissues and by cell lines as well as by the scarcity and deficiency of commercially available antibodies. While prevalence of ULBP4 transcripts indicates a strong expression bias toward tissues and cell lines of ectodermal origin, physiologic and cellular expression of ULBP4 glycoproteins by such cells remains to be addressed by future research. We here provide evidence that the aminoterminus of mature ULBP4 molecules is recessed when compared with other ULBP molecules and that also the carboxyterminus of at least a substantial portion of ULBP4 molecules substantially differs from the originally reported sequence due to previously unrecognized alternative splicing, resulting in a shortened mature ULBP4 polypeptide which we termed isoform 0. It remains to be shown which isoform is more physiological relevant. Our results also suggest that some previous results obtained with mAb 6E6 and mAb 709116 should be considered with caution. Furthermore, we here demonstrate that ectopically expressed ULBP4 is shed from the cell surface by metalloproteases giving rise to sULBP4 in a manner similarly to many other human NKG2DL. However, in HeLa cells release of sULBP4 was only in part dependent on metalloprotease activity, and possibly a major portion of sULBP4 secreted by HeLa cells is due to the alternatively spliced isoform 3. Of note, most ULBP4 glycoproteins were intracellularly retained in HeLa cells as evident from their lack of EndoH resistance and lack of surface expression, and it remains to be determined by future studies whether this is

a peculiar feature of HeLa cells or a general property of ULBP4. In addition, it will be of considerable interest to determine occurrence of sULBP4, either proteolytically shed or as secreted isoform 3, in healthy or diseased state such as in patients with skin or cervical tumors. Altogether, we propose that ULBP4 should not regarded as just another NKG2DL, but that the unique cellular expression and processing of ULBP4 together with the early evolutionary separation from other ULBP family members indicates a peculiar function within the framework of NKG2D-mediated immunosurveillance.

## ETHICS STATEMENT

Study includes analyses of buffy coats obtained from the Blood Donor Service Baden-Wuerttemberg-Hessen, Frankfurt am Main, after approval by the Ethics committee of the Goethe University of Frankfurt. Study includes generation of monoclonal antibodies based on immunization of mice which was carried out in accordance with current laws for animal research and approved by the Regierungspräsidium Tübingen and Darmstadt.

## AUTHOR CONTRIBUTIONS

TZ, MH, and MW designed and performed experiments and analyzed data. AS conceptualized study, designed experiments, and wrote the manuscript with the support of TZ and MH.

## ACKNOWLEDGMENTS

The authors thank Christina Born for excellent technical assistance.

## FUNDING

This work was funded by institutional funds of the Institute for Molecular Medicine.

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**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: 23 April 2018
doi: 10.3389/fimmu.2018.00827

# The NKG2D/NKG2DL Axis in the Crosstalk Between Lymphoid and Myeloid Cells in Health and Disease

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Natural killer group 2, member D (NKG2D) receptor is a type II transmembrane protein expressed by both innate and adaptive immune cells, including natural killer (NK) cells, CD8+ T cells, invariant NKT cells, γδ T cells, and some CD4+ T cells under certain pathological conditions. NKG2D is an activating NK receptor that induces cytotoxicity and production of cytokines by effector cells and supports their proliferation and survival upon engagement with its ligands. In both innate and T cell populations, NKG2D can costimulate responses induced by other receptors, such as TCR in T cells or NKp46 in NK cells. NKG2D ligands (NKG2DLs) are remarkably diverse. Initially, NKG2DL expression was typically attributed to stressed, infected, or transformed cells, thus signaling "dysregulated-self." However, many reports indicated their expression under homeostatic conditions, usually in the context of cell activation and/or proliferation. Myeloid cells, including macrophages and dendritic cells (DCs), are among the first cells sensing and responding to pathogens and tissue damage. By secreting a plethora of soluble mediators, by presenting antigens to T cells and by expressing costimulatory molecules, myeloid cells play vital roles in inducing and supporting responses of other immune cells in lymphoid organs and tissues. When activated, both macrophages and DCs upregulate NKG2DLs, thereby enabling them with additional mechanisms for regulating lymphocyte responses. In this review, we will focus on the expression of NKG2D by innate and adaptive lymphocytes, the regulation of NKG2DL expression on myeloid cells, and the contribution of the NKG2D/NKG2DL axis to the crosstalk of myeloid cells with NKG2D-expressing lymphocytes. In addition, we will highlight pathophysiological conditions associated with NKG2D/NKG2DL dysregulation and discuss the putative involvement of the NKG2D/NKG2DL axis in the lymphocyte/myeloid cell crosstalk in these diseases.

**Keywords: NKG2D, NKG2D ligands, myeloid cells, natural killer cells, NKG2D+ T cells**

OPEN ACCESS

***Edited by:***
*Nadia Guerra, Imperial College London, United Kingdom*

***Reviewed by:***
*Alessandra Zingoni, Sapienza Università di Roma, Italy*
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***Specialty section:***
*This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology*

***Received:*** *08 February 2018*
***Accepted:*** *04 April 2018*
***Published:*** *23 April 2018*

***Citation:***
*Stojanovic A, Correia MP and Cerwenka A (2018) The NKG2D/NKG2DL Axis in the Crosstalk Between Lymphoid and Myeloid Cells in Health and Disease. Front. Immunol. 9:827. doi: 10.3389/fimmu.2018.00827*

## THE NATURAL KILLER GROUP 2, MEMBER D (NKG2D) RECEPTOR

Natural killer group 2, member D is a type II transmembrane protein with a C-type lectin-like extracellular domain, expressed on the cell surface as a disulfide-linked homodimer. The receptor possesses a short intracellular tail with charged amino acid residues that enable its association with the adaptor molecules DAP10 and/or DAP12, which is essential for NKG2D surface expression (1–3). In mice,

alternative splicing of NKG2D results in two distinct isoforms: NKG2D-short and NKG2D-long. The NKG2D-short isoform can associate with both DAP10 and DAP12, whereas the NKG2D-long isoform associates only with DAP10 (2). Freshly isolated naïve natural killer (NK) cells express only the NKG2D-long isoform that forms complexes with DAP10. Activated NK cells express both NKG2D isoforms and can therefore pair with both adapters (2). However, human NK cells only express the NKG2D-long isoform, and consequently, NKG2D appears to only use DAP10 for signal transduction. NKG2D triggering induces cytotoxic responses, secretion of cytokines and chemokines, and supports proliferation and survival of responding effector cells (4).

The NKG2D receptor gene (*KLRK1*, killer cell lectin-like receptor K1) was first described in 1991 in human NK cells (5). Since then, several other lymphocytes have been shown to express NKG2D, namely, all αβCD8+ T cells in human, activated, but not naïve, αβCD8+ T cells in mice, γδ T cells, invariant NKT (*i*NKT) cells, and some CD4+ T cells under certain pathological conditions. More recently, NKG2D was detected on the surface of innate lymphoid cells (ILCs), including helper ILC1 and ILC3 (6–8). Importantly, those different NKG2D-expressing immune cell types reside in distinct morphological sites, such as lymphoid organs, skin, and epithelial and sub-epithelial tissues. Indeed, NKG2D-expressing cells and upregulation of NKG2D ligands (NKG2DLs) were shown to play important roles in inflammation, anti-tumor, and anti-viral responses in different organs (9, 10). In addition, the pathophysiology of several autoimmune conditions, as well as acute and chronic allograft rejection, involved dysregulated expression or/and activation of NKG2D and its ligands (11–17).

Expression of NKG2D on immune effector cells is regulated by cell activation and microenvironmental factors. In NK cells, which constitutively express NKG2D, the cytokines IL-2, IL-12, and IL-15 were shown to upregulate, while IL-21, IFN-γ, and TGF-β negatively regulated NKG2D surface expression (18). The triggering of the NKG2D receptor in mouse and human NK cells can induce effector functions, such as cytotoxicity and cytokine production. However, several studies revealed that pre-activation with cytokines, such as IL-2 or IL-15, was required to trigger NK cell responses upon NKG2D engagement (19, 20). This feature might have evolved to assure NKG2D activation only in cases when potential "danger signals" are present in the context of inflammation, characterized by the production of inflammatory cytokines. In addition, NKG2D can act as a costimulatory molecule, able to induce cytolytic activity in resting NK cells, when cotriggered with other activating receptors, such as NKp46 or 2B4 (9). Several mechanisms account for cytokine-mediated priming of NKG2D responsiveness, such as IL-2-mediated activation of mTORC1 and upregulation of amino acid transporters (20), or IL-15-induced phosphorylation of the adaptor molecule DAP10 (19) and activation of cytosolic phospholipase $A_2$ accompanied by production of arachidonic acid (21). In human NK cells and resting mouse NK cells, which express the NKG2D-long isoform, the signal is transduced *via* DAP10 and propagates through Grb2/Vav and the PI3K pathway, similar to the costimulatory molecule CD28 (22), which might explain the need for additional signals for cell activation. In mouse, activation induces changes in *Nkg2d* mRNA alternative splicing, leading to the expression of the NKG2D-short isoform. Its coupling to the ITAM-bearing adaptor DAP12, which recruits and activates signaling *via* Syk and ZAP70 protein kinases, has been implicated in the triggering of the NKG2D-short isoform without the need of cytokine "priming" or coreceptor activation (22). These results signify that NKG2D function on NK cells depends on the NK cell activation status and tightly correlates with the presence of additional microenvironmental signals, such as cytokines or ligands of other receptors expressed on target or neighboring cells. Therefore, it is not surprising that NKG2D-deficient mice do not show a major phenotype until crossed to TRAMP or Eμ-Myc mice, which spontaneously develop prostate cancer and lymphoma, respectively (23).

## NKG2DLs: EXPRESSION AND REGULATION

Although NKG2D is largely not polymorphic (only two alleles with a single aa difference exist in human) and shows strong evolutionary conservation with ~70% sequence identity between mouse and human, this receptor is able to bind a broad range of stress-induced ligands that, in contrast, show a high degree of polymorphism (24, 25). In the context of transplantation, polymorphic NKG2DLs can cause donor–recipient incompatibility and lead to allograft rejection by inducing the formation of antibodies directed against NKG2DL epitopes and complement-dependent cytotoxicity (26–29).

NKG2D ligands comprise several MHC class I-like molecules, which include murine UL16-binding protein-like transcript 1 (MULT1), retinoic acid early transcripts α-ε (RAE-1 α-ε) and H60 a-c in mice, and MHC class I-related genes A and B (MICA and MICB) and UL16-binding protein (ULBP) family in human. All NKG2DLs have α1α2 domains responsible for binding to the NKG2D receptor, however, only low sequence similarity can be observed between various ligands, indicating a significant level of variability. It was proposed that the variability of these ligands increased with coevolution with pathogens, allowing their differential expression patterns among cells and tissues, distinct intracellular trafficking, and differential affinity for the NKG2D receptor, which might influence the strength of the delivered signal (24).

NKG2D ligand expression is most frequently associated with infection, cell stress, and transformation, thus alerting for "stressed- and damaged-self." Distinct forms of cell stress can induce cell surface expression of NKG2DLs, including DNA damage, oxidative stress, heat-shock, or the ER stress response (30–35). For example, the p53 pathway, involved in the DNA damage response, was shown to strongly upregulate ULBP1 and ULBP2 at both mRNA and protein level. Similarly, heat-shock-induced transcription factor HSF1 can drive MICA promoter activation (36). Other transcription factors, including E2F, NF-kB, ATF4, the Sp-family, and AP-1, were also shown to be involved in NKG2DL mRNA transcription (36–38). Sauer et al. showed that histone acetylation and binding of acetyltransferases CBP and p300 to NKG2DL promoter regions increased NKG2DL

expression by tumor cells (39), suggesting the importance of an open chromatin state in the regulation of NKG2DL expression. In addition, NKG2DL expression has been associated with viral infections, including CMV, influenza, hepatitis B, Epstein–Barr, and adenovirus (40), as well with as some bacterial infections (e.g., *E. coli*, *M. tuberculosis*) (24). Accordingly, triggering of toll-like receptors (TLRs), that sense microbial products, also induced NKG2DL expression in macrophages and dendritic cells (DCs) (41, 42). Certain viruses, such as HIV, engage the DNA damage pathway, while other viruses, such as MCMV, induce NKG2DL expression *via* PI3K-mediated activation (35).

NKG2D ligand expression is regulated at several levels (transcriptional, post-transcriptional, and post-translational) and depends on the cell type, its activation and metabolic state, and the microenvironmental context (35, 36). Cytokines, such as IFN-γ, IFN-α, and TGF-β, were reported to regulate NKG2DL expression on mouse and human cell lines (10). NKG2DL surface expression is further controlled by miRNAs and mRNA-binding proteins that target NKG2DLs at the transcript level (43–45), by alternative splicing (37), and by Ub-mediated degradation of NKG2DL protein(s) (46, 47). In various cell types and tissues, NKG2DL RNA transcripts were detected in the absence of protein (40). In gut and bronchial epithelia, ligands can be stored intracellularly and transferred to the cell surface upon stimulation (40). In addition, NKG2DLs can be delivered to effector cells *via* exosome secretion, which represents a potent mechanism exploited by cancer cells to downregulate NKG2D expression on effector cells (48). NKG2DL surface expression can be downregulated by proteolytic shedding at the plasma membrane; however, this process depends on the type of ligand, and it seems to be regulated by the microenvironment (49). For example, elevated serum levels of soluble MIC proteins were detected in patients with different types of cancer and correlated with unfavorable prognosis. Soluble MIC was shown to induce NK and CD8+ T cell dysfunction by inducing loss of the CD3ζ signaling molecule, or by decreasing NKG2D surface expression through endocytosis and degradation upon its engagement (50). By contrast, soluble MULT1, a high affinity mouse NKG2DL, was shown to counteract NK cell desensitization and to cause NK cell activation and tumor rejection *in vivo* (51). Opposite to cancer, in autoimmune disease, although increased levels of MIC proteins in serum have been detected, NKG2D expression was not affected in the analyzed patients (50).

Although most frequently associated with infection, cellular stress, and transformation, NKG2DLs are also detected on certain cell types in the absence of pathophysiological conditions [reviewed in Ref. (40)]. These include subsets of uncommitted thymocytes, activated T and myeloid cells, class-switching B cells, regulatory T cells, myeloid-derived suppressor cells (MDSCs), bone marrow stromal cells, committed myelomonocytic progenitors, pluripotent mesenchymal cells, and epithelial cells of the respiratory and gut mucosa. Other cells, such as myoblasts, hepatocytes, neurons, and mouse embryonic cells, were also reported to express NKG2DLs, but their regulation and function on these cells is less clear. In the case of hematopoietic cells, it has been reported that NK cells can eliminate activated immune cells in an NKG2D-dependent manner, thereby restraining T cell responses or excessive inflammation mediated by myeloid cell activation. In the thymus, NKG2D might be involved in the regulation of the T cell repertoire (40), while at the epithelial barriers, NKG2D/NKG2DL expression can be linked to continuous presence of commensal microbiota and constitutive state of low-grade, the so-called physiological inflammation (52). Recently, Thompson and colleagues reported that endothelial cells in lymph nodes constitutively expressed Rae-1, whose engagement at steady state led to downmodulation of NKG2D expression and function in circulating NK cells (53). Human activated NK cells can themselves upregulate certain ULBP family members after culture with the cytokines IL-12/15/18 (54). In this case, intercellular NKG2D activation led to ADAM17-mediated release of TNF-α, thus promoting NK cell cytokine release. By contrast, NKG2DLs expressed on NK cells from diabetic NOD mice were postulated to negatively regulate expression of the NKG2D receptor (55). Moreover, NK cells could acquire NKG2DLs *via* trogocytosis upon interaction with target cells, which led to their fratricide, thereby resulting in downmodulation of the immune response (56).

## EXPRESSION OF NKG2DLs BY MYELOID CELLS

In both human and mice, various myeloid cell subsets were reported to express NKG2DLs (**Figure 1**). In many cases, NKG2DL induction on myeloid cells is a direct consequence of infection. For example, human macrophages upregulate NKG2DL expression upon influenza or Sendai virus infection (57). In infection settings, macrophages can directly sense pathogens using various innate immune receptors, some of which have been shown to directly regulate NKG2DL expression when triggered *in vitro*. Signaling *via* different TLRs can induce NKG2DLs in both monocytes and macrophages, but the nature of the induced ligand(s), the levels of their expression, and the magnitude of subsequent NKG2D-driven responses might vary. It is tempting to speculate that such differential responses were evolutionarily driven and tailored to fit the defense strategy against an invading pathogen. In primary human macrophages, TLR triggering induces MICA and MICB expression. Eissmann et al. showed that LPS not only induced MICA expression, but also decreased the levels of miRNAs involved in targeting MICA transcripts for degradation (58). In addition, while TLR4 signaling was required for *MICA* gene transcription, the ATM/ATR pathway, involved in the DNA damage response, controlled the expression of the MICA protein (58), highlighting the complexity of regulation of NKG2DL expression. The Davis lab also showed that TLR7/8, but not TLR3 ligation, induced both MICA and MICB, and that MICA expression correlated with macrophage activation, measured by the production of proinflammatory cytokines (58). Similar to macrophages, TLR-stimulated monocytes upregulated MICA, but not other NKG2DLs, along with CD80 and MHC class I and II (59). Besides MICA/B, monocytes and macrophages can express other NKG2DLs. TLR2-driven ULBP1 expression was reported on *M. tuberculosis* infected monocytes and alveolar macrophages, leading to their NKG2D-dependent lysis by NK cells (60). ULBP1 could also be induced on monocytes by growth factors, but not

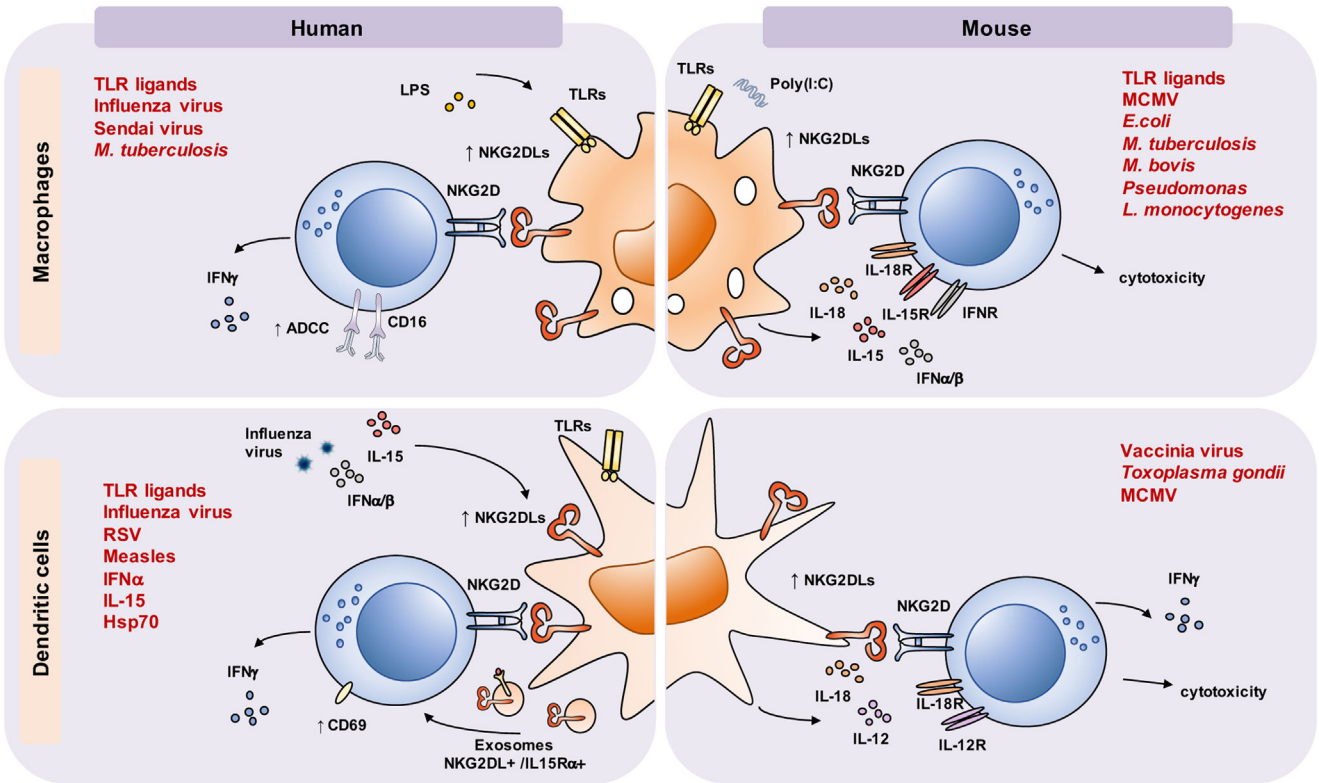


**FIGURE 1** | Macrophages and dendritic cells (DCs) activated by toll-like receptor (TLR) ligands, cytokines, viral or bacterial infection upregulate NKG2D ligands (NKG2DLs) and regulate natural killer (NK) cell effector responses. In both mouse and human, TLR activation and viral and bacterial infection were shown to upregulate NKG2DLs on macrophages and DCs. In addition, cytokines produced by myeloid cells upon infection, such as IL-15 or type I IFNs, can also induce NKG2DL expression on DCs and, importantly, increase natural killer group 2, member D (NKG2D) expression on interacting lymphocytes. Induced NKG2DLs interact with NKG2D expressed by NK cells and lead to their activation, resulting in secretion of IFN-γ, cytotoxicity, CD69 upregulation, and increased killing of antibody-coated cells by antibody-dependent cellular cytotoxicity (ADCC). NKG2DLs and IL-15Rα/IL-15 can also be delivered to NKG2D+ effector cells *via* exosomes. NK cell responses are further supported by soluble factors released by myeloid cells, including IL-12 and IL-18, which strongly synergize in IFN-γ induction. In turn, IFN-γ released by NK cells supports myeloid cell activation and release of soluble factors, creating a myeloid-lymphoid feedback activation loop. In some instances, activated NK cells can kill NKG2DL-expressing macrophages and DCs, thereby limiting their numbers, their responses, or improper stimulation. Abbreviations: RSV, respiratory syncytial virus; MCMV, mouse cytomegalovirus.

by the cytokines TNF-α, IL-1β, and IFN-α, or by the TLR4 ligand LPS (61).

Similar to human monocytes and macrophages, mouse macrophages were reported to express NKG2DLs in the context of infection, such as *M. tuberculosis* (62) or *P. aeruginosa* (63). Murine peritoneal macrophages expressed Rae-1, but not other NKG2DLs, upon stimulation *via* TLRs, both *in vitro* and *in vivo* (42). Exposure to G− (*E. coli*) and G+ (*S. aureus, L. monocytogenes*) bacteria, *Mycobacterium bovis* BCG or infection with CMV, all induced Rae-1 expression (42, 64), reinforcing that macrophage activation and pathogen recognition is linked to NKG2DL upregulation. Cytokines produced upon response to pathogens, such as TNF-α, type I IFNs, or IFN-γ, were not required for Rae-1 expression in these settings.

Besides infection, evidence exists of NKG2DL association with myeloid cell differentiation and acquisition of an activated effector phenotype. This is not surprising, as induction of NKG2DLs was correlated with cell proliferation (65) and DNA damage (30, 31), which might occur during effector responses in tissues. It was shown that human CD34+ hematopoietic stem cells (HSC) expressed low levels of NKG2DLs, which were increased upon commitment to the myeloid lineage (61, 66). In mice, HSC transplantation in irradiated animals gave rise to Gr1+ and CD11b+ cells expressing Rae-1 and H60 in the bone marrow (29). Similarly, myeloid cells with immunosuppressive function that accumulated in tumor-bearing mice were reported to express Rae-1 (51, 67). So far, the reason for NKG2DL expression by immature myeloid cells is unclear. In tumor settings, engagement of NKG2D on NK cells led to NK cell activation and cytolysis of Rae-1-expressing myeloid cells (67). Whether NK cells can control myeloid lineage differentiation *via* regulating the numbers of developing progenitors is, so far, elusive.

Of note, besides expressing NKG2DLs, there are some indications that activated macrophages could also upregulate the NKG2D receptor, although these observations remain controversial. Thioglycolate-induction *in vivo* or stimuli, such as LPS, type I IFNs, and IFN-γ, were shown to drive mRNA and protein NKG2D expression on peritoneal and bone marrow-derived

macrophages, respectively. When stimulated with immobilized ligands or ligand-expressing cells, NKG2D+ macrophages produced nitric oxide and TNF-α (2, 68, 69), indicating that this system might enhance macrophage-mediated elimination of pathogens, pathogen-infected or tumor cells. Indeed, it was also suggested that crosstalk with certain NKG2DL+ tumor cells might contribute to macrophage activation and lower the threshold for their responses (70). However, this effect might also be exploited in tumor settings and facilitate cancer immune evasion. For example, Qian et al. detected NKG2D expression on Gr1+ CD11b+ myeloid cells accumulating in blood, spleen, and bone marrow of tumor-bearing mice. Blockade of NKG2D *in vitro* impaired IL-4 and IL-10 production by these cells, while *in vivo* neutralization reduced their accumulation in mice bearing Rae-1+ tumors (71).

In DCs, NKG2DL upregulation was also associated with activation caused by infection and/or cellular stress (**Figure 1**). In mouse, vaccinia virus infection induced Rae-1 (72), while pulse with *Toxoplasma gondii* lysates led to both Rae-1 and MULT1 expression on the DC surface (73). In infected human monocyte-derived DCs, influenza virus induced ULBP proteins (74). Other RNA viruses, such as respiratory syncytial virus, led to ULBP1 upregulation, while measles virus, as well as exposure to poly(I:C), upregulated ULBP2 (41). These data indicate that different mechanisms can be employed by DCs to induce expression of distinct NKG2DLs in response to various virus types. IFN-α, which is primarily produced during viral infections, was reported to induce MIC ligand expression on human DCs. A similar effect was attributed to IL-15, while stimulation with LPS, poly(I:C), CD40L, TNF-α, IL-12, and IL-18 did not affect NKG2DL expression (75, 76). MICA expression was also induced by Hsp70 (77), although bacterial products or other molecules might as well have contributed to this effect (78). TLR ligands, such as LPS and poly(I:C), were able to upregulate ULBPs on human monocyte-derived DCs (41). ULBP1 was also detected on mature DCs in T cell areas of lymph nodes *in situ*, in close proximity to NKG2D-expressing CD8+ T cells (79), indicating its possible involvement in T cell priming.

## THE NKG2D/NKG2DL AXIS IN THE CROSSTALK OF MYELOID CELLS AND NK CELLS

Myeloid cells and innate lymphocytes form the first line of defense against invading pathogens. Their activation leads not only to the direct elimination of pathogens and their products, but also to the activation of proper adaptive immune responses. Moreover, it is becoming appreciated that these cells also respond to cues indicating tissue damage and, in addition to defense, orchestrate mechanisms of tissue repair (80). Since (i) both infection and damage were shown to upregulate NKG2DLs on myeloid cells, and (ii) factors produced in response to these events (by stroma, parenchymal, and immune cells) can induce or upregulate NKG2D on lymphocytes, an NKG2D-driven lymphocyte-myeloid cell crosstalk is expected to play an important role in these processes. Indeed, in many animal models, NKG2D genetic deletion or Ab-mediated blockade *in vivo* affected disease development and tissue repair, including tumor progression, autoimmunity, and wound healing (12, 13, 23, 65). However, the contribution of the individual cell populations to these observations remain not fully addressed. This particularly applies to cells expressing NKG2DLs, whose depletion or conditional targeting is experimentally challenging. In addition, the generation of NKG2DL-deficient mice is complicated by the existence of multiple ligands organized as gene families. However, recent success in creating Rae-1-deficient mice (51) opens the possibility to generate bone marrow chimeras and to address the contribution of Rae-1 expression, at least in the hematopoietic versus non-hematopoietic compartment, in disease settings *in vivo*.

In the case of myeloid cells, while their role in activating NKG2D-expressing cells *in vivo via* NKG2DLs remains largely unknown, *in vitro* data provide convincing evidence of an NK cell crosstalk with DCs and macrophages (**Figure 1**). For example, in the MCMV model, virus-infected mouse DCs play a crucial role in NK cell activation by both inducing IFN-γ release (through IL-12/18 production) and NK cell cytotoxic responses *via* IFNα and NKG2D engagement (81). In human *in vitro* system, it was shown that DCs infected with influenza virus supported CD69 upregulation and IFN-γ production by NK cells *via* NKG2D and NKp46 (74). In addition, it was shown that human DC-derived exosomes displayed ULBP1, together with IL-15Rα, on their surface and were able to promote NKG2D-dependent activation of NK cells (82). Also, MICA and MICB, induced on DCs upon IFN-α or IL-15 treatment, contributed to NK cell activation (75, 76). In these studies, the authors showed that type I IFNs and IL-15 induced MIC molecules on DCs, which was impaired in patients with chronic hepatitis C infection. Similarly, coculture of NK cells and DCs pulsed with *T. gondii* lysate increased DC IL-12 production and their ability to prime Ag-specific CD8+ T cell responses, which was impaired by NKG2D blockade (73). Besides mutual activation, the NK/DC crosstalk can also result in an NK cell cytolytic response, leading to DC elimination, which is considered essential in the regulation of the numbers and quality of the activated DCs and, consequently, the extent of the overall immune response. The outcome of the NK/DC crosstalk depends on the activation/maturation status of both interacting cell types and their relative abundance. Accordingly, human IL-2-activated NK cells increased mature DC responses, measured by the level of IL-12 release and ability to induce CD4+ T cell activation (83). In coculture with immature DCs (iDCs), activated NK cells were shown to support autologous DC maturation and activation at low NK/DC ratios, while increased numbers of NK cells resulted in iDC lysis (84). In these settings, cytotoxic NK cell responses were mainly mediated *via* the activating receptors NKp30 and DNAM-1 (85), although a partial contribution of NKG2D has also been observed (86). However, treatment of mature DCs with IL-10 induced their elimination by NK cells, which was mediated *via* NKG2D, while IL-10-treated iDCs resisted to NK-mediated cytotoxicity (87). As the authors of this study suggested, aberrant accumulation of iDCs in patients with chronic infections that are frequently associated with increased levels of IL-10 production, such as HIV, thus might be the consequence of IL-10-induced DC resistance to NK cell elimination (87). Remarkably, Schulz

and colleagues showed that IL-10 also rendered autologous human macrophages susceptible to NK cell lysis, which involved NKG2D. In the presence of IL-10, NKG2D receptor expression on NK cells increased, while macrophages induced NKG2DL expression (88). Thus, IL-10 might exert its immunomodulatory pleotropic effect, not only *via* suppressing T cell responses, but also *via* inducing NK cell-mediated killing of activated myeloid cells, including antigen-presenting cells (APCs).

Similar to DCs, monocyte-derived macrophages were reported to express MICA and ULBP1-3 upon stimulation with LPS, rendering them susceptible to NK cell-mediated lysis (89). In alveolar macrophages, *M. tuberculosis* infection led to ULBP1 induction and their NKG2D-dependent lysis by NK cells (60). However, it was also reported that TLR-stimulated monocytes, which upregulate MICA, promoted IFN-γ release *via* interaction with NKG2D-expressing NK cells (59). In the presence of IL-12, NKG2DL-expressing monocytes were shown to not only stimulate IFN-γ release, but also to enhance antibody-dependent cellular cytotoxicity toward Ab-coated target cells (90). Results from the Davis lab indicated that NK cells and autologous human macrophages can engage two distinct types of interactions. On the one hand, low-dose LPS-stimulated macrophages can trigger NK cell proliferation, secretion of cytokines and increased killing of tumor targets *via* the 2B4/CD48 axis, while, on the other hand, macrophages activated with high doses of LPS expressed MICA and ULPBs, formed the so-called lytic synapse, and were lysed by NK cells *via* NKG2D (89). These data indicate that the activation status of macrophages can determine the outcome of their crosstalk with NK cells and that NKG2DL expression might be a signal for removal of activated macrophages to prevent exaggerated inflammation and tissue damage. In mice, activation of peritoneal macrophages with poly(I:C) induced Rae-1, H60, and MULT1 expression, along with IL-15, IL-18, and type I IFN production. These soluble factors could increase NKG2D expression on NK cells, leading to increased cytotoxicity in response to tumor cells expressing NKG2DLs. However, macrophages remained resistant to NK cell lysis due to the expression of Qa-1, a surface molecule engaging the inhibitory NK cell receptor NKG2A (91).

Besides a direct interaction that triggers immediate effector responses, such as cytokine production and cytotoxicity, myeloid cells expressing NKG2DLs can indirectly control the function of NKG2D expressed on effector lymphoid cells. It was shown that NKG2DL engagement can lead to NKG2D downregulation and that the constitutive presence of NKG2DLs can cause long-term desensitization of the NKG2D pathway (53, 92, 93). This phenomenon can be mediated by both membrane bound ligands and by ligands released in soluble form upon proteolytic shedding or *via* exosomes. While the effect of tumor-released soluble ligands/exosomes has been extensively studied, mainly as a mechanism of immune evasion, the contribution of myeloid cells to this phenomenon remains largely unrevealed. Only recently, a study showed that *in vivo* overexpression of Rae-1 on CD11c$^{high}$ cells, comprising mainly DCs in mice, led to reduced NK cell-mediated cytotoxicity toward NKG2DL+ or MHC class I-deficient targets, compromising the ability of these animals to reject NKG2DL-expressing tumor cells, while the control of viral infection (MCMV) remained unaffected (94). Of note, continuous engagement of NKG2D was shown not only to affect NKG2D-dependent responses, but also to desensitize the signaling downstream of other activating receptors, such as NK1.1 and NKp46 (92, 95). Accordingly, Rae-1 expression on lymph node endothelial cells in steady state (53) or by myeloid cells in tumor-bearing animals (51) is responsible for NKG2D downregulaton and global desensitization of NK cells. Similarly, in cancer patients, the presence of MICA- and ULBP1-expressing myeloid cells in blood and tumor correlated with reduced NKG2D expression on NK cells (96). However, it will be important to further improve the mechanistic understanding of NKG2D downmodulation in disease conditions and to dissect whether NKG2D downregulation in patients truly results from the engagement with soluble and cell-expressing ligands or if other factors, such as TGF-β, might contribute in these settings.

In the past few years, ILCs, which mainly reside in tissues, have been identified as novel players in the regulation of tissue homeostasis, regeneration, and response to infection (80). Since helper ILC1 and ILC3 populations express NKG2D (6–8), investigating the importance of the NKG2D/NKG2DL axis in the putative crosstalk between ILCs and myeloid cells, particularly in tissues and/or pathologies where ILC populations play an important role, would be of high relevance.

## NKG2D EXPRESSION ON T CELL SUBSETS: WHEN AND WHERE?

As referred above, NKG2D is a widely expressed receptor detectable on NK cells and several subsets of T cells, including CD8+ T cells, subsets of γδ T cells and NKT cells in steady state, and CD4+ T cells under certain pathological conditions. This transversal expression from innate to adaptive immune lymphocytes makes NKG2D a remarkable NK receptor. Thus, the focus on the dynamics of a NKG2D/NKG2DL axis should go beyond the NK cell-myeloid cell crosstalk. Below, we discuss the so far described NKG2D-expressing T cell subsets (see **Figure 2**).

**αβ CD8+ T Cells**. CD8+ T cells are the most representative T cell subset expressing NKG2D. While in mice expression of NKG2D is restricted to activated CD8+ T cells, in humans, all CD8+ T cells express NKG2D constitutively (97). In human, as with NK cells, CD8+ T cells do not express the short NKG2D isoform. In mice, although activated CD8+ T cells express both NKG2D isoforms, they usually lack expression of DAP12, contrarily to NK cells. Thus, for both species, NKG2D in CD8+ T cells seems to primarily signal *via* DAP10 (2, 3). It was reported that DAP12 could also be expressed by T cells and that, unlike NK cells, human activated CD8+ T cells required simultaneous signaling mediated by both DAP10 and DAP12 pathways (98). However, several studies support that DAP10 appears to be the most important adaptor for NKG2D signaling in CD8+ T cells (3, 22, 99).

In conventional CD8+ T cells, NKG2D has been shown to mainly serve as a costimulatory receptor for TCR-induced signaling (22, 69, 99–101). Although the costimulatory

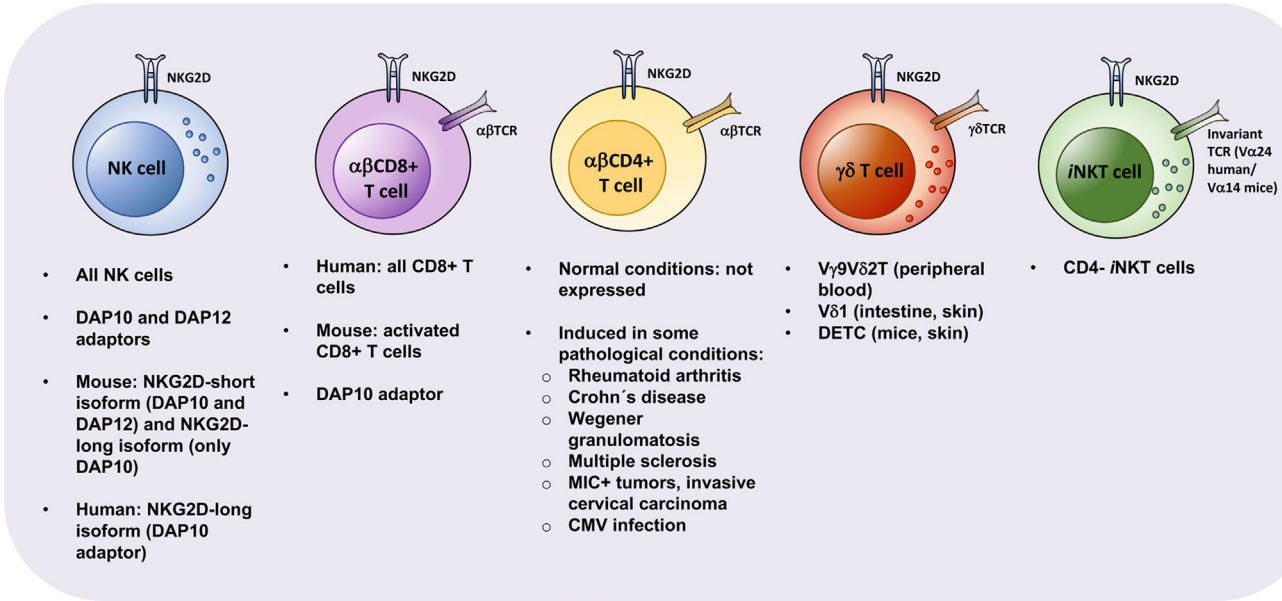


**FIGURE 2** | Properties of natural killer group 2, member D (NKG2D)-expressing lymphocytes. NKG2D can be expressed by natural killer (NK) cells, αβ CD8+ T cells, γδ T cells, invariant NKT (*i*NKT) cells, and αβ CD4+ T cells. However, its expression, alternative splicing, and coupling to adapter molecules vary between the indicated lymphoid subsets. Expression of NKG2D on certain lymphocytes correlates with activation (mouse αβ CD8+ T cells) or can be detected only in disease settings (αβ CD4+ T cells). While NK cells, *i*NKT cells and CD8+ T cells are circulatory and can be detected in peripheral blood, other lymphoid cells, such as helper innate lymphoid cells (data not shown) and certain γδ T cells, are tissue-resident and are involved in local tissue immune responses. Similarly, pathologic αβ CD4+ T cells are enriched in affected tissues in depicted autoimmune conditions. Abbreviations: DETCs, dendritic epidermal T cells; CMV, cytomegalovirus.

function of NKG2D was more evident for activated CD8+ T cells that lack CD28 (99, 100), it was also observed in naïve CD8+ T cells (101). In fact, the cytoplasmic domain of DAP10 comprises a signaling motif similar to CD28, which activates PI3K and leads to similar, though not identical effects on T cell costimulation (102, 103). Besides its role in decreasing the TCR threshold in CD8+ T cells, as a costimulatory receptor, several studies have shown that NKG2D can also work as an activating receptor *per se* on CD8+ T cells under certain conditions, namely upon prolonged exposure to IL-15 (104–106). In fact, IL-15 appears to be a key factor in arming the NKG2D-mediated cytolysis of effector CD8+ T cells. Meresse et al. showed that, in celiac disease, NKG2D expressed on intraepithelial intestinal lymphocytes could mediate direct cytolysis, putatively due to overexpression of IL-15 in this disease (104). Other studies demonstrated that prolonged exposure of human peripheral blood CD8+ T cells to IL-15 *in vitro* led to a functional NKG2D receptor *per se*, without the need of TCR coengagement for activation. IL-15, besides being involved in NKG2D and DAP10 induction and upregulation (104–108), was demonstrated to synergize with the NKG2D downstream signaling pathway through activation of PI3K, JNK, ERK, and cPLA2 (21, 104, 109, 110), thereby enabling NKG2D to mediate direct activation and cytolysis in a TCR-independent manner. Also, it was shown that NKG2D could enhance IL-15-mediated PI3K signaling in activated CD8+ T cells, promoting CD8+ T cell survival and memory formation (111), showing a bi-directional importance of the IL-15–NKG2D downstream signaling interaction. Moreover, NKG2D on CD8+ T cells was shown to rescue CD4-unhelped CD8+ T cell memory recall responses, but not effector responses, by repressing the transcription factor T-bet (112). Accordingly, besides activating CD8+ T cells and driving cytotoxicity, NKG2D has also been implied in the survival and memory formation of CD8+ T cells.

**αβ CD4+ T Cells**. Under physiological conditions, expression of NKG2D is not detectable on conventional αβCD4+ T cells. However, NKG2D-expressing CD4+ T cells have been described in human under certain pathological conditions. NKG2D+ CD4+ T cells were initially found on peripheral blood and synovial fluid from rheumatoid arthritis (RA) patients, putatively as result of increased TNF-α and IL-15 levels in this disease. Groh et al. showed that those cells promoted the cytotoxic damage against synoviocytes with anomalous expression of NKG2DLs (14). Later on, NKG2D+ CD4+ T cells have been associated with several other autoimmune diseases, such as Crohn's disease (108, 113, 114), Wegener granulomatosis (115, 116), type 2 diabetes (117), multiple sclerosis (118), and systemic lupus erythematosus (SLE) (119, 120). Moreover, NKG2D+ CD4+ T cells accumulated in patients with MIC+ tumors (121) and cervical carcinoma (122–124). Also, NKG2D+ CD4+ T cells were identified in patients suffering from a human T cell lymphotropic virus type I-associated neurological disease (125) and linked with human cytomegalovirus infection (126).

Of note, NKG2D+ CD4+ T cells appear to have similar features to a previously described CD4+ CD28− T cell phenotype, which prevails in several pathologies, namely autoimmune disorders (127–132). In fact, NKG2D+ CD4+ T cells have been detected mainly within the CD4+ CD28− T cell population (14, 126, 133). Thus, previous studies describing the CD4+ CD28− T cell population should be revisited by addressing a putative role of NKG2D in disease progression and severity.

Similar to CD8+ T cells, NKG2D plays a major role as a costimulatory receptor, enhancing TCR-mediated responses in NKG2D+ CD4+ T cells. Several studies showed the involvement of this population in the pathology of autoimmune diseases contributing to disease progression or severity in an NKG2D-dependent manner. However, increased frequencies of NKG2D+ CD4+ T cells inversely correlated with disease activity in juvenile-onset SLE, suggesting that these T cells may also have regulatory effects (134). Moreover, a recent study showed that NKG2D+ CD4+ T cells were involved in Treg killing in an NKG2D–NKG2DL-dependent manner in SLE (120).

Besides the existence of mounting evidence about the association of NKG2D+ CD4+ T cells and pathological conditions, shown to correlate with an increase in NKG2DLs, the functional crosstalk between myeloid cells expressing NKG2DL and NKG2D+ CD4+ T cells remains mainly unaddressed.

**γδ T Cells**. In both humans and mice, γδ T cells comprise a small population of peripheral blood cells (around 2–5%), but are abundant in tissues, particularly in the intestine, reproductive tract, and skin (135–137). Vγ9Vδ2 T cells (also known as Vγ2Vδ2), are the most abundant population in human peripheral blood (50–95%) (138), while Vδ1 γδ T cells are mainly enriched in the intestine (together with Vδ3 γδ T cells) and in the skin. Both Vγ9Vδ2 and Vδ1 γδ T cell subsets are described to express NKG2D on their cell surface, associating with DAP10 for signal triggering. In human, the IL-17+ Vγ9Vδ2 T population was reported to lack the expression of NKG2D, although the majority of circulating Vγ9Vδ2 T cells expressed NKG2D receptor (139).

In 1998, Groh and colleagues described that MICA and MICB could be recognized by intestinal Vδ1 γδ T cells through their γδ TCR (140). Shortly after, the same group showed that the cell surface NKG2D expressed on intestinal Vδ1 γδ T cells recognized MICA, and that NKG2D–MICA engagement resulted in target cell recognition and killing, suggesting that NKG2D might function as a costimulatory receptor on γδ T cells (141). MICA was later confirmed to be directly recognized by Vδ1 T cells through their γδ TCR, although weakly compared to recognition by NKG2D (142). Several studies showed that Vδ1 γδ T cells can recognize NKG2DLs expressed on cancer cells, triggering their cytotoxicity against targets (143, 144).

Das and colleagues showed that infection with *M. tuberculosis* induced MICA on the surface of dendritic and epithelial cells, both *in vitro* and *in vivo* (145). Moreover, MICA engagement by NKG2D expressed on Vγ9Vδ2 T cells resulted in a considerable increase of the TCR-dependent Vγ9Vδ2 T cells response (145). In the absence of antigen, NKG2D+ Vγ9Vδ2 T cells did not lyse MICA+ targets, indicating that the NKG2D–MICA interaction is not sufficient to trigger Vγ9Vδ2 T cell-mediated lysis (145). By contrast, a subsequent study showed that NKG2D expressed on Vγ9Vδ2 T cell could induce by itself Vγ9Vδ2 T cell activation and NKG2D-dependent cytolysis of target cells (146). NKG2D has been shown to be involved in Vγ9Vδ2 T cell recognition of leukemia and lymphoma (147), as well as of solid tumors (148, 149). Similar to Vδ1 γδ T cell recognition of MICA, ULBP4 was described to bind to both NKG2D and γδ TCR of Vγ9Vδ2 T cells, mediating their activation and cytotoxicity (150). Thus, altogether those studies indicate that tumor-expressed NKG2DLs can be specifically recognized by both TCR and NKG2D expressed on human γδ T cells.

Dendritic epidermal T cells (DETCs) are epithelial γδ T cells that reside in murine skin. While it is described that only 25% of splenic γδ T cells and 5% of thymic γδ T cells express NKG2D, basically all DETCs are NKG2D+ in mice (69). So far, human counterparts of mouse skin γδ T cells with the same dendritic-like characteristics have not been identified. Girardi and colleagues have shown that DETCs can kill carcinoma cells in an NKG2D-dependent manner (151), providing the first evidence of NKG2D-mediated DETC activation. Afterward, it was found that DETCs displayed impaired wound healing properties upon NKG2DL blocking (65, 152) and, as well, that the NKG2D/NKG2DL interaction was involved in allergen-induced activation of DETCs in contact hypersensitivity (153).

As with the other γδ T cell subsets, whether NKG2D expressed on DETCs works as a costimulatory receptor or as an activation receptor by itself is still controversial (153–156). Ibusuki et al. showed that NKG2D engagement alone was sufficient to trigger degranulation, but not cytokine production, in DETCs, which was mainly mediated *via* the DAP10–PI3K-dependent signaling pathway (157). However, in this study, DETCs were expanded in culture with IL-2, which may account for the observed surpass of the need for TCR cotriggering. In fact, NKG2D could not trigger cytotoxicity in freshly *ex vivo* isolated DETCs (157). Of note, since it is known that cytokines, such as IL-15, can synergize with the NKG2D downstream signaling pathway, it is important to consider that the majority of protocols using γδ T cells are preceded by an expansion phase including IL-2 or IL-15 cytokines. This fact might account for controversial views of NKG2D as costimulatory or stimulatory receptor by itself in NKG2D-expressing populations.

***i*NKT Cells**. Invariant NKT cells represent a small population of blood cells (0.01–1% among peripheral blood T cells) in human that, however, can be found highly enriched in the liver, particularly in mice. Those cells express a semi-invariant TCR, characterized by Vα14-Jα18 in mice and Vα24-Jα18 in humans, which recognizes lipid-based antigens in the context of CD1d molecules. *i*NKT cells can be functionally distinguished by the expression of CD4 on the cell surface, both in human and mice. In general, CD4+ *i*NKT cells display a Th2-like profile, whereas CD4− *i*NKT are rather skewed toward Th1-like responses (158, 159). NKG2D surface expression is restricted mainly to the CD4 negative subset of *i*NKT cells (158, 160). Kuylenstierna and colleagues found that NKG2D stimulation in CD4− NKT cells could act as a costimulatory signal in response to suboptimal anti-CD3 triggering or CD1d-presented ligands. In the same

study, NKG2D stimulation in CD4− NKT cells also mediated a direct NKG2D-dependent lysis of target cells, independent of invariant TCR engagement, thus demonstrating both a stimulatory and costimulatory role of NKG2D in those cells (160). Wang et al. showed that tumor-derived soluble MICs downregulated NKT cell NKG2D expression and consequently tumor cell killing *in vitro* (161), supporting an anti-tumor function of NKG2D+ NKT cells.

Besides playing a role in cancer, NKG2D+ NKT cells were shown to be enriched in certain pathologies, namely autoimmune diseases. Patients with type 2 diabetes showed increased NKG2D+ NKT cells in peripheral blood, when compared with healthy controls (162). Early onset SLE was associated with changes in the ratio of NKG2D/NKG2A expression in multiple cell types, including NKT cells (163). NKG2D+ NKT cells with a Th1-like profile were also increased in pre-eclampsia (164). Further studies focusing on the specific role of NKG2D expressed by *i*NKT cells, in both cancer and other pathologies, and on the potential crosstalk with myeloid cells *via* interaction with NKG2DLs, would be important.

Noteworthy, besides the existence of mounting evidence associating several subsets of NKG2D+ T cells and pathological conditions characterized by an increase in NKG2DLs, the functional crosstalk between myeloid cells expressing NKG2DL and NKG2D+ T cell subsets remains mainly unaddressed. In this regard, it would be important on the one hand, to determine the specific role of NKG2D in conditional knockout mice, where NKG2D could be selectively deleted on different cell populations; and on the other hand, to dissect in parallel the importance of the NKG2DL-expressing myeloid cells. Since myeloid cells are important APCs, the putative crosstalk between myeloid cells and T cells expressing NKG2D might be highly relevant. Particularly in pathological conditions, myeloid cells could not only trigger NKG2D expressed by T cells *via* NKG2DL but could also in parallel activate T cells *via* antigen presentation by MHC molecules, further enhancing or controlling specific T cell-mediated responses. As such, further studies focusing on the impact of NKG2DL expressed on myeloid cells in the crosstalk with T cells would be of major relevance.

Importantly, a connection between NKG2D, autoimmunity, and IL-15 is getting increasingly evident. First, several autoimmune diseases have been correlated with increased IL-15 cytokine levels (165, 166). Second, NKG2D induction, upregulation and its role in autoimmunity has been extensively demonstrated (167). Finally, a role of IL-15 in NKG2D upregulation and enhancement of downstream signaling has been shown. Interestingly, myeloid cells are widely regarded as main producers of IL-15, particularly under pathological conditions, trans-presenting IL-15 to responding cells. On the one hand, it is known that IL-15 is involved in NKG2DL upregulation (104, 110) and, on the other hand, it has been shown that this cytokine can upregulate or induce NKG2D expression (104–106, 110). In this regard, it is tempting to imagine a scenario where myeloid cells trans-present IL-15 to effector cells, leading to NKG2D induction and/or support of its signaling, while these cells at the same time trigger NKG2D *via* NKG2DL interaction.

## NKG2DLs AND MYELOID CELLS IN DISEASE SETTINGS

Multiple diseases are associated with NKG2DL upregulation, where its expression might be either protective or detrimental. Dysregulated receptor/ligand expression was reported in various autoimmune diseases, such as RA, colitis, celiac disease, multiple sclerosis, type 1 diabetes, or atherosclerosis, where their involvement was postulated to mainly promote inflammation (11–17, 104). By contrast, in conditions such as wound healing, although NKG2DLs were induced by initial tissue damage and cellular stress, NKG2D was shown to support tissue remodeling and regeneration (65). However, in many of the studied diseases and respective animal models, it remains unclear which cells express NKG2DLs in the affected tissues and what is the myeloid cell contribution to the overall ligand expression and NKG2D-driven responses.

In tumor-bearing mice, Rae-1 was detected on a subset of MDSCs and contributed to NK cell activation (67). In glioblastoma multiforme (GBM) patients, MICA and ULBP1 were detected on microglia, tumor-infiltrating myeloid cells, and circulating monocytes (96). In this study, the authors showed that lactate dehydrogenase isoform 5 (LDH5), secreted by tumor cells, was elevated in GBM patients' sera and responsible for NKG2DL upregulation on healthy monocytes. *In vitro*, IL-2-activated NK cells degranulated, produced IFN-γ, and induced apoptosis of autologous NKG2DL-bearing monocytes. However, *in vivo*, LDH5-mediated induction of NKG2DLs might serve as a cancer immune evasion mechanism, as NK cells from GBM patients displayed reduced surface NKG2D expression and impaired function (96). Similarly, NKG2DL-expressing monocytes were detected in the blood of breast, prostate, and virus-induced liver cancer patients (96).

In *atherosclerotic plaques*, both endothelial cells and macrophages have been reported to express MICA/B (15). In line with that, exposure of monocyte-derived macrophages to acetylated low-density lipoproteins *in vitro* led to MICA/B induction (168). In a mouse model of atherosclerosis, Rae-1 expression was detected on macrophages, not only in plaques but also in the liver, which is affected by metabolic changes associated with disease (15). The liver is enriched in NKG2D-expressing ILCs and NKT cells, whose crosstalk with the myeloid compartment might play a significant role in atherosclerosis. Consistent with that hypothesis, it was shown that NKG2D-deficient animals displayed smaller plaques in aortas, reduced liver damage, and reduced levels of proinflammatory cytokines, cholesterol, and triglycerides in serum (15).

La Scaleia et al. reported increased NKG2DL expression in the colon mucosa of pediatric patients with active *inflammatory bowel disease* (IBD). Their results indicated that the NKG2DL+ cells displayed a macrophage-like morphological phenotype (17). In addition, in active ulcerative colitis, MICA and MICB expression was significantly upregulated in peripheral blood monocytes (17). MICA/B+ macrophages were also detected in the duodenal tissue of patients with celiac disease, where those ligands were distributed intracellularly in the form of cytoplasmic aggregates (169). These data suggest that the crosstalk of myeloid cells with

NKG2D+ innate and adaptive lymphocytes might play a significant role in IBD-associated inflammation. Moreover, intracellular NKG2DLs might have a specific, so far unappreciated function, not only in myeloid cells, but also in enterocytes, where peri- and supra-nuclear NKG2DL aggregates were also detected (169).

In *experimental autoimmune encephalomyelitis*, a mouse model of multiple sclerosis, Rae-1δ and Rae-1γ were induced at mRNA and protein level in spinal cord early upon disease onset. Djelloul et al. demonstrated that myeloid cells, including macrophages and microglia, expressed both Rae-1 and MULT1 (170). Expression of these ligands correlated with myeloid cell recruitment to affected tissue and their proliferation. Furthermore, this study identified M-CSF as factor driving NKG2DL expression on microglia.

As in the diseases discussed above NKG2D-expressing lymphocytes play significant roles, displaying either protective or detrimental properties, and myeloid cells exert potent regulatory roles in their activation, it would be of great importance to determine the contribution of NKG2D to their interaction. Moreover, NKG2D seems to play a costimulatory role in lymphocyte activation, acting often in the context of the proinflammatory environment. As myeloid cells contribute to an inflammatory environment *via* both soluble factors and NKG2DLs, it is tempting to speculate that their therapeutic targeting in combination with existing treatments might help to reduce tissue damage, especially in context of autoimmune diseases.

## CONCLUSION AND FUTURE PERSPECTIVES

NKG2D is considered a major lymphocyte receptor detecting dysregulated cell homeostasis induced by infection or transformation. Distinct pathways activated by cellular stress can upregulate various NKG2DLs, thus conveying an alert-signal of potential cell dysfunction to the immune system. Importantly, NKG2DL upregulation is frequently accompanied by specific microenvironmental milieus that support NKG2D upregulation, NKG2D function, and NKG2DL induction. These milieus are characterized by the presence of proinflammatory cytokines, among which, IL-15 plays a central role. In tissues, tissue-resident and recruited NKG2D+ lymphocytes are crucial for detecting and eliminating infected and transformed cells. However, this function is greatly supported by the myeloid immune compartment. Similar mechanisms, leading to NKG2DL upregulation in infected and transformed cells, operate to induce their expression in myeloid cells as well. Thus, besides their classical functions, that include Ag presentation to T cells or cytokine-mediated activation of innate lymphocytes, myeloid cells use the NKG2D/NKG2DL axis to support a regulatory loop, leading to lymphocyte activation *via* NKG2D, which in turn can activate or eliminate myeloid cells. The NKG2D-mediated myeloid–lymphocyte interaction can have a dual effect on the lymphoid effector cell. On the one hand, it can lead to cell activation, which promotes cytotoxicity, survival, and/or cytokine production. On the other hand, it causes cell inactivation, as a consequence of ligand-induced receptor internalization and desensitization of activating pathways beyond NKG2D. Although some of the factors that regulate these processes are known, such as soluble ligands and chronic stimulation, it would be highly relevant to define molecular events that can shift NKG2D engagement toward activation, e.g., during anti-tumor responses, or inactivation in the case of autoimmunity.

In many experimental settings, especially in disease, the importance of the myeloid–lymphocyte activation/inactivation loop has not been fully addressed, namely due to the existence of a broad variety of NKG2DLs and due to their expression beyond the myeloid compartment, including stromal and parenchymal cells. Accordingly, it remains a major challenge to address the contribution of the myeloid compartment to NKG2D-dependent lymphocyte activation, especially in pathological settings. So far, conditional knockout animals lacking all NKG2DLs were not generated, but mice overexpressing Rae-1 ligands exist and their overexpression in specific cell types was obtained by the use of Cre-lox technology (94). However, the complexity of the NKG2DL system, concerning their differential expression, differential shedding susceptibility and the ability to induce NKG2D downregulation, might compromise the full understanding of complex pathologies. In addition, recent data indicating that the continuous engagement of the NKG2D receptor in steady state regulates its activity (53), suggest that the use of inducible systems of deletion or overexpression would be a better experimental choice.

Although the NKG2D-mediated crosstalk of myeloid cells and NK cells is relatively well understood, the outcome of the interaction of the different NKG2D-expressing T cell subsets with NKG2DL+ myeloid cells remain largely unknown. In many diseases, especially in autoimmune disorders, that are often associated with aberrant activation of myeloid cells, the presence of NKG2D-expressing T cells is well documented. It would be of great value to gain understanding of the relative contribution of NKG2D-expressing T cells in these settings and the involvement of NKG2D, especially for therapeutic targeting. For this purpose, mice carrying a conditional NKG2D deletion specifically in T cells would be valuable tools. In addition, novel populations of tissue-resident lymphocytes are emerging and their importance in regulating inflammation, tissue homeostasis, and regeneration is now eminent. Their topological position and fast responses to tissue damage, in cooperation with the myeloid compartment, along with the fact that NKG2DLs are the messengers of damage, impose them as attractive candidates that might utilize the NKG2D system to perform their functions. It is known that such scenarios operate in skin with tissue-resident γδ T cells being major players. Other barrier sites are awaiting further evaluation in the context of the NKG2DL/NKG2D crosstalk.

## AUTHOR CONTRIBUTIONS

AS and MPC wrote the manuscript; MPC generated the figures; AC read the manuscript and provided critical input.

## ACKNOWLEDGMENTS

We thank all the scientist who performed important work in the NKG2D field and inspired the writing of this review article, and we apologize to those ones we could not cite.

## FUNDING

The authors of this review are supported by Deutsche Krebshilfe (German Cancer Aid) grants no. 70112233 and 110442 and DFG Priority Program 1937.

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**Conflict of Interest Statement:** AC is a member of the Scientific Advisory Board of Dragonfly Therapeutics.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 May 2018
doi: 10.3389/fimmu.2018.00991

# Murine CMV Expressing the High Affinity NKG2D Ligand MULT-1: A Model for the Development of Cytomegalovirus-Based Vaccines

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The development of a vaccine against human cytomegalovirus (CMV) has been a subject of long-term medical interest. The research during recent years identified CMV as an attractive vaccine vector against infectious diseases and tumors. The immune response to CMV persists over a lifetime and its unique feature is the inflationary T cell response to certain viral epitopes. CMV encodes numerous genes involved in immunoevasion, which are non-essential for virus growth *in vitro*. The deletion of those genes results in virus attenuation *in vivo*, which enables us to dramatically manipulate its virulence and the immune response. We have previously shown that the murine CMV (MCMV) expressing RAE-1γ, one of the cellular ligands for the NKG2D receptor, is highly attenuated *in vivo* but retains the ability to induce a strong $CD8^+$ T cell response. Here, we demonstrate that recombinant MCMV expressing high affinity NKG2D ligand murine UL16 binding protein-like transcript (MULT-1) (MULT-1MCMV) inserted in the place of its viral inhibitor is dramatically attenuated *in vivo* in a NK cell-dependent manner, both in immunocompetent adult mice and in immunologically immature newborns. MULT-1MCMV was more attenuated than the recombinant virus expressing RAE-1γ. Despite the drastic sensitivity to innate immune control, MULT-1MCMV induced an efficient $CD8^+$ T cell response to viral and vectored antigens. By using *in vitro* assay, we showed that similar to RAE-1γMCMV, MULT-1 expressing virus provided strong priming of $CD8^+$ T cells. Moreover, MULT-1MCMV was able to induce anti-viral antibodies, which after passing the transplacental barrier protect offspring of immunized mothers from challenge infection. Altogether, this study further supports the concept that CMV expressing NKG2D ligand possesses excellent characteristics to serve as a vaccine or vaccine vector.

**Keywords: murine CMV, cytomegalovirus, vaccine, NKG2D, murine UL16 binding protein-like transcript 1, NK cells, $CD8^+$ T cells**

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***Edited by:***
*Nadia Guerra, Imperial College London, United Kingdom*

***Reviewed by:***
*Aimee Beaulieu, Rutgers New Jersey Medical School, United States*
*Michael G. Brown, University of Virginia, United States*

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

***Received:*** *15 February 2018*
***Accepted:*** *20 April 2018*
***Published:*** *07 May 2018*

***Citation:***
*Hiršl L, Brizić I, Jenuš T, Juranić Lisnić V, Reichel JJ, Jurković S, Krmpotić A and Jonjić S (2018) Murine CMV Expressing the High Affinity NKG2D Ligand MULT-1: A Model for the Development of Cytomegalovirus-Based Vaccines. Front. Immunol. 9:991. doi: 10.3389/fimmu.2018.00991*

## INTRODUCTION

Human cytomegalovirus (HCMV) is a member of herpesvirus family with high seroprevalence rate worldwide (1). After acute infection, cytomegaloviruses (CMVs) establish life-long latency from which periodic reactivations can occur. In immunocompetent individuals CMV is usually asymptomatic, whereas infection in immunocompromised or immunologically immature individuals can

cause a severe morbidity (2, 3). In healthy individuals, CMV is controlled by the combined effort of innate and adaptive immunity. While NK cell control is critical during the first days of infection, long-term virus control is maintained predominantly by T cells and antibodies (4–6). Despite several decades of intensive work, no HCMV vaccine is still approved (7).

NKG2D is an activating immune receptor expressed on NK cells, activated and antigen-experienced T cells, and a proportion of NKT and γδ T cells (8). When expressed on NK cells, NKG2D acts as a strong activating receptor, while its engagement on T cells provides co-stimulation (9, 10). Ligands for NKG2D are structurally similar to MHC class I molecules and normally expressed at very low levels. However, upon cellular stress caused by infection or cell transformation, their expression increases leading to NKG2D engagement and activation of immune cells (11, 12). In humans, NKG2D ligands include highly polymorphic MHC-I-related proteins MICA and MICB and UL16-binding proteins (ULBPs) (13). In mice, ligands for NKG2D receptor belong to the family of retinoic acid inducible early transcripts 1 (RAE-1), histocompatibility 60 (H60), and murine UL16 binding protein-like transcript (MULT-1). Although binding to the same receptor, NKG2D ligands differ in their regulation and affinity for the receptor. It is still not fully understood which consequence has the engagement of different NKG2D ligands on the functional outcome of NK and T cell response.

The best evidence that NKG2D engagement plays an important role in immunosurveillance of CMV is the fact that CMV possesses several immunoevasion genes, which prevent the surface expression of NKG2D ligands (14, 15). In MCMV, the majority of these genes belong to the m145 family of immunoevasins which includes *m145*, *m152*, and *m155* gene products targeting MULT-1, RAE-1, and H60, respectively (16–18). In addition, viral FcRγ receptor encoded by the *m138* gene has been shown to downmodulate the expression of H60, MULT-1, and RAE-1ε ligands (19, 20).

Murine CMV mutants lacking proteins involved in the regulation of NKG2D ligands are attenuated *in vivo* by NK cells. We exploited this knowledge of NKG2D immunoevasion to develop novel CMV-based vaccine vectors. Recombinant MCMV expressing NKG2D ligand RAE-1γ, inserted in a place of its viral regulator *m152* is severely attenuated *in vivo*, but nevertheless induces strong antigen-specific $CD8^+$ T cell response to CMV and vectored antigens, providing long-term protection against bacterial infection and tumors (21–23). Likewise, HCMV expressing ULBP2 in place of its viral regulator is susceptible to control by NK cells, but preserved the ability to stimulate HCMV-specific T cells (24).

In this work, we constructed new MCMV-based vaccine vector expressing NKG2D ligand MULT-1 in place of its viral regulator *m145*. Based on our previous results on RAE-1γMCMV, we hypothesized that MULT-1MCMV would also be efficiently controlled while retaining ability to induce potent $CD8^+$ T cell response. Indeed, MULT-1MCMV was dramatically attenuated *in vivo* by NK cells and virus was cleared more rapidly than RAE-1γMCMV. Nevertheless, MULT-1MCMV induced a strong $CD8^+$ T cell response and anti-viral antibodies. This study further supports our previous results showing that recombinant CMVs expressing NKG2D ligands can be utilized as efficient vaccines and vaccine vectors.

# MATERIALS AND METHODS

## Construction of Recombinant MCMV Viruses

Wild-type (WT) MCMV refers to a bacterial artificial chromosome (BAC)-derived mouse cytomegalovirus, MW97.01, previously shown to be biologically equivalent to the MCMV Smith strain (VR-1399). Construction of WT MCMV, Δ*m152*MCMV, and RAE-1γMCMV expressing SIINFEKL was described previously (21, 22, 25). MULT-1MCMV, MULT-1MCMV expressing SIINFEKL and Δ*m145*MCMV expressing SIINFEKL were constructed according to published procedure (26). Briefly, for construction of MULT-1MCMV an ORF encoding FLAG-tagged MULT-1 was first cloned into a plasmid containing kanamycin resistance gene (KanR), I-SceI restriction site, and HCMV immediate early promoter (hMIEP) upstream of the cloning site (kind gift from Martin Messerle). The MULT-1 expression cassette containing KanR was PCR amplified using primers 5′-GGGTTAAA ACCGCACACAGATGTAGGGGCAGACTCTGAGGACCGGT GTTTCAACTCCGCGGTTGACATTGATTATTGACT-3′ and 5′-GTGAGGGGATTATGTCCTGTTTATTGTC-TCACGACA GACATACAGAGATTCGGACAGTCATCATGGGATCCCG TCGATGT-3′, which contained 60 nucleotides at their 5′ ends homologous to the intended integration site in the BAC-cloned MCMV genome, thereby replacing the *m145* ORF following the homologous recombination. To swap the sequence of $D^d$ restricted antigenic $m164_{167–175}$ peptide AGPPRYSRI with $K^b$ restricted peptide SIINFEKL, linear DNA fragment was generated using KanR as a template and primers 5′-GCCGTTCGGAAAGG ACTACTGTCGGACGTGGGGCGCTGACAGTATAATCA ACTTTGAAAAACTGAGGATGACGACGATAAGT-3′ and 5′-AAGGTCTCCTCGCCCGCTGCCACGATGG-CCTGGTTG TTGACGGCCCAGAACAGTTTTTCAAAGTTGATTATACT GTCAGCGCCCCACCAACCAATTAACCAATTC-3′ for PCR amplification. Δ*m145*MCMV expressing SIINFEKL was constructed by deletion of MULT-1 from MULT-1MCMV expressing SIINFEKL according to the same procedure using 5′-GGGTTAAA ACCGCACACAGATGTAGG-GGCAGACTCTGAGGACCG GTGTTTCAACTCCGCGAGGATGACGACGATAAGTAG-3′ and 5′-GATTATGTCCTGTTTATTGTCTCACGACAGACATA CAGAGATTCGGACAGCGCGGAG-TTGAAACACCGG TCCTCAGAGTCTGCCCCTACACAACCAATTAACCAA TTCTG-3′ primers resulting in *m145* deletion.

## Cells and Virus Propagation

BALB/c mouse embryonic fibroblasts (MEF) were grown according to published procedure (27). MEF and SVEC4-10 cells were infected with 1.5 or 3 PFU/cell, respectively. Viruses were propagated on MEF and concentrated by sucrose gradient ultracentrifugation (28). To assess virus replication *in vitro* by multi-step growth kinetics assay, MEF were infected with 0.1 PFU/cell of WT MCMV, RAE-1γMCMV, and MULT-1MCMV. Supernatants were harvested at indicated times after infection and virus titers were determined by plaque assay (28).

## Mice and Infection

C57BL/6, congenic C57BL/6 (Ly5.1/CD45.1$^+$), NKG2D$^{-/-}$ (29), BALB/c, TCR transgenic mice specific for M38 (Maxi) (30), and SIINFEKL (OT-1) (31) were bred under specific pathogen-free conditions at the Faculty of Medicine, University of Rijeka. All experiments performed in this study were approved by the Animal Welfare Committee of the University of Rijeka. Unless otherwise noted, gender matched mice at age of 8–16 weeks were infected with $2 \times 10^5$ PFU of tissue culture derived recombinant MCMV either in the footpad (f.p.) or intravenously (i.v.). Newborn BALB/c mice were infected intraperitoneally (i.p.) with 500 PFU of indicated viruses 6 h after birth. Newborn C57BL/6 mice were infected i.p. with 200 or 500 PFU of indicated viruses 24 h post-partum. *In vivo* blocking of NKG2D and depletion of NK cells was performed by i.p. injection of mouse α-mouse NKG2D blocking antibody (generated by Center for Proteomics, University of Rijeka, Faculty of Medicine, clone NKG2D.03) or mouse α-mouse NK1.1 (clone PK136) (32) and rabbit α-asialo GM1 antiserum (αAGM1) (Wako Chemicals), respectively. Viral titers from organs were determined by a plaque assay (28).

## Adoptive Transfer

For adoptive transfer experiments C57BL/6 or C57BL/6 CD45.1$^+$ mice were immunized f.p. with $2 \times 10^5$ PFU of indicated viruses. After 6 weeks, total CD8$^+$ T cells were enriched from splenocytes using CD8a$^+$ T Cell Isolation Kit (Miltenyi) and sorted on BD FACSAriaII. Adult C57BL/6 recipients were administrated i.p. with 250 μg of depleting NK1.1 antibody (PK136) 1 day prior to sublethal irradiation with 7 Gy. Next day, mice were i.v. injected with $10^6$ sorted CD8$^+$ T cells. Newborn C57BL/6 mice were i.p. injected with $10^5$ sorted CD8$^+$ T cells 1 day prior to infection.

## *In Vitro* Stimulation of CD8$^+$ T Cells

Bone marrow cells isolated from both femurs and tibias of C57BL/6 mice were differentiated into bone marrow-derived dendritic cells (BMDCs) in the presence of J558 supernatant for 6 days (33). BMDCs were infected with 2 PFU/cell of indicated viruses. After 24 h of infection corresponding amount of either Maxi or OT-1 splenocytes was added at different T:E (BMDCs:CD8$^+$) ratios, together with Brefeldin A (Sigma). After 6 h of co-incubation, intracellular staining for IFN-γ production was performed.

## Flow Cytometry and Immune Assays

For staining of cell surface expression of NKG2D ligands, mouse NKG2D protein fused with human Fc fragment was used, followed by conjugated donkey α-human IgG Fc secondary antibody (Jackson Immunoresearch). Alternatively, mouse α-mouse RAE-1γ (generated by Center for Proteomics, University of Rijeka, Faculty of Medicine, clone RAE-1γ0.01) or rat α-mouse MULT-1 (clone 1D6) (18) was used, followed by fluorochrome conjugated goat α-mouse IgG (BD Pharmingen) and goat α-rat IgG F(ab′)$_2$ (Santa Cruz) secondary antibody, respectively. As isotype control irrelevant protein fused with human Fc was used (human PVR-Fc) or antibodies of same isotype originating from the same host, respectively. Splenocytes from immunized C57BL/6 mice were either immediately stained with fluorescently labeled antibodies for assessment of CD8$^+$ T cell phenotype or incubated in the presence of 5 μg/ml H-2$^b$ restricted custom synthesized peptides of M45 ($_{985}$HGIRNASFI$_{993}$), M38 ($_{316}$SSPPMFRV$_{323}$), and ovalbumin ($_{257}$SIINFEKL$_{264}$) (JPT Peptide Technologies) in presence of Brefeldin A (Sigma) for cytokine production. After 4 h of stimulation, cells were stained for viability, expression of surface markers, and intracellular cytokines. Reagents used in flow cytometry analysis were purchased from eBioscience/Thermo Fischer Scientific and included: Fixable Viability Dye, αCD8 (clone 53-6.7), αCD44 (clone IM7), αCD62L (clone MEL-14), αCD127 (clone SB/199), αIFNγ (clone XMG1.2), and αTNF (clone MP6-XT22) antibodies. Following reagents were obtained through NIH Tetramer Core Facility: H-2K(b) MCMV M38 ($_{316}$SSPPMFRV$_{323}$), H-2L(d) MCMV IE1 ($_{168}$YPHFMPTNL$_{176}$), and H-2D(d) MCMV m164 ($_{257}$AGPPRYSRI$_{265}$). SIINFEKL-specific multimer H-2Kb/SIINFEKL MHC multimer was kindly provided by Dirk Busch (Munich). Samples were analyzed on BD FACSAriaII using FACSDiva and FlowJo (Tree Star, Inc.).

## ELISA

For detection of MCMV-specific total IgG in immune sera, high binding ELISA plates were coated with lysate of Δ*m138*MCMV infected MEF as previously described (34). Shortly, sera of mice were incubated overnight at +4°C, followed by extensive washing and detection with peroxidase conjugated goat α-mouse IgG (H + L) (Jackson Immunoresearch).

## Statistical Analysis

Statistical significance was calculated by unpaired two-tailed Student's *t*-test or Mann–Whitney *U* test for statistical analyses of the virus titers. Differences in MCMV-specific antibody titers in sera of immune animals were analyzed using two-way-ANOVA and Bonferroni *post hoc* test. *P* values less than 0.05 were considered significant. Only statistically significant differences are indicated in figures. All data were analyzed using GraphPad Prism 5 software.

# RESULTS

## Construction and *In Vitro* Characterization of Recombinant MCMV Expressing MULT-1

MULT-1MCMV was constructed by replacing the *m145* gene, encoding a viral inhibitor of MULT-1, with the gene encoding MULT-1 under the control of hMIEP (**Figure 1A**). The construction of RAE-1γMCMV was described previously (21, 22). To study CD8$^+$ T cell response induced by these recombinant viruses against well characterized CD8$^+$ T cell epitope, all viruses used in this study expressed the immunodominant K$^b$ epitope SIINFEKL in place of D$^d$ restricted antigenic m164 epitope (**Figure 1A**). MCMV expressing only SIINFEKL was used as a control (hereby referred as WT MCMV) (25). MULT-1MCMV replication *in vitro* was comparable to WT MCMV and RAE-1γMCMV (**Figure 1B**). To measure the expression of NKG2D ligands on the surface of infected cells, MEF were infected with WT MCMV, Δ*m152*MCMV, RAE-1γMCMV, Δ*m145*MCMV,

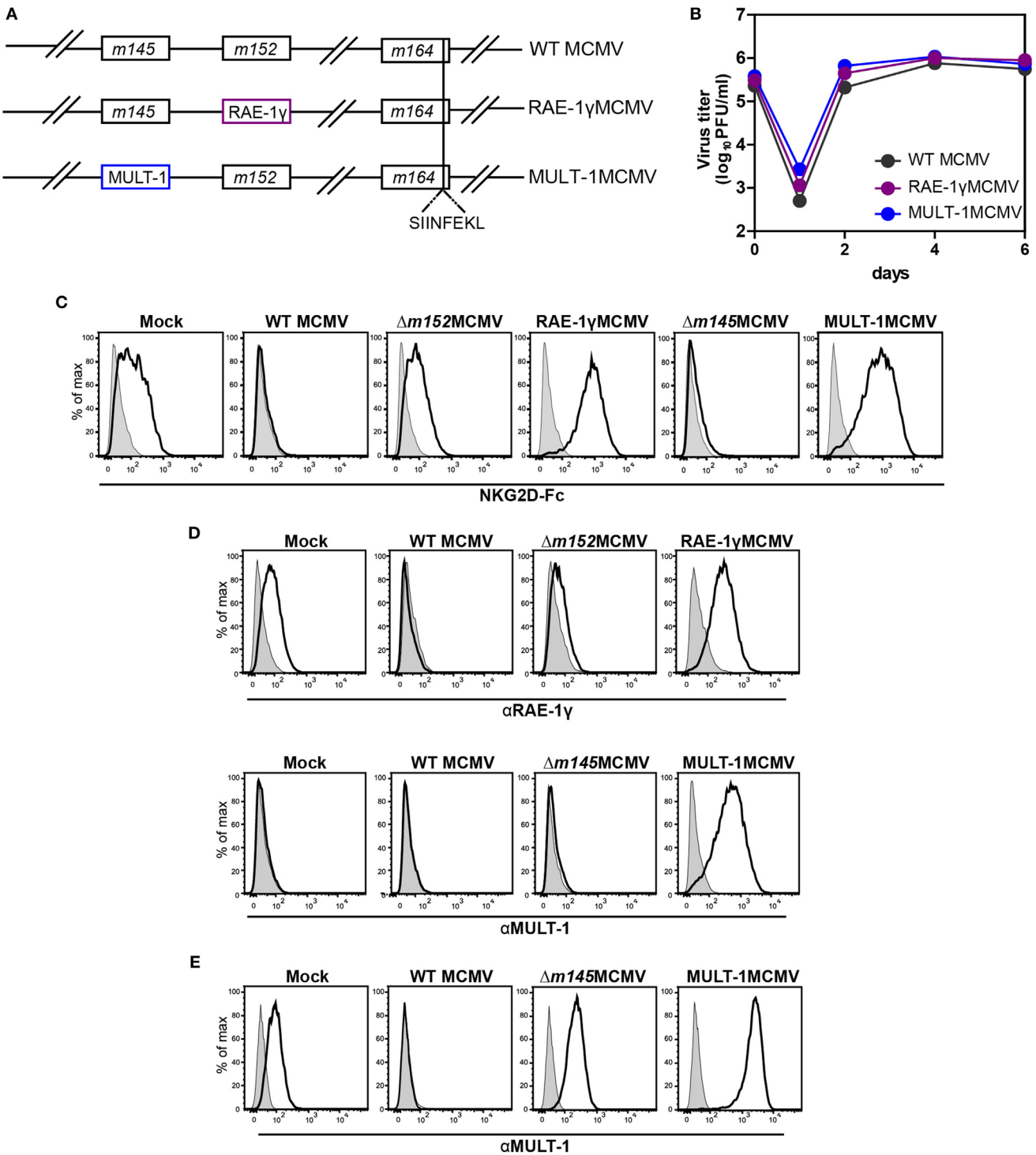


**FIGURE 1** | Recombinant viruses used in this study. **(A)** Recombinant murine CMV (MCMV) was made by insertion of genes for NKG2D ligands, RAE-1γ, and murine UL16 binding protein-like transcript-1 (MULT-1) in place of *m152* and *m145*, respectively. OVA-derived $K^b$ restricted $CD8^+$ T cell epitope SIINFEKL was swapped with $D^d$ restricted viral $CD8^+$ T cell epitope of m164 $_{167}$AGPPRYSRI$_{175}$. **(B)** Multi-step growth kinetics assay on mouse embryonic fibroblasts (MEF) comparing wild-type (WT) MCMV, RAE-1γMCMV, and MULT-1MCMV is shown. **(C,D)** MEFs were infected with 1.5 PFU/cell of indicated viruses and expression of NKG2D ligands was evaluated 24 h after infection by staining either with **(C)** mouse NKG2D-Fc fusion protein (black line) or **(D)** αRAE-1γ (upper row, black line), αMULT-1 (lower row, black line), and appropriate isotype controls (gray). **(E)** SVEC4-10 cells were infected with 3 PFU/cell of WT MCMV, Δ*m145*MCMV, and MULT-1MCMV for 16 h. Surface expression of MULT-1 was detected with αMULT-1 (black line) or isotype control (gray).

and MULT-1MCMV for 24 h. To assess the expression of exogenous NKG2D ligands as well as endogenous NKG2D ligands affected by the deletion of viral evasins, staining was performed using mouse NKG2D-Fc fusion protein (**Figure 1C**). Expression of inserted NKG2D ligands on the surface of RAE-1γMCMV or MULT-1MCMV infected cells was confirmed by staining with specific antibodies against RAE-1γ and MULT-1, respectively (**Figure 1D**). As previously published, WT MCMV downregulated NKG2D ligands from the cell surface and deletion of *m152* substantially restored RAE-1 surface expression (35). However, infection of MEF with MULT-1MCMV resulted in high surface expression of MULT-1. Furthermore, both RAE-1γMCMV and MULT-1MCMV infected cells showed similar binding of NKG2D-Fc (**Figure 1C**). Since MEF constitutively express very low level of MULT-1, the effect of *m145* deletion on the expression of this ligand was hardly detectable. However, the impact of m145 on the surface expression of MULT-1 was detectable after infecting SVEC4-10 cells that constitutively express MULT-1 (**Figure 1E**) (18, 19). Altogether, these data demonstrate that cells infected with MULT-1MCMV express high levels of MULT-1 on their surface and its expression was not abrogated by function of other viral regulator of MULT-1.

## MULT-1MCMV Is Strongly Attenuated in Adult and Neonatal Mice

To determine how expression of high affinity NKG2D ligand MULT-1 affects viral control *in vivo*, BALB/c mice were infected with WT MCMV, RAE-1γMCMV, and MULT-1MCMV i.v. and viral titers were analyzed at different times after infection. By the day 4 after infection both RAE-1γMCMV and MULT-1MCMV were heavily attenuated in spleen, lungs, and liver (**Figures 2A,C**). Moreover, in liver MULT-1MCMV was even more attenuated as compared to RAE-1γMCMV (**Figure 2C**). Both RAE-1γMCMV and MULT-1MCMV were also attenuated in lungs at day 14 post infection compared to WT MCMV and were completely undetectable in salivary glands (**Figure 2A**). As shown previously (18), Δ*m145*MCMV was attenuated as compared to WT MCMV, but attenuation of MULT-1MCMV was even stronger (Figure S1A in Supplementary Material).

We have previously shown that RAE-1γMCMV was attenuated in immunologically immature newborns (21). Here, we show that MULT-1MCMV was severely attenuated in newborn mice as well (**Figure 2B**). Surprisingly, MULT-1MCMV was controlled more rapidly than RAE-1γMCMV in infected newborns, with virus undetectable at the day 8 after infection.

To identify the immune mechanism responsible for the efficient control of MULT-1MCMV, groups of infected BALB/c mice were treated either with αAGM1 to deplete NK cells or NKG2D blocking antibody. Blocking of NKG2D receptor abolished the differences between mutant viruses and WT MCMV suggesting that early attenuation of recombinant viruses expressing NKG2D ligands is exclusively NKG2D-dependent. Depletion of NK cells by αAGM1 also resulted in the abrogation of differences in viral titer between the groups (**Figure 2C**). It has been well established that NK cells in MCMV resistant C57BL/6 mice control virus *via* a direct recognition of viral m157 protein by Ly49H activating receptor (36). Notably, viruses expressing NKG2D ligands were more sensitive to NK cell-dependent control even in C57BL/6 mice but this phenotype was lost in NKG2D$^{-/-}$ mice (**Figure 2D**).

## MULT-1MCMV Induces a Strong and Functional Antigen-Specific CD8$^+$ T Cell Response

We have already shown that MCMV expressing NKG2D ligand RAE-1γ induces a strong CD8$^+$ T cell response despite its attenuation *in vivo* (21, 22). To examine CD8$^+$ T cell response after MULT-1MCMV infection, C57BL/6 mice were infected into footpad with WT MCMV, RAE-1γMCMV, and MULT-1MCMV. Antigen-specific CD8$^+$ T cell response was evaluated in the early (7 days) and late (2 months) phase of infection by measuring IFN-γ and TNF-α production after stimulation with non-inflationary peptide M45, inflationary peptide M38, as well as OVA-derived peptide SIINFEKL (**Figure 3A**; Figure S2 in Supplementary Material). At both time points after infection the frequencies and absolute numbers of virus-specific CD8$^+$ T cells were comparable or higher in MULT-1MCMV immunized animals compared to WT MCMV. While virus-specific CD8$^+$ T cell response was similar in RAE-1γMCMV and MULT-1MCMV, RAE-1γMCMV induced more CD8$^+$ T cells specific for the vectored epitope SIINFEKL. Furthermore, RAE-1γMCMV induced more effector memory SIINFEKL-specific CD8$^+$ T cells than MULT-1MCMV, while frequency of M38-specific memory subsets was comparable between viruses (Figure S3 in Supplementary Material). Of note, although MULT-1MCMV was attenuated compared to Δ*m145*MCMV, both viruses induced similar CD8$^+$ T cell response (Figure S1B in Supplementary Material).

To circumvent the impact of a different antigenic load, we performed an *in vitro* stimulation of CD8$^+$ T cells. BMDCs were infected with WT MCMV, RAE-1γMCMV, and MULT-1MCMV and co-incubated with splenocytes isolated from naive TCR transgenic mice possessing CD8$^+$ T cells specific for the M38 epitope (Maxi) or SIINFEKL (OT-1) (**Figure 3B**). MULT-1MCMV infected BMDCs stimulated a higher proportion of Maxi CD8$^+$ T cells to produce IFN-γ compared to WT MCMV and equal to RAE-1γMCMV. In accordance with data presented in **Figure 3A**, RAE-1γMCMV infected BMDCs provided superior stimulation of OT-1 CD8$^+$ T cells. However, MULT-1MCMV infected BMDCs stimulated OT-1 CD8$^+$ T cells better than WT MCMV. Altogether, MULT-1MCMV induced a robust antigen-specific CD8$^+$ T cells response to both viral and vectored epitopes in both acute and latent phase of infection.

Long-term CD8$^+$ T cell memory formation is critical for protection upon challenge infection later in life. To assess the protective capacity of virus-specific CD8$^+$ T cells induced by MULT-1MCMV, adoptive transfer experiments were performed. CD8$^+$ T cells were sorted from C57BL/6 CD45.1$^+$ donor mice latently infected with WT MCMV, RAE-1γMCMV, and MULT-1MCMV and adoptively transferred into NK depleted and irradiated C57BL/6 CD45.2$^+$ recipients infected

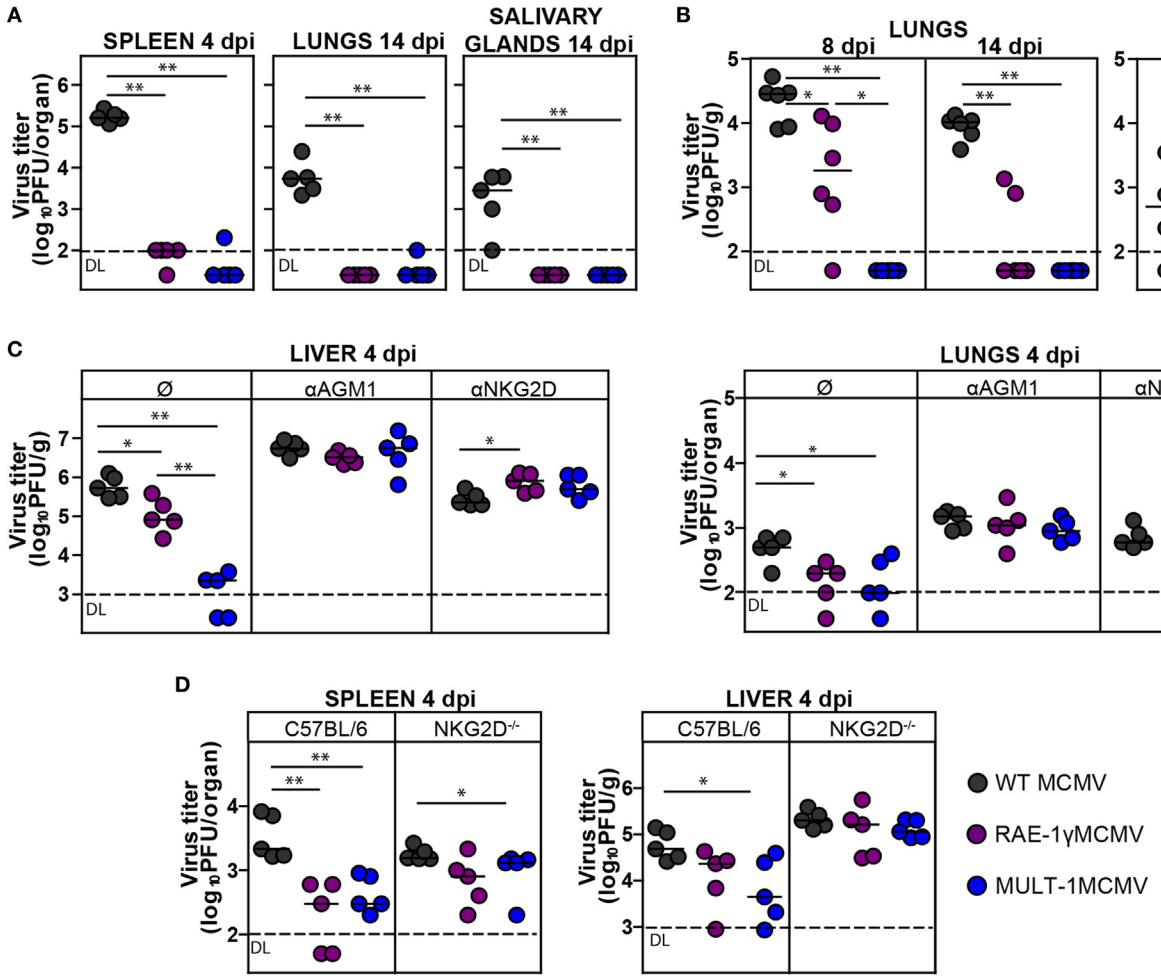


**FIGURE 2** | Recombinant murine CMV (MCMV) expressing MULT-1 is controlled by NK cells in NKG2D-dependent manner. **(A)** BALB/c mice were infected intravenously (i.v.) with $2 \times 10^5$ PFU of wild-type (WT) MCMV, RAE-1γMCMV, and MULT-1MCMV. On day 4 and 14 after infection viral titer was determined in organs by plaque assay. **(B)** Newborn C57BL/6 mice were infected with 200 PFU of WT MCMV, RAE-1γMCMV, and MULT-1MCMV intraperitoneally (i.p.) 24 h after birth. Viral titer was determined in organs by plaque assay on day 8 and 14 after infection. **(C)** BALB/c mice received 20 µl of αAGM1 or 250 µg of NKG2D blocking antibody i.p. 2 h before infection and additional dose of NKG2D blocking antibody on day 2 after i.v. infection with $2 \times 10^5$ PFU of WT MCMV, RAE-1γMCMV, and MULT-1MCMV. Viral titer was determined in organs by plaque assay on day 4 after infection. **(D)** C57BL/6 and NKG2D$^{-/-}$ mice were i.v. infected with $5 \times 10^5$ PFU of WT MCMV, RAE-1γMCMV, and MULT-1MCMV. On day 4 after infection viral titer was determined in organs by plaque assay. Each circle represents an individual animal and lines represent medians. Data were analyzed using Mann–Whitney *U* test. Asterisks denote significant values: $^*P < 0.05$; $^{**}P < 0.01$. Abbreviation: DL, detection limit.

with WT MCMV (**Figure 4A**). CD8$^+$ T cells from MULT-1MCMV immunized donors controlled the infection in lungs and liver of challenged recipients more efficiently than non-immune CD8$^+$ T cells and equally efficient as CD8$^+$ T cells obtained from WT MCMV and RAE-1γMCMV immunization. By using a similar approach, we evaluated the protective capacity of MULT-1MCMV immunization by transferring immune CD8$^+$ T cells into newborn mice infected with WT MCMV (**Figure 4B**). CD8$^+$ T cells derived from WT MCMV, RAE-1γMCMV, and MULT-1MCMV infected mice provided a similar level of protection in this model as well. Altogether, our results demonstrated that antigen-specific CD8$^+$ T cells induced by highly attenuated MULT-1MCMV are functional and protective against MCMV infection.

## Immunization of Female Mice With MULT-1MCMV Provides Antibody Mediated Protection to Their Offspring

Induction of protective antibody response is a favorable feature of any vaccine. To determine whether MULT-1MCMV can induce MCMV-specific antibodies in immunized mothers which could protect their offspring from MCMV disease, BALB/c females were infected with WT MCMV, RAE-1γMCMV, and MULT-1MCMV 2 weeks prior mating (**Figure 5A**). Analysis of immune sera showed that MULT-1MCMV immunized mothers, as well as their offspring, had a substantial level of MCMV-specific antibodies (**Figure 5B**). There was no difference in levels of anti-viral antibodies between MULT-1MCMV compared to WT MCMV.

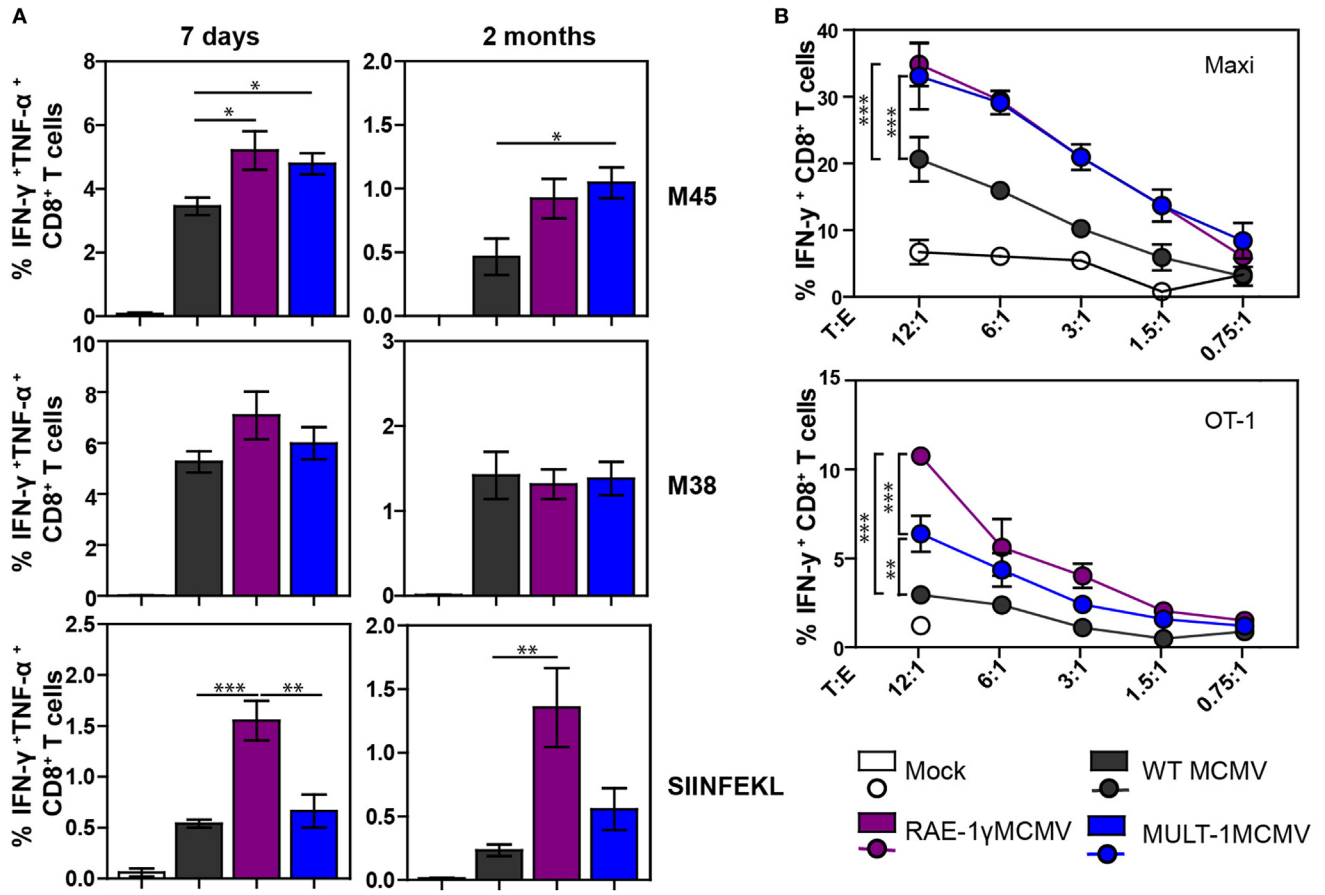


**FIGURE 3** | Attenuated MULT-1MCMV induces strong antigen-specific CD8+ T cell response and efficiently stimulates CD8+ T cells *in vitro*. C57BL/6 mice were infected footpad with $2 \times 10^5$ PFU of wild-type (WT) MCMV, RAE-1γMCMV, and MULT-1MCMV. **(A)** At different times after infection CD8+ T cells from spleen were analyzed after peptide stimulation for cytokine production ($n = 5$). **(B)** C57BL/6 bone marrow-derived dendritic cells (BMDCs) were infected with 2 PFU/cell of WT MCMV, RAE-1γMCMV, or MULT-1MCMV. After 24 h, infected BMDCs were co-incubated with splenocytes from Maxi or OT-1 mice for 6 h and IFN-γ production was measured by flow cytometry. Representative data from two to five independent experiments are shown. Data are presented as mean ± SEM and were analyzed using Student's *t*-test. Asterisks denote significant values: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

As shown previously, slightly lower antibody titer was observed in mice immunized with RAE-1γMCMV (21). Moreover, newborns from MULT-1MCMV immunized mothers were completely protected upon infection with WT MCMV, as well as newborns from WT MCMV and RAE-1γMCMV immunized dams (**Figure 5C**). Altogether, these data show that despite a strong attenuation, MULT-1MCMV immunization of mothers induces a strong production of anti-viral antibodies, which can pass the placenta and protect their offspring from MCMV disease.

## DISCUSSION

After resolution of primary CMV infection, CD8+ T cells specific for certain immunodominant epitopes are not maintained as a low abundant memory population, but rather gradually increase in frequency acquiring an effector-like phenotype, a phenomenon known as "memory inflation" (37, 38). This characteristic of CMV induced CD8+ T cell response could be exploited in the development of CD8+ T cell-based live vaccines (39–42). Live attenuated vaccines imitate natural infection by inducing a broad cellular and humoral response to variety of antigens, which makes them superior to subunit vaccines. Still, there are concerns about using live virus as a vaccine as it might cause unwanted virulence, especially in high-risk individuals such as immunocompromised patients. CMV has many nonessential genes involved in subversion of immune response and the deletion of those genes enables the manipulation of its virulence and the quality of immune response. In our previous studies we took advantage of virus lacking the NKG2D immunoevasin to generate recombinant immunologically attenuated vaccine vector RAE-1γMCMV that retained the ability to induce a strong adaptive response (21–23). NKG2D ligands are differently expressed and regulated in tissues, they differ in the affinity for the receptor and certain ligands are restricted to the particular mouse strains (11, 43–45). MULT-1 has the highest affinity for the receptor among all mouse NKG2D ligands and its transcripts were found in most of the healthy tissues, but its protein expression was strictly regulated on posttranslational

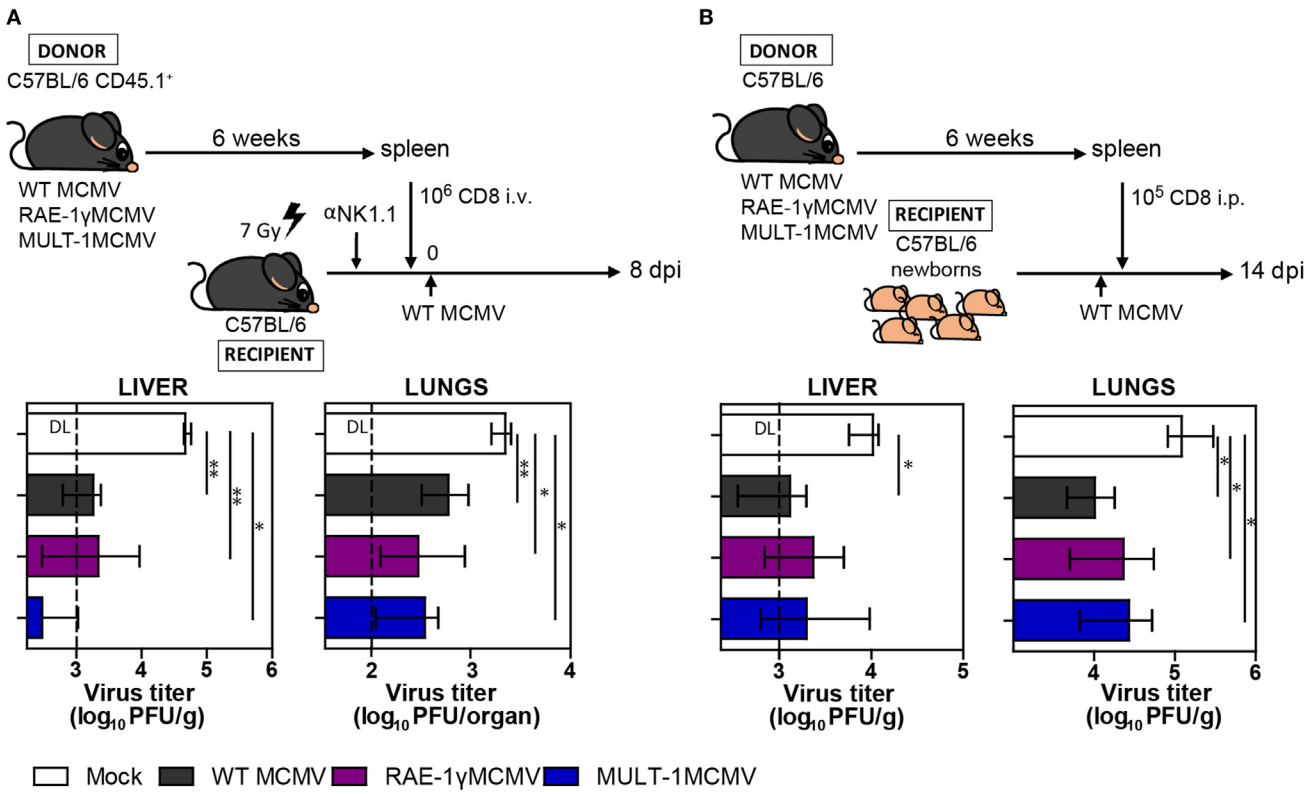


**FIGURE 4** | MULT-1MCMV induces protective anti-viral CD8+ T cell response. **(A)** C57BL/6 CD45.1+ mice were infected footpad (f.p.) with $2 \times 10^5$ PFU of wild-type (WT) MCMV, RAE-1γMCMV, and MULT-1MCMV. After 6 weeks $10^6$ CD8+ T cells sorted from spleens were intravenously transferred into NK depleted, irradiated C57BL/6 recipients challenged f.p. with $10^5$ PFU of WT MCMV. Eight days after infection organs of recipient animals were collected and viral titer was determined by plaque assay ($n = 4$–8). **(B)** C57BL/6 mice were infected f.p. with $2 \times 10^5$ PFU of WT MCMV, RAE-1γMCMV, and MULT-1MCMV and after 6 weeks $10^5$ CD8+ T cells sorted from spleen were transferred into C57BL/6 newborn mice challenged i.p. with 500 PFU of WT MCMV. On day 14 after challenge infection viral titer was determined in organs by plaque assay ($n = 4$–6). Representative data of two independent experiments are shown. Data are presented as medians with interquartile range and analyzed using Mann–Whitney *U* test. Asterisks denote significant values: $^*P < 0.05$; $^{**}P < 0.01$. Abbreviation: DL, detection limit.

level (46). To exploit those differences in the context of a MCMV-based vaccine, we constructed MULT-1MCMV and compared it with WT MCMV and RAE-1γMCMV. MULT-1MCMV was highly immunologically attenuated, but nevertheless induced a strong and protective antigen-specific CD8+ T cell and antibody response. Thus, this study further supports the idea that attenuated recombinant CMVs expressing NKG2D ligands can be used as efficient vaccines and vaccine vectors.

It is well known that NK cell response can also shape the adaptive immune response (47). The most extensively studied example of strong NK control of MCMV infection is the direct recognition of viral protein m157 by the activating NK receptor Ly49H, leading to efficient virus control (36, 48). In most cases, a strong NK control would impair CD8+ T cell responses presumably due to the lower viral burden and cytokine milieu diminishing antigen presentation (49, 50). Indeed, during MCMV infection the lower viral burden reduces the magnitude of long-term CD8+ T cell response but does not impact its kinetics (51, 52). Paradoxically, it has been shown that blocking viral replication increases CD8+ T cell response to non-inflationary epitopes presented by preserved dendritic cells (DCs) which would otherwise be depleted due to type I IFNs (53, 54). Interestingly, this effect was not evident in the population of inflationary CD8+ T cells. While convectional non-inflationary CD8+ T cells are preferentially primed by cross-presented antigens on DCs (55), inflationary CD8+ T cells are originating from cells primed early in infection as well as from constantly replenished short-lived effectors (56) maintained at high levels by sporadic virus reactivations in non-hematopoietic cells (30, 57). Differential requirements in generation of non-inflationary and inflationary CD8+ T cells in MCMV infection might explain these findings (58). Here we show that despite efficient NK-mediated virus control, MULT-1MCMV induces CD8+ T cell response of similar or greater magnitude compared to WT MCMV to both non-inflationary and inflationary epitopes. While all viruses induced comparable CD8+ T cell response to viral antigens, RAE-1γMCMV induced a higher number of SIINFEKL-specific CD8+ T cells. Somewhat better response to non-inflationary epitope M45 in mice infected with recombinant viruses expressing NKG2D ligands could be explained either by a better cross-presentation due to preserved DCs in absence of strong infection or immune function of these proteins (21, 22, 54). The strong engagement of NKG2D and activation of NK cells might result in an environment favoring antigen presentation. Alternatively, the co-stimulation signal

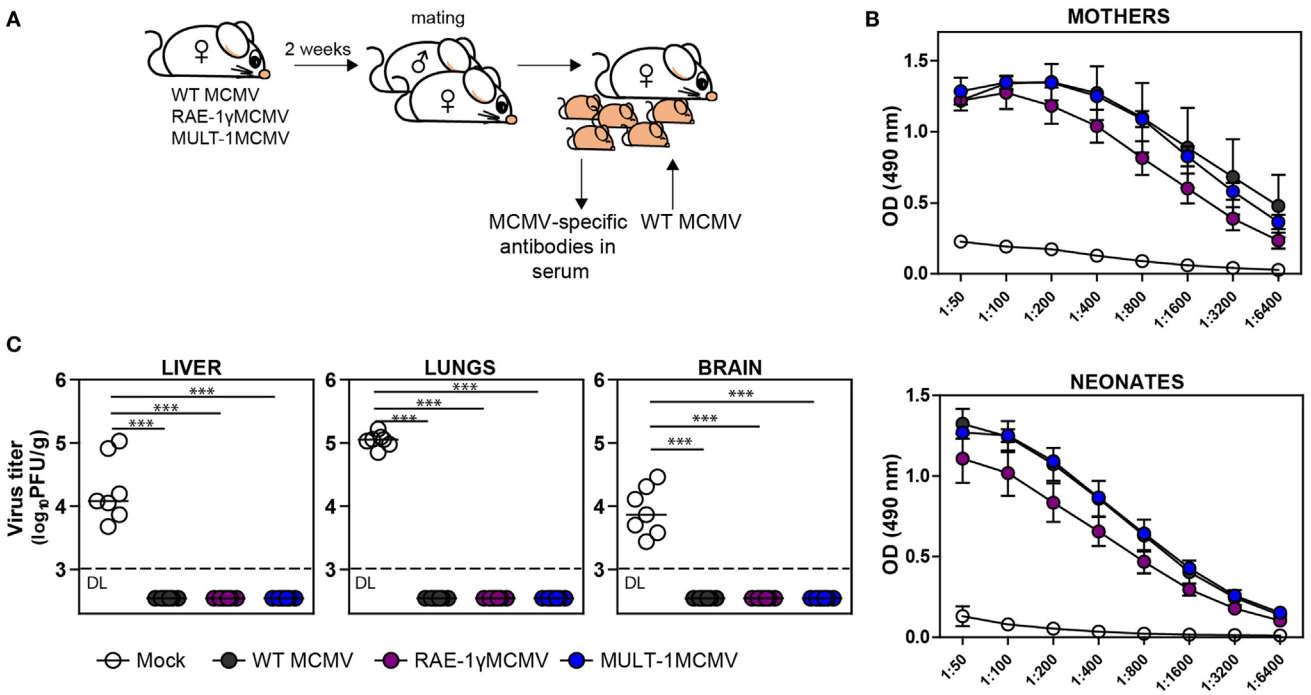


**FIGURE 5** | Vaccination of female mice with MULT-1MCMV induces MCMV-specific antibodies which protect their offspring from MCMV challenge. **(A)** BALB/c females were immunized intravenously with $2 \times 10^5$ PFU of wild-type (WT) MCMV, RAE-1γMCMV, and MULT-1MCMV 2 weeks prior mating. Six hours after birth, their offspring were either bled to collect sera or intraperitoneally challenged with 500 PFU of WT MCMV. **(B)** Titers of MCMV-specific antibodies in sera of immunized mothers (in average 1.5 month after infection) and their offspring (6 h after birth) are shown. **(C)** WT MCMV challenged newborn mice were sacrificed 9 days after infection and viral titer was determined in their organs by plaque assay. Each circle represents an individual animal. Results are presented as pooled data from two independent experiments. Data are presented as mean ± SEM **(B)** or medians **(C)** and were analyzed using two-way ANOVA **(B)** and Mann–Whitney *U* test **(C)**. Asterisks denote significant values: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Abbreviation: DL, detection limit.

by engagement of NKG2D on CD8$^+$ T cells could rescue T cell responses which would otherwise be weaker due to strong viral control and action of other viral evasins. The importance of CD8$^+$ T cell co-stimulation is evident from the fact that TCR signaling without an appropriate co-stimulation signal drives CD8$^+$ T cells to the state of anergy rather than activation (59). CMVs have developed multiple mechanisms to evade CD8$^+$ T cell recognition of infected cells. Indeed, MCMV possess several genes encoding proteins which interfere with antigen presentation by downregulating the expression of MHC I molecules (6). Ligands for the major T cell co-stimulatory receptor CD28 are also targeted by viral immunoevasion (59–61). CD28/B7-mediated co-stimulation is indispensable in acute infection and for the establishment of CD8$^+$ T cell memory in MCMV (62). In LCMV infection, the absence of B7-mediated co-stimulation can be substituted with other co-stimulatory pathways (63). Though this was not shown for CD8$^+$ T cell response in herpes viruses, it suggests that under certain conditions co-stimulatory pathways might act compensatory to each other. Our *in vitro* studies showed that RAE-1γMCMV and MULT-1MCMV infected BMDCs have an improved capacity to stimulate CD8$^+$ T cells compared to WT MCMV. The engagement of co-stimulatory receptor NKG2D on CD8$^+$ T cells was reported to promote proliferation and cytotoxic capacity of antigen-specific CD8$^+$ T cells in various experimental settings (64–66), as well as rescue memory of unhelped CD8$^+$ T cells (67). Moreover, it was shown recently that engagement of NKG2D on NK cells augments their expansion during MCMV infection (68). Therefore, we hypothesize that co-stimulatory function of NKG2D on CD8$^+$ T cells might overcome multiple ways of viral interference with antigen presentation and downregulation of co-stimulatory molecules resulting in a better stimulation and improved CD8$^+$ T cell responses *in vivo*.

Apart from prompting durable CD8$^+$ T cell memory, the ability to induce humoral response is another vital feature of CMV vaccine, since antigen-specific antibodies are able to cross the placental barrier and are the first to protect the fetus and newborns against congenital infection (69, 70). Despite the lower antigenic load, MULT-1MCMV immunization induced MCMV-specific antibodies capable of protecting offspring against a challenge infection.

Another interesting finding of this study was the rapid attenuation of MULT-1MCMV pronounced in perinatally infected newborns. This is in agreement with our recent study showing that MULT-1MCMV failed to reach the brain of mice infected as newborns resulting in the absence of brain inflammation and establishment of tissue-resident memory CD8$^+$ T cells (71). NK cells are immature in newborns, at least partially due to the presence of high levels of immune suppressive TGF-β, which makes newborns highly susceptible to viral infections (72). Since NKG2D is expressed early in ontogeny of NK cells (73), we speculate that the engagement of NKG2D with a high

affinity ligand might overcome the suppressive environment in newborn mice, but the exact mechanism of MULT-1MCMV control in newborns remains elusive. Yet, this study demonstrates the feasibility of CMV-based vaccine vectors to be used even in newborns.

Altogether, we show for the first time that MCMV expressing the high affinity NKG2D ligand MULT-1 has numerous favorable features of a vaccine such as being highly attenuated, but still able to induce both cellular and humoral adaptive immunity. We have established the vaccine properties of MULT-1MCMV against MCMV infection, which does not exclude the prospect to test its capacity as a vector vaccine. In conclusion, by comparing recombinant viruses expressing different NKG2D ligands, we confirmed the dual role of NKG2D in mediating a strong virus control, while retaining $CD8^+$ T cell response equivalent or better than WT virus infection, which can serve as a model approach for the development of a similar HCMV-based vaccine vector.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Regulations on the protection of animals used for scientific purposes (Official Gazette of the Republic of Croatia, 55/2013). Ethics Committee of the Veterinary Department of the Ministry of Agriculture and Animal Welfare Committee of the University of Rijeka Faculty of Medicine approved all animal experiments.

## AUTHOR CONTRIBUTIONS

LH and IB designed the study, performed the experiments, analyzed the data, and wrote the manuscript. TJ and JR designed and generated recombinant viruses. VL and SJu contributed to performing experiments and critical reading of the manuscript. AK and SJo designed and oversaw the study and wrote the manuscript.

## ACKNOWLEDGMENTS

We thank Karmela Miklić, Dijana Rumora, Edvard Ražić, Kristina Vuković, and Jelena Železnjak for their technical support.

## FUNDING

The study was supported by European Research Council Advanced Grant (no. 322693) (to SJ) and by the grant "Strengthening the capacity of CerVirVac for research in virus immunology and vaccinology," KK.01.1.1.01.0006, awarded to the Scientific Centre of Excellence for Virus Immunology and Vaccines and co-financed by the European Regional Development Fund (to SJ).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00991/full#supplementary-material.

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**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.

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ORIGINAL RESEARCH
published: 08 May 2018
doi: 10.3389/fimmu.2018.00917

# The Donor Major Histocompatibility Complex Class I Chain-Related Molecule A Allele rs2596538 G Predicts Cytomegalovirus Viremia in Kidney Transplant Recipients

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**Edited by:**
*Nadia Guerra, Imperial College London, United Kingdom*

**Reviewed by:**
*Miguel López-Botet, Institut Hospital del Mar d'Investigacions Mèdiques, Spain*
*Amir Horowitz, Icahn School of Medicine at Mount Sinai, United States*

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

**Received:** *01 February 2018*
**Accepted:** *13 April 2018*
**Published:** *08 May 2018*

**Citation:**
*Rohn H, Tomoya Michita R, Schwich E, Dolff S, Gäckler A, Trilling M, Le-Trilling VTK, Wilde B, Korth J, Heinemann FM, Horn PA, Kribben A, Witzke O and Rebmann V (2018) The Donor Major Histocompatibility Complex Class I Chain-Related Molecule A Allele rs2596538 G Predicts Cytomegalovirus Viremia in Kidney Transplant Recipients. Front. Immunol. 9:917. doi: 10.3389/fimmu.2018.00917*

The interaction of major histocompatibility complex class I chain-related protein A (MICA) and its cognate activating receptor natural killer (NK) group 2 member D (NKG2D) receptor plays a significant role in viral immune control. In the context of kidney transplantation (KTx), cytomegalovirus (CMV) frequently causes severe complications. Hypothesizing that functional polymorphisms of the MICA/NKG2D axis might affect antiviral NK and T cell responses to CMV, we explored the association of the MICA-129 Met/Val single nucleotide polymorphism (SNP) (affecting the binding affinity of MICA with the NKG2D receptor), the MICA rs2596538 G/A SNP (influencing MICA transcription), and the NKG2D rs1049174 G/C SNP (determining the cytotoxic potential of effector cells) with the clinical outcome of CMV during the first year after KTx in a cohort of 181 kidney donor-recipients pairs. Univariate analyses identified the donor MICA rs2596538 G allele status as a protective prognostic determinant for CMV disease. In addition to the well-known prognostic factors CMV high-risk sero-status of patients and the application of lymphocyte-depleting drugs, the donor MICA rs2596538 G allele carrier status was confirmed by multivariate analyses as novel-independent factor predicting the development of CMV infection/disease during the first year after KTx. The results of our study emphasize the clinical importance of the MICA/NKG2D axis in CMV control in KTx and point out that the potential MICA transcription in the donor allograft is of clinically relevant importance for CMV immune control in this allogeneic situation. Furthermore, they provide substantial evidence that the donor MICA rs2596538 G allele carrier status is a promising genetic marker predicting CMV viremia after KTx. Thus, in the kidney transplant setting, donor MICA rs2596538 G may help to allow the future development of personal CMV approaches within a genetically predisposed patient cohort.

**Keywords: major histocompatibility complex class I chain-related molecule A, natural killer group 2 member D ligands, natural killer group 2 member D receptor, cytomegalovirus, kidney transplantation, major histocompatibility complex class I chain-related molecule A-129 dimorphism, major histocompatibility complex class I chain-related molecule A rs2596538, natural killer group 2 member D rs1049174**

## INTRODUCTION

The major histocompatibility complex class I chain-related molecule A (MICA), belongs to the family of non-HLA molecules and is recognized by its cognate activating natural killer (NK) group 2 member D (NKG2D) receptor, a C-type lectin-like transmembrane protein. Both molecules strongly influence the activation and regulation of innate and adaptive immunity. NKG2D is expressed by most natural killer cells (NK cells), CD8+ αβ T cells as well as γδ T cells, and plays a pivotal role in the recognition of damaged, stressed, or infected cells (1–3). The binding of NKG2D to MICA stimulates NK cell activation and subsequent cytotoxicity. Additionally, it provides co-stimulatory signals for T cells, enhancing their cytolytic activity and cytokine production. The NKG2D ligand MICA is encoded in the MHC locus located on chromosome 6 and exhibits a tissue-specific expression pattern (4). Under conditions of cellular stress like in the course of viral infections or malignant transformations, it is significantly upregulated (5–8), defining the MICA/NKG2D axis important for immune surveillance (9). Consequently, deregulation of MICA or NKG2D is associated with viral immune escape or tumor growth, but it is also implicated to autoreactive T cell stimulation (10, 11).

Viral infections result in a strong induction of MICA expression (5, 12). Based on the selective pressure elicited by the MICA/NKG2D axis, certain viruses like cytomegalovirus (CMV) have evolved immune evasive proteins (e.g., pUL142, pUS9, pUS18, or pUS20) targeting MICA expression (13–17). Together with other immune antagonists (18, 19), these mechanisms enable CMV to persist lifelong despite the presence of a functional immune system.

In spite of their potency, such immune antagonists fail to completely eliminate the control mediated by their cognate targets (12, 20). Consistently, the genetic variability in coding and non-coding regions of MICA and NKG2D genes affects the efficiency of the antiviral immune surveillance during CMV infections (21–23). According to their binding affinity to NKG2D, the 107 currently known allelic MICA variations (https://www.ebi.ac.uk/ipd/imgt/hla/stats.html) can be stratified into two classes based on the functionally relevant single nucleotide polymorphism (SNP) rs1051792 A > G at position 454 in exon 3 of the MICA gene. This single nucleotide exchange of G with A results in a substitution of valine (Val) by methionine (Met) at position 129 in the α2 domain of the MICA protein, which increases the binding affinity to NKG2D (24, 25). Disease association studies focusing on the MICA-129 Met/Val dimorphism suggest a complex role in the immune system in which the high affinity allele MICA-129 Met is associated with strong immune activation, reducing the likelihood of infections or cancer but also stimulating autoimmunity (26–28). However, these studies do not allow a uniform simplified interpretation of the data and appear partly controversial. One of the reasons discussed is the internalization of NKG2D receptor due to persistent exposure to ligand-expressing cells impairing the MICA/NKG2D-driven functionality of effector cells (12, 29, 30). Additionally, the expression levels of MICA display the second layer of polymorphisms of the MICA/NKG2D axis: The SNP rs2596538 G/A located at 2.8 kb upstream of the MICA coding sequence regulates MICA expression strength. The transcription factor specificity protein 1 (SP1) exhibits an increased binding affinity to the G allele of the SNP rs2596538 resulting in higher transcriptional activity compared to the A allele of the rs2596538 SNP (31). Consistent with the decreased expression of the A allele of the SNP rs2596538 and a relevant role of MICA for virus control, this allele constitutes a risk factor for hepatitis C virus (HCV) -related hepatocellular carcinoma (31).

Besides the functional consequences of these polymorphic variations of MICA, other genetic factors influencing NKG2D regulation need to be considered for the functional outcome of T and NK cell effectors. Recently, the microRNA (miR)-1245 was identified as a negative regulator of NKG2D (32), which targets a binding site located within the 3′-untranslated region (3′-UTR) of the NKG2D gene. The interaction of miR-1245 with the NKG2D 3′-UTR results in downregulation of NKG2D expression and impaired NKG2D-driven immune functions, including cytotoxicity and cytokine secretion. As shown for human papillomavirus (33), expression of miR-1245 can be upregulated under the influence of viruses. Importantly, the rs1049174 G/C SNP is located within the recognition site of miR-1245, dividing NK cells into high (carrying the rs1049174 C allelic variant) and low cytotoxic responders (33). Thus, rs1049174G/C SNP is a relevant determinant of viral infection surveillance.

Viral infections, especially CMV, cause severe and fatal complications in immune-compromised transplant recipients (34, 35). Given the relevance of MICA and NKG2D in immune activation and surveillance against CMV infection, we hypothesized that the functional relevant allelic variation of MICA and NKG2D might influence the clinical occurrence of CMV infection or disease after kidney transplantation (KTx). A CMV transmission to allograft recipients may occur *via* donor organs as the virus is able to infect several types of human kidney cells and thus can reside in the graft (36). In contrast to other tissues, a notably strong MICA protein expression has been described in kidney allografts (37, 38). Consequently, MICA/NKG2D axis polymorphisms of the donor organ or the recipient may affect immune antiviral NK and T cell responses against CMV.

Thus, we determined if and which allelic MICA and/or NKG2D variations predispose patients to increased risk of CMV replication. To this end, we analyzed (i) the MICA-129 Met/Val SNP (affecting the binding affinity of MICA to the NKG2D receptor), (ii) the MICA rs2596538 A/G SNP (influencing MICA expression levels), and (iii) the NKG2D rs1049174 G/C SNP (determining the cytotoxic potential of effector cells) in 181 living-donor kidney transplant pairs and associated the allele status of donor and recipient to occurrence of CMV viremia in the first year.

## PATIENTS, MATERIALS, AND METHODS

### Patients

A total of 181 living-donor kidney transplant recipient and donor pairs from the transplant program at the University Hospital Essen, Germany, were included in the study. Written informed consent was obtained from every recipient–donor pair, and the local Ethics Committee approved the study protocol in compliance with the Declaration of Helsinki Principles. Patient- and transplant-related variables were collected by

chart review. Patient-related variables comprised age at time of transplantation and gender. Transplant-related variables included donor age and gender, HLA-A/B/DR mismatch, and donor-recipient CMV IgG status. Occurrence of CMV infection or disease was monitored during the first year after transplantation, and classified according to recent recommendations as follows (39): (i) CMV infection was defined as CMV viremia (polymerase chain reaction >400 copies/mL or >1/100 pp65/pUL83 antigen positive cells), (ii) CMV disease was defined as CMV viremia in combination with attributable symptoms, such as fever, malaise, leukopenia, thrombocytopenia, or elevation of liver enzymes. CMV complications were analyzed within the first 12 months after transplantation. Incidence of first episode of clinically significant CMV viremia or disease within the 12 months follow-up was 11% ($N = 20$). The CMV infection/disease rate found in the present single-centre study is concordant with rates described in previous studies of risk-adapted CMV prophylaxis (40–42). Patients with CMV disease ($N = 2$ with CMV gastrointestinal disease, $N = 1$ with CMV pneumonia) were initially treated with intravenous ganciclovir and continued with oral valganciclovir; CMV replication without apparent CMV disease was treated with valganciclovir. Due to low event rates, recipients with CMV infection or disease were combined and analyzed together.

Pre-transplant CMV naïve recipients receiving a CMV positive kidney allograft have the highest risk of symptomatic and disseminated CMV replication and were therefore considered as CMV high-risk patients. Before the end of 2011, centre-specific CMV-prophylaxis regimen consisted of (val-)ganciclovir during first 100 days for high-risk CMV recipients and for patients receiving lymphocyte-depleting induction therapy. Because emerging data suggests that the incidence of CMV is lower among patients receiving prolonged antiviral prophylaxis, the duration of the prophylactic CMV regimen was prolonged to 200 days for high-risk population in the year 2012 (43). All other patients (CMV intermediate- and low-risk) were pre-emptively monitored for CMV. For CMV high-risk patients, the screening for CMV was performed during prophylaxis only if CMV infection and/or disease were clinically suspected and thereafter on monthly basis. For intermediate- and low-risk patients, regular screening for CMV was performed weekly for 3 months and later on monthly.

Standard local immunosuppressive protocol, consisting of calcineurin inhibitors, mycophenolate mofetil, or mycophenolic acid (MPA), and steroids was administered. The distribution of patient-related variables among the cohort split by cytomegalovirus (CMV) infection/disease is summarized in **Table 1**.

## MICA and NKG2D Genotyping

Major histocompatibility complex class I chain-related molecule A genotypes were utilized to determine the MICA-129 Met/Val and the rs2596538 A/G SNP and the donor-recipient MICA mismatches. Allele frequencies of MICA were calculated by direct gene counting.

Genotyping of MICA-129 Met/Val polymorphism (rs1051792) was determined by a modified nested PCR method followed by *RsaI* restriction enzyme (New England Biolabs) digestion (44) using the following primers MICA1-F 5′-CAGGGAGGCATACCCCCTG-3′ and MICA1-R 5′-TCCGGGACCCCTGACCTG-3′ for the first PCR, and MICA2-F 5′-GGGTCTGTGAGATCCATGA-3′ and MICA2-R 5′-TGAGCTCTGGAGGA

**TABLE 1** | Demographic and transplant-related characteristics at baseline.

| | A | B | C | |
|---|---|---|---|---|
| | **Total** | **No CMV infection** | **CMV infection** | ***P* value BvsC** |
| Donor | *N* = 181 | *N* = 161 | *N* = 20 | |
| Gender (men/women) | 73/108 | 66/95 | 7/13 | 0.6† |
| Age (y) ± SD | 51.3 ± 10.1 | 41.5 ± 15.4 | 45.1 ± 18.5 | 0.3§ |
| Recipient | *N* = 181 | *N* = 161 | *N* = 20 | |
| Gender (men/women) | 107/74 | 97/64 | 10/10 | 0.4† |
| Age (y) ± SD | 41.9 ± 15.8 | 51.0 ± 9.8 | 53.7 ± 12.2 | 0.3§ |
| **Cause of end-stage renal disease** | | | | |
| Diabetes mellitus | 9 | 8 | 1 | 1§ |
| Chronic glomerulonephritis | 76 | 70 | 6 | 0.3§ |
| Polycystic kidney disease | 25 | 23 | 2 | 1§ |
| Others or unknown | 71 | 60 | 11 | 0.1§ |
| **KTx-related parameters** | | | | |
| AB0 incompatible yes/no | 19/162 | 16/145 | 3/17 | 0.5† |
| HLA-A/B mismatches mean ± SD | 3.0 ± 1.5 | 1.9 ± 1.2 | 2.2 ± 0.8 | 0.3§ |
| HLA-DR mismatch mean ± SD | 1.1 ± 0.6 | 1.1 ± 0.7 | 1.1 ± 0.6 | 0.8§ |
| Cold ischemia time mean ± SD; minutes | 132.2 ± 44.1 | 132.5 ± 41.9 | 129.5 ± 60.5 | 0.7§ |
| Acute cellular rejection yes/no | 41/140 | 35/126 | 6/14 | 0.4† |
| **Baseline CMV characteristics** | | | | |
| CMV positive recipient (R+) | 92 | 86 | 6 | 0.048† |
| CMV positive donor (D+) | 104 | 86 | 18 | 0.002† |
| CMV high risk situation (yes/no) | 41/140 | 27/134 | 14/6 | <0.0001† |

†*Fisher's exact test.*

§*Wilcoxon rank-sum test (Mann–Whitney U Test).*

*y, years; HLA, human leukocyte antigen; CMV, cytomegalovirus; KTx, kidney transplantation.*

CTGGGGTA-3′ for the second PCR. The first PCR yields an 864 base pairs (bp) fragment, which was used as a template for the second PCR reaction resulting in a final fragment of 127 bp. A 2.5% (w/v) agarose gel electrophoresis was used to visualize digestion patterns and to determine MICA 454G/A genotypes: 454GG = Val/Val (106 and 21 bp), 454GA = Val/Met (127, 106, and 21 bp), and 454AA = Met/Met (127 bp). For reasons of clarity and to follow the published nomenclature, the alleles were designated here as MICA-129 Met (454A) and MICA-129 Val (454G).

Genotyping of the rs2596538 A/G SNP in the gene promoter region was performed by PCR-RFLP method using the following PCR primers (31): MICA538F 5′-GTGAGTGCATGG GGTATAAGGC-3′ and MICA538R 5′-GTGCCAGCTCCAGCA AAGGAT-3′. The resulting PCR product size is 339 bp. All PCR amplifications were checked in 1% (w/v) agarose gel and submitted to *AluI* (New England Biolabs) restriction enzyme digestion according to manufacturer's instructions. The amplified sequence has four recognition sites for *AluI*, including the polymorphic site. Thus, the presence of the G allele disrupts the recognition site of one site and results in segment of 170 bp. A 3.5% (w/v) agarose gel electrophoresis was used to visualize digestion patterns and to determine MICA rs2596538 A/G genotypes: AA (139, 92, 78, 23, and 7 bp), AG (170, 139, 92, 78, 23, and 7 bp), and GG (170, 139, 23, and 7 bp).

The *NKG2D* rs1049174 G/C SNP was performed by PCR-RFLP as previously described by Asadi-Saghandi et al. with the following forward and reverse primers: 5′-TTAAGGCTGGAGAA TAATGC-3′ and 5′-TCAGTGAAGGAAGAGAAGG-3′ (45).

## Statistics

Statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) or BIAS 11.01 (http://www.bias-online.de/). Baseline characteristics of donors and recipients were compared with two-sided Fisher's exact or Wilcoxon rank-sum test, as indicated in the table legend. The contribution of allelic variants as risk factors of CMV was evaluated by Fisher's exact test. Joint genotype analysis was performed using a Mantel–Haenszel test. The analysis of the time to the first CMV event was assessed by the method of Kaplan–Meier and compared using log-rank test. Bonferroni–Holm correction was applied were appropriate to account for multiple hypothesis testing. Multivariate Cox proportional hazards' modeling was used to assess the risk of CMV infection after transplantation. Risk factors for CMV were screened with unadjusted Cox models. Variables with a *p*-value lower than 0.10 in univariate analysis were included in the multivariate Cox-regression model. Two-sided *p*-values of 0.05 or lower were considered statistically significant.

# RESULTS

## No Significant Difference of MICA-129 Met/Val, MICA rs2596538 G/A, and NKG2D rs1049174 G/C Allelic and Genotype Distributions Between Living-Kidney Recipients and Donors

No significant statistical differences in the overall distribution of the MICA-129 Met/Val, the MICA rs2596538 G/A, and the rs1049174 G/C allele and genotype frequencies were observed between recipients and their corresponding donors in our cohort (**Figure 1**; Figure S1 and Table S1 in Supplementary Material).

With 34.5% (125 out of 362) for the MICA-129 Met allele and 65.5% (237 out of 362) for the MICA-129 Val, the recipient MICA-129 Met/Val allelic frequencies were very similar to the corresponding donor frequencies of MICA-129 Met allele (30.1%; 109 out of 362), and of MICA-129 Val allele (69.9%; 253 out of 362).

The recipient MICA rs2596538 G/A gene promoter allelic frequencies were 67.1% (243 out of 362) for the MICA rs2596538 G allele and 32.9% (119 out of 362) for the MICA rs2596538 A allele and also did not differ from the donor frequencies [66.0% (239 out of 362) MICA rs2596538 G allele, 34.0% (123 out of

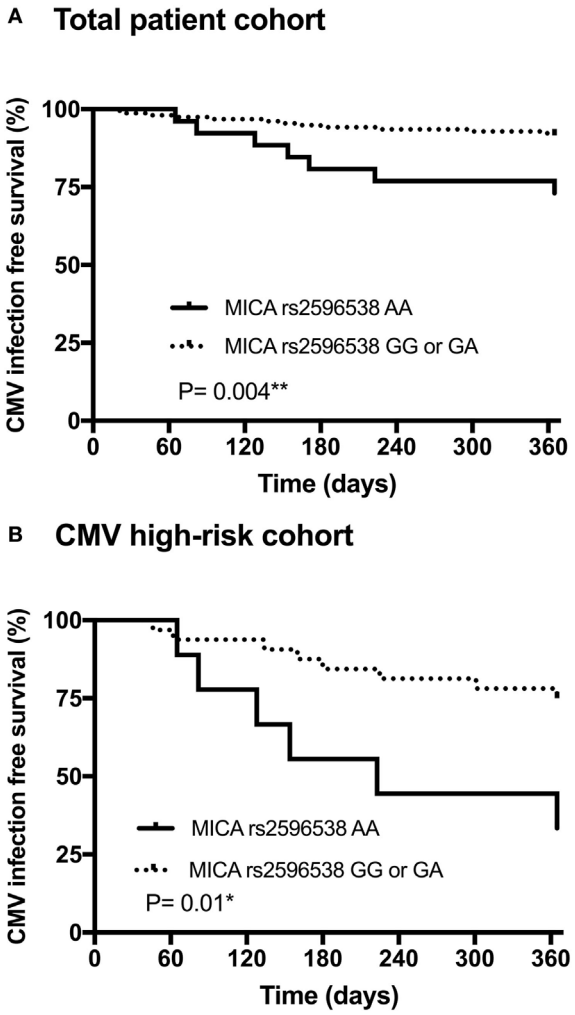


**FIGURE 1** | Association between the donor major histocompatibility complex class I chain-related molecule A (MICA) rs2596538 G/A allelic variants, and occurrence of cytomegalovirus (CMV) infection/disease within 12 months after living-donor kidney transplantation. Patients with a donor MICA rs2596538 G allele carrier status had a significantly decreased likelihood of CMV replication in the overall cohort **(A)** and in the high-risk CMV cohort **(B)**. The donor MICA rs2596538 G allele carrier status was tested for association with CMV infection or disease using Kaplan–Meier curves and log-rank testing.

362) MICA rs2596538 A allele]. Moreover, the recipient NKG2D rs1049174 G/C allelic frequency was with 65.5% (237 out of 362) for the NKG2D rs1049174 G allele and 34.5% (119 out of 362) for NKG2D rs1049174 C allele being again similar to the corresponding donor frequencies with 69.0% (250 out of 362) for the NKG2D rs1049174 G allele and NKG2D rs1049174 C allele 31.0% (112 out of 362).

Furthermore, with respect to patient CMV risk status groups, no significant differences were found in the MICA-129 Met/Val, MICA rs2596538 G/A, or NKG2D rs1049174 G/C allelic frequencies between recipients or donors (see Table S2 in Supplementary Material).

## Donor-Specific Association of CMV Infection/Disease, MICA rs2596538 G Carrier Status, and MICA rs2596538 GG Genotype in KTxs

In total, 11% (20 of 181) of the recipients exhibited at least one episode of CMV infection or disease requiring antiviral treatment during the first year after KTx.

The MICA dimorphism in the coding region (MICA-129 Met/Val SNP) in the genotype and allele frequencies of recipients as well as of donors were not statistically different among patients suffering from CMV infection as compared to patients without CMV detection during the first year after transplantation (**Tables 2** and **3**). Thus, the MICA-129 Met/Val status which is described to be associated with differential binding affinities of MICA to the NKG2D receptor (26) does not seem to play an apparent role in the development of CMV infection in the first year after living-KTx in our collective.

Although no association of MICA rs2596538 G/A gene promoter polymorphism and CMV infection/disease was observed in the recipients, the donors exhibited a significant difference in the MICA rs2596538 G/A allele distribution: the MICA rs2596538 G allele was significantly less frequent in case of CMV infection/disease (69 vs. 42.5%; $p = 0.0013$; odds ratio OR = 0.3; 95% CI 0.2–0.7; **Table 3**). Additionally, a joint analysis of the three MICA rs2596538 G/A genotypes was performed using the Mantel–Haenszel test confirming the MICA rs2596538 genotype frequencies to differ with regards to CMV infection/disease [$p = 0.006$; $Chi^2 = 10.18$; degrees of freedom (df) = 2]. Multiple comparisons of genotypes showed that MICA rs2596538 GG genotype is significantly less frequent in case of CMV infection/disease compared to the MICA rs2596538 AA genotype ($p = 0.001$ and after Bonferroni–Holm correction $^*p = 0.003$). Thus, the MICA rs2596538 G/A gene promoter status of the donor being a regulatory element for the MICA expression in the allograft appears to be associated with CMV infection/disease after living-KTx.

Taking the longitudinal course into consideration, the results of Kaplan–Meier curve analysis combined with those of the log-rank test (**Figure 1A**) indicated that during the first year after KTx the probability of CMV infection/disease was significantly higher among patients receiving a donor kidney graft negative for MICA rs2596538 G allele variant (i.e., AA homozygous), compared to

**TABLE 2** | Recipient genotype and allele frequencies association with cytomegalovirus (CMV) infection.

| | No CMV infection | CMV infection | *P* | Odds ratio (95%) |
|---|---|---|---|---|
| **Genotype MICA-129 methionine (Met)/valine (Val)** | | | | |
| Met/Met | 23 | 3 | 1.0 | 0.9 (0.3–3.2) |
| Met/Val | 63 | 10 | 0.47 | 0.6 (0.2–1.7) |
| Val/Val | 75 | 7 | 0.35 | 1.6 (0.6–4.1) |
| **Allele MICA-129 Met/Val** | | | | |
| Met | 109 | 16 | 0.48 | 0.8 (0.4–1.5) |
| Val | 213 | 24 | | |
| **Genotype MICA rs2596538 G/A** | | | | |
| GG | 78 | 8 | 0.6 | 0.7 (0.2–1.8) |
| GA | 64 | 7 | 0.8 | 1.2 (0.5–3.1) |
| AA | 19 | 5 | 0.15 | 0.4 (0.1–1.1) |
| **Allele MICA rs2596538 G/A** | | | | |
| G | 220 | 23 | 0.2 | 0.6 (0.3–1.3) |
| A | 102 | 17 | | |
| **Genotype natural killer (NK) group 2 member D (NKG2D) rs1049174 G/C** | | | | |
| CC | 22 | 2 | 1 | 0.7 (0.2–3.1) |
| CG | 66 | 11 | 0.2 | 0.6 (0.2–1.4) |
| GG | 73 | 7 | 0.3 | 1.8 (0.6–4.9) |
| **Allele NKG2D rs1049174 G/C** | | | | |
| C | 110 | 15 | 0.6 | 0.7 (0.4–1.6) |
| G | 212 | 25 | | |

*The genotype and allele frequencies of the MICA-129 Met/Val (rs1051792), the MICA rs2596538 G/A, and the NKG2D rs1049174 G/C polymorphisms in kidney transplant recipients.*

**TABLE 3** | Donor genotype and allele frequencies association with cytomegalovirus (CMV) infection.

| | No CMV infection | CMV infection | *P* | Odds ratio (95%) |
|---|---|---|---|---|
| **Genotype major histocompatibility complex class I chain-related molecule A (MICA)-129 methionine/valine (Met/Val)** | | | | |
| Met/Met | 22 | 5 | 0.19 | 0.5 (0.2–1.3) |
| Met/Val | 48 | 7 | 0.62 | 0.8 (0.3–2.0) |
| Val/Val | 91 | 8 | 0.23 | 1.9 (0.8–4.9) |
| **Allele MICA-129 Met/Val** | | | | |
| Met | 92 | 17 | 0.09 | 0.5 (0.3–1.1) |
| Val | 230 | 23 | | |
| **Genotype MICA rs2596538 G/A** | | | | |
| GG | 80 | 4 | 0.016* | 0.25 (0.1–0.7) |
| GA | 62 | 9 | 0.6 | 0.8 (0.3–1.8) |
| AA | 19 | 7 | 0.012* | 4.0 (1.5–11.7) |
| **Allele MICA rs2596538 G/A** | | | | |
| G | 222 | 17 | 0.0013** | 0.3 (0.2–0.7) |
| A | 100 | 23 | | |
| **Genotype natural killer (NK) group 2 member D (NKG2D) rs1049174 G/C** | | | | |
| CC | 15 | 2 | 1 | 0.9 (0.2–4.3) |
| CG | 73 | 5 | 0.09 | 2.5 (0.9–6.4) |
| GG | 73 | 13 | 0.15 | 2.3 (0.9–5.7) |
| **Allele NKG2D rs1049174 G/C** | | | | |
| C | 103 | 9 | 0.27 | 0.6 (0.3–1.3) |
| G | 219 | 31 | | |

*The genotype and allele frequencies of the MICA-129 Met/Val (rs1051792), the MICA rs2596538 G/A, and the NKG2D rs1049174 G/C polymorphisms in kidney transplant donors. *P < 0.05; **P < 0.01.*

allografts positive for G allele variant ($p = 0.004$; OR = 0.3; 95% CI 0.08–1.0). Similar results were obtained when patients with a high-risk of CMV were analyzed separately ($p = 0.01$; OR = 0.16; 95% CI 0.04–0.7; **Figure 1B**). Kaplan–Meier curve analysis of the three MICA rs2596538 G/A genotypes confirmed the MICA rs2596538 GG donor genotype to be associated with a lower probability of CMV infection/disease compared to the MICA rs2596538 AA genotype ($p = 0.0007$; $^*p = 0.002$; RH = 0.15; 95% CI 0.05–0.5; Figure S2 in Supplementary Material).

Taken together, the results suggest that the donor MICA rs2596538 G allele variant as well as MICA rs2596538 GG genotype, known to be associated with higher expression, may be protective against CMV infection/disease after KTx.

## No Significant Association of CMV Infection/Disease and NKG2D rs1049174 G/C Polymorphism in KTx

To elucidate whether rs1049174 G/C polymorphism in the 3′-UTR region of NKG2D is involved in the susceptibility to CMV infection, the genotype, and allele distribution of donors and recipients was determined. The NKG2D rs1049174 G/C status of recipients as well as of donors was not different in patients with or without CMV infection/disease in the first year after transplantation (**Tables 2** and **3**).

Of note, focusing exclusively on recipients who were NKG2D rs1049174 C positive (exhibiting higher NKG2D-mediated effector cell cytotoxicity) and additionally received an allograft positive for the MICA-129 Met allelic variant (exhibiting increased receptor binding affinity), chi-square test revealed a significant hazard from CMV infection/disease ($p = 0.023$; OR = 4.4; 95% CI 1.1–15.5). On the contrary, the patients with a recipient NKG2D rs1049174 C allelic variant in combination with a donor MICA rs2596538 G allelic variant (exhibiting higher MICA expression) were protected from CMV ($p = 0.003$; OR = 0.16; 95% CI 0.04–0.6), suggesting an additional MICA allele-specific role of the NKG2D receptor expression in CMV infection after KTx.

Univariate analysis determined that CMV high-risk status, use of lymphocyte-depleting induction therapy and donor MICA rs2596538 G allele carrier status were prognostic factors for CMV infection or disease (**Table 4**). Concordantly, a multivariate Cox-regression analysis was performed with the categorical covariates CMV high-risk status, use of lymphocyte-depleting induction therapy of the patient, the donor's MICA rs2596538 G allele carrier status as well as the recipient NKG2D rs1049174 C allele carrier status. In addition to the well-known CMV high-risk sero-status or the application of lymphocyte-depleting induction therapies ($p < 0.0001$; hazard ratio HR = 8.73; 95% CI 3.2–24.0), the MICA rs2596538 G allele carrier status ($p = 0.009$; HR = 0.3; 95% CI 0.1–0.7) was confirmed as a novel significant-independent prognostic factor for CMV infection/disease during the first year after KTx.

# DISCUSSION

To our knowledge, this is the first study demonstrating that (i) the donor MICA rs2596538 G carrier status, which is known to be associated with a higher transcription, represents an independent genetic prognostic factor for protection against CMV infection/disease in the first year after transplantation and (ii) that the other herein assessed functional genetic markers affecting the MICA/NKG2D axis by either differential binding affinity to the NKG2D receptor or receptor expression itself, appear to be less relevant regarding the control of productive CMV infection in the first year after KTx. Thus, the combined results of this study point out

**TABLE 4** | Univariate analysis and multivariate Cox-regression analysis for prediction factors of cytomegalovirus (CMV) infection within 12 months after living kidney transplantation.

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| **Risk factor** | ***P*-value** | **Hazard ratio (HR) [95% confidence interval (CI)]** | ***P*-value** | **HR (95%CI)** |
| Recipient gender | 0.38 | 0.67 (0.28–1.61) | | |
| Donor gender | 0.61 | 0.8 (0.31–1.97) | | |
| Recipient age | 0.34 | 1.0 (0.99–1.04) | | |
| Donor age | 0.27 | 1.0 (0.98–1.0) | | |
| Cold ischemia time | 0.77 | 0.99 (0.99–1.0) | | |
| Acute cellular rejection within 12 months after transplantation | 0.39 | 1.5 (0.6–3.93) | | |
| Highest historical panel reactive antibody | 0.41 | 0.91 (0.72–1.14) | | |
| Human leukocyte antigen (HLA)-DR MM | 0.82 | 1.1 (0.55–2.1) | | |
| HLA-A/B MM | 0.29 | 1.22 (0.84–1.79) | | |
| CMV high risk status and/or lymphocyte-depleting induction therapy | <0.0001*** | 8.73 (3.17–24.03) | <0.0001*** | 8.72 (3.2–24.0) |
| Recipient major histocompatibility complex class I chain-related molecule A (MICA)-129 Met pos | 0.35 | 0.65 (0.26–1.62) | | |
| Donor MICA-129 Met pos | 0.16 | 0.53 (0.21–1.29) | | |
| Recipient MICA rs296538 G pos | 0.11 | 2.3 (0.84–6.33) | | |
| Donor MICA rs296538 G pos | 0.007** | 0.29 (0.1–0.7) | 0.009** | 0.3 (0.1–0.7) |
| Recipient NKG2D rs1049174 C pos | 0.39 | 1.4 (0.6–3.7) | 0.54 | 1.3 (0.5–3.4) |
| Donor NKG2D rs1049174 C pos | 0.096 | 2.18 (0.87–5.5) | | |

*CI, confidence interval; HR, hazard ratio; PRA, panel reactive antibody, HLA, human leukocyte antigen; MM, mismatch; CMV; cytomegalovirus.*
***P < 0.01; ***P < 0.001.*

that the potential transcriptional activity of MICA in the donor allograft is of predominant importance in CMV immune control in this allogeneic situation.

In the course of solid organ transplantation, the ubiquitous β-herpesvirus CMV is frequently transmitted *via* the donor allograft (46), endangering especially previously CMV-naïve transplant recipients lacking CMV-specific immunity. Despite the availability of antiviral therapies, CMV remains a significant cause of life-threatening diseases in immunocompromised hosts (47–50). CMV encodes an enormous arsenal of immune evasion mechanisms in order to avoid elimination by the host immune system. Several of them inhibit the MHC class I antigen presentation pathway (18). Decreased MHC/HLA antigen presentation by virus-infected cells provides protection from T cell recognition (18), but renders the CMV-infected cells more prone to NK cell-mediated lysis, owing to missing self-recognition of MHC class I-specific inhibitory NK cell receptors (51). Consequently, NK cells play a pivotal role in CMV infection control with the MICA/NKG2D axis representing a very important functional mediator (5, 52).

Major histocompatibility complex class I chain-related molecule A expression is induced by several stress factors including viral infections or pro-inflammatory cytokines (5, 53). The interplay between CMV and the MICA/NKG2D pathway has been explored in *in vitro* studies. Their combined results demonstrate that viral infections efficiently induce MICA transcription in infected cells, and thereby mediate activating NKG2D signaling (5, 6). Of particular interest in the immediate early phases of CMV infection is the direct regulation of MICA by the viral transcriptional regulators (54). The enhanced MICA transcription is deployed by the host to induce an antiviral immune response potentially leading to elimination of virus-infected cells.

Conversely, numerous viral immune evasion strategies target disruption of MICA cell surface expression, and thereby limiting immune control mediated by the MICA/NKG2D axis (13–17). Because NKG2D is expressed both on NK, CD8+ and certain subsets of CD4+ T cells, targeting its ligands is particularly beneficial for CMV, since both the innate and the adaptive immune responses are impaired. In line with this hypothesis, a multitude of studies has demonstrated that CMV-specific T cells play a crucial role in the control of viral replication in the transplant setting (55, 56). Especially, CMV-specific CD8+ T cells have been associated with protection from CMV in immune-compromised hosts (57, 58). In addition, it has been shown that expansion of NKG2D expressing cytotoxic CD4+ T cells lacking co-stimulatory CD28 (CD4 + CD28 null cells) is associated to latent CMV infection and that this CD4+ T cells can induced endothelial injury—a process being mitigated by NKG2D blocking (59). Our data provide compelling evidence that these viral evasion mechanisms do not completely protect human CMV from MICA/NKG2D-mediated immune control, at least in the presence of certain MICA alleles.

In the past few decades, various studies have shown that the MICA/NKG2D axis may have relevance to the KTx outcome (26–28, 60). However, the clinical impact of these interactions is still unclear and conflicting. It is well established that NKG2D expression is mainly modulated by ligand-dependent and -independent signals (61–63) thus, making NKG2D expression dynamic in many clinical settings, including transplantation (2, 10, 11, 64–69). NKG2D expressing immune cells (i.e., NK cells, γδ T cells, CD8+ αβ T cells and subsets of CD4+ T cells) can be detected in the circulation, but are also all known to migrate to the renal allograft under the influence of homing markers like e.g. CXCR4 and SDF1 (70–73). Stress conditions following transplantation cause a general inflammatory status in recipients, which could increase MICA production. In kidney allografts, an enhanced MICA expression has been reported on epithelial and endothelial cells in response to ischemia-reperfusion injury, acute rejection, or viral infection (27, 37). Thus, the presence of MICA in the donor organ could elicit NK and T cell activating responses *via* the NKG2D receptor. Accumulation of CD56+ NK cells and CD8+ T cells can be observed in kidney allograft biopsies upon acute rejection (74, 75). Seiler et al. described the presence of CD8+ NKG2D+ cells in tubulointerstitial areas in kidney biopsies with acute cellular rejection (76) reported and elevated NKG2D mRNA expression to be associated with poorer graft survival (76). It would be of interest to analyze MICA and NKG2D expression levels as well as the distribution of infiltrating cells in kidney transplant biopsy specimens in our cohort. Whereas the analysis of circulating immune effector cells in our opinion would not mirror the situation properly for following reasons: (i) the observed association of CMV infection/disease with the functional MICA gene promotor polymorphism is donor and not recipient specific and (ii) investigation of circulating NKG2D expressing immune effector cells outside the donor tissue and its corresponding microenvironment during CMV infection/disease will not locally mirror the phenotype of infiltrating effectors and their mode of action. Due to ethical reasons and our center-specific guidelines rule out transplant biopsies in absence of allograft dysfunction, which was not the instance in the recipients with CMV infection/disease.

It is reasonable to hypothesize that polymorphisms of MICA and NKG2D in recipients and donors shape the MICA/NKG2D axis, and may have functional implication, since allele-dependent variations in MICA expression or NKG2D receptor avidity may affect antiviral NK and T cell immune responses (27).

Previous studies conducted on non-transplant cohorts have identified a relationship between MICA polymorphisms and outcomes of infections, such as human immunodeficiency virus (HIV), hepatitis B and C viruses, and CMV (21, 26, 31, 77–79). In this study, we provided compelling evidence that the donor MICA rs2596538 G allele ensures protection from CMV infection after KTx. In line with our data, the MICA rs2596538 G allele variant was also identified as protective factor for hepatocellular carcinoma (HCC) in HCV-infected patients (31). The MICA rs2596538 G is located in the 5′-flanking region of the MICA, and proved to affect the binding affinity of the transcription factor SP1 which in turn, is a strong regulator of MICA expression (31, 80). CMV, HCV as well as other viruses have been shown to utilize SP1 to obtain efficient early viral gene expression in specific cell types (81–83). An overexpression of SP1 remarkably induced MICA expression in cells carrying the MICA rs2596538 G allele, exhibiting higher affinity to the SP1 binding (31). The MICA rs2596538 G/A SNP is in strong linkage disequilibrium with an additional MICA SNP rs2596542 C/T located in the MICA promoter region, affecting serum levels of soluble MICA

and being likewise associated with HCC development prediction in HCV infection (31, 77, 78). Due to the linkage disequilibrium between the MICA rs2596538 G/A and rs2596542 C/T SNP (31) described for a Japanese population, a combined effect of both SNPs might conjointly influence MICA transcription levels. The MICA rs2596538 G/A and the rs2596542 C/T SNP have been described to impact on soluble MICA levels. Besides those two SNPs, further MICA SNPs and certain alleles have been associated with high or low soluble MICA status. A combined analysis of these different MICA SNPs and alleles would be of interest, but has to our knowledge not been performed so far. In terms of function, the collectivity of results of these studies indicate that enhanced MICA expression in CMV- and HCV-infected individuals may elicit stronger immune responses and thus lead to an elimination of virus-infected cells by NK and CD8+ T cells.

Regarding the influence of MICA expression on viral infection, it could be demonstrated that a triplet repeat microsatellite polymorphism (GCT) in the transmembrane region (exon 5) of the MICA gene (MICA-A5.1) by negatively affecting MICA cell surface stability, and thus expression levels has been associated with disease outcome in immune compromised host. The presence of the MICA-A5.1 allele in HIV1-infected patients was identified as a risk factor for recurrence of CMV (21).

Not only MICA expression levels, but also receptor binding avidity might impact on the effector cell potential against infected cells. In this context, the MICA-129 Met/Val SNP is of specific interest (79, 84). However, no association was observed between the MICA-129 Met/Val dimorphism and CMV infection in our transplant cohort during the first year after KTx. This could partly be explained by experimental *in vitro* data indicating that expression intensity can change the biological effect of the MICA-129 Met/Val SNP: MICA-129 Met allelic variants elicit strong NKG2D responses at low expression intensities, but, however, stimulate at higher expression intensities a downregulation of NKG2D, leading to impaired function, whereas MICA-129 Val variant elicits more NKG2D effects at high expression (30).

With respect to the MICA/NKG2D axis, the NKG2D receptor has to be additionally considered for the functional outcome of NK and T cells effectors. Immunosuppression in the transplant setting as well as CMV infection induce an adaptive reconfiguration of the NK cell repertoire, although the expression modulation of NKG2D seemed to be less affected (85–87). Thus, the genetic variation of NKG2D would influence the effector cell phenotype dominantly. NKG2D rs1049174 G/C SNP located within the binding site of the negatively regulating miR-1245 allows the stratification of effector cells in high and low cytotoxic responders (33, 88). Of importance, as shown for human papillomavirus (33) the expression of miR-1245 can be upregulated under the influence of viruses, making NKG2D rs1049174 G/C SNP of pivotal interest in viral infection surveillance.

The important finding of our study is the identification of donor MICA rs2596538 G allelic variant and the MICA rs2596538 GG genotype as independent genetic protective prognostic factors for CMV infection/disease. Thus, our data provided strong evidence that the MICA transcription encoded by the MICA rs2596538 G/A SNP is dominant to the polymorphisms impacting the MICA binding affinity to the cognate NKG2D receptor or the receptor expression levels *in vivo* for susceptibility to CMV infection/disease in kidney transplanted patients. Moreover, our data support the functional importance of the MICA/NKG2D axis in CMV immune control. However, due to its retrospective nature and the low number of CMV events observed in this single-center analysis; the findings should be independently confirmed by future prospective studies. Furthermore, because of the low event rates, recipients with CMV infection or disease were combined and analyzed together. Therefore, it cannot be discriminated if the MICA rs2596538 G/A SNP is differentially associated with protection against subclinical CMV infection and CMV disease. This point remains to be evaluated in larger patient cohorts. Considering the clinical negative impact of CMV infection on allograft outcome after KTx and in the absence of an effective and preemptive CMV vaccine, identification of additional predictive markers for detection of CMV prone transplant recipients benefiting from alternative monitoring strategies or alternative therapeutic approach is urgently needed. In this context, donor MICA rs2596538 G allelic variant represents a useful genetic marker helping physicians to identify individuals within the CMV high-risk transplant population.

## CONCLUSION

Taken together, our findings contribute to improve the understanding of the mechanisms underlying the regulation of MICA/NKG2D axis interaction in CMV infection/disease in the context of KTx. In addition, the donor MICA rs2596538 G allelic variant is a prospective protective prediction marker for CMV infection after transplantation potentially allowing the future development of individually tailored CMV therapy approaches for this genetically predisposed patient cohort.

## ETHICS STATEMENT

The protocol was approved by the University Hospital Essen ethics committee (12-5312-BO). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

## AUTHOR CONTRIBUTIONS

HR, PH, AK, OW, VR: conceived and designed research. HR, RM, ES: performed the experiments. FH: contributed reagents. SD, AG, BW, JK: collected and provided clinical data. HR, RM, SD, MT, VL-T, VR: interpreted data and HR, RM, AG, BW, JK, VR: performed statistical analysis. HR, MT, VL-T, VR: wrote the initial draft. HR, RM, ES, SD, AG, MT, VL-T, BW, JK, FH, PH, AK, OW, VR: read and approved the final article.

## ACKNOWLEDGMENTS

We especially thank the patients participating in this study and the kidney transplant unit team. We are grateful to Sabine Schramm and Monika Collenburg as well as the contributing members of the diagnostic section of the Institute for Virology for their excellent technical support. Statistical assistance was provided by Jean-Marie Bois from the Institute for Medical Informatics, Biometry, and Epidemiology; University Duisburg-Essen. HR was supported by the "Deanery

of the Faculty of Medicine, University Duisburg-Essen;" by the "IFORES Research fellowship program of the University Duisburg-Essen Medical School (grant D/107-40620)". RM was supported by the "CAPES Foundation, Ministry of Education of Brazil, Brasília – DF Brazil (99999.000124/2016-08)," CNPq (142475/2015-7), and DAAD. We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00917/full#supplementary-material.

**FIGURE S1** | Distribution of major histocompatibility complex class I chain-related molecule A (MICA)-129 methionine/valine, MICA rs2596538 G/A and rs1049174 G/C genotypes between recipients and corresponding donors.

**FIGURE S2** | Association between donor major histocompatibility complex class I chain-related molecule A (MICA) rs2596538 G/A genotypes and occurrence of cytomegalovirus (CMV) infection/disease within 12 months after living-donor kidney transplantation. Method of Kaplan–Meier was applied in order to estimate the probability of CMV infection/disease. The *p*-value over all three genotypes was 0.005 (see Figure S2 in Supplementary Material). Multiple comparison of genotypes revealed that the homozygous genotypes, i.e., GG vs AA, were significantly different with a *p*-value = 0.0007; and after Bonferroni–Holm correction *p = 0.002; relative hazard (RH) = 0.15; 95% CI 0.05–0.5.

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**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: 09 May 2018
doi: 10.3389/fimmu.2018.01009

# IL-15 Overcomes Hepatocellular Carcinoma-Induced NK Cell Dysfunction

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NK cells have potent antitumor capacity. They are enriched in the human liver, with a large subset specialized for tissue-residence. The potential for liver-resident versus liver-infiltrating NK cells to populate, and exert antitumor functions in, human liver tumors has not been studied. We examined liver-resident and liver-infiltrating NK cells directly *ex vivo* from human hepatocellular carcinomas (HCCs) and liver colorectal (CRC) metastases, compared with matched uninvolved liver tissue. We found that NK cells were highly prevalent in both HCC and liver CRC metastases, although at lower frequencies than unaffected liver. Up to 79% of intratumoral NK cells had the $CXCR6^{+}CD69^{+}$ liver-resident phenotype. Direct *ex vivo* staining showed that liver-resident NK cells had increased NKG2D expression compared to their non-resident counterparts, but both subsets had NKG2D downregulation within liver tumors compared to uninvolved liver. Proliferation of intratumoral NK cells (identified by Ki67) was selectively impaired in those with the most marked NKG2D downregulation. Human liver tumor NK cells were functionally impaired, with reduced capacity for cytotoxicity and production of cytokines, even when compared to the hypo-functional tissue-resident NK cells in unaffected liver. Coculture of human liver NK cells with the human hepatoma cell line PLC/PRF/5, or with autologous HCC, recapitulated the defects observed in NK cells extracted from tumors, with downmodulation of NKG2D, cytokine production, and target cell cytotoxicity. Transwells and conditioned media confirmed a requirement for cell contact with PLC/PRF/5 to impose NK cell inhibition. IL-15 was able to recover antitumor functionality in NK cells inhibited by *in vitro* exposure to HCC cell lines or extracted directly from HCC. In summary, our data suggest that the impaired antitumor function of local NK cells reflects a combination of the tolerogenic features inherent to liver-resident NK cells together with additional contact-dependent inhibition imposed by HCC itself. The demonstration that IL-15 can recover hepatic NK cell function following tumor exposure supports its inclusion in immunotherapy strategies.

**Keywords: NK cells, tissue-resident NK cells, hepatocellular carcinoma, liver tumors, NKG2D, tumor escape, immunotherapy, IL-15**

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***Edited by:***
*Nadia Guerra, Imperial College London, United Kingdom*

***Reviewed by:***
*Francisco Borrego, BioCruces Health Research Institute, Spain*
*Michael R. Verneris, University of Colorado Denver, United States*

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

***Received:*** *05 February 2018*
***Accepted:*** *23 April 2018*
***Published:*** *09 May 2018*

***Citation:***
*Easom NJW, Stegmann KA, Swadling L, Pallett LJ, Burton AR, Odera D, Schmidt N, Huang W-C, Fusai G, Davidson B and Maini MK (2018) IL-15 Overcomes Hepatocellular Carcinoma-Induced NK Cell Dysfunction. Front. Immunol. 9:1009. doi: 10.3389/fimmu.2018.01009*

## INTRODUCTION

Hepatocellular carcinoma (HCC) remains a very difficult to treat tumor with an exceptionally poor prognosis. It is the second leading cause of cancer deaths worldwide and the fastest increasing cause of cancer-related mortality in Europe (1). The liver is also a common site for metastases from colorectal cancer (CRC); liver metastases carry a very poor prognosis, being typically resistant

to chemotherapy and even to early immunotherapy trials with checkpoint inhibitors (2). New approaches able to harness local antitumor immunity are therefore urgently needed for both primary and secondary liver tumors. These require an understanding of the constraints on protective immunity inherent to the liver environment that might be co-opted by tumors in this organ.

Most research into tumor immunology, including in the setting of the liver, has focused on understanding, and attempting to reverse, defects in CD8 T cells (3). However, NK cells also have potent capacity to recognize and kill tumor cells (4, 5). This is evidenced by the multiple NK cell evasion mechanisms exhibited by tumors. NK cell functionality is tuned by the dynamic balance of signals it receives from its environment through a complex array of activatory and inhibitory receptors. One of the major pathways through which NK cells provide immunosurveillance of tumors is through recognition of DNA damage response-induced NKG2D-ligands (NKG2D-L) by the activatory receptor, NKG2D (6, 7). However, the NKG2D pathway plays a complex role in tumor immunity, with some evidence it can also mediate the opposing effect of downmodulating NK cell tumor immunity or even promoting HCC progression (8, 9). Some studies suggested that shedding of tumor NKG2D ligands serves as an escape mechanism by rendering the tumor invisible to NK cells through this receptor and/or by inhibitory effects of the soluble ligands (10–12). Other murine studies have demonstrated that repetitive engagement of NKG2D by its ligands on tumors (13), tumor-associated infiltrates (14), or endothelial cells within tumor vasculature (15) can drive downregulation of this receptor, with consequent NK cell desensitization.

NK cells are a major component of the immunological landscape within human liver, constituting 30–40% of all intrahepatic lymphocytes (16, 17). We and others have recently discovered that a variable fraction (up to 80%) of intrahepatic NK cells are liver-resident, characterized by expression of CXCR6 and CD69, with a distinct transcriptional and functional signature (18–20). These tissue-resident NK cells survive long term in the human liver and are unable to recirculate, while the remaining liver-infiltrating fraction have the transcriptional profile of peripheral NK cells (20, 21). Tissue-resident lymphocytes are adapted for frontline defense, being well positioned to sense and respond immediately to damaged or transformed epithelial cells (22). In the case of tissue-resident T cells, recent work shows they can infiltrate into lung tumors, correlating with a good prognosis, suggesting their propensity for efficient pathogen defense can extend to tumor protection (23, 24). Conversely, we have recently reported that liver-resident T and NK cells have some features reflective of the tolerogenic properties of the liver, such as reduced cytotoxicity (20, 25), which could facilitate tumor immune escape. It is therefore timely to examine the NK cell response to liver tumors to see whether it is influenced by tissue-resident NK cells and their specialized adaptations.

Here, we use direct *ex vivo* analysis of freshly isolated human tissue lymphocytes to compare the contribution of liver-resident and liver-infiltrating NK cells to the composition and functional features of the intratumoral pool. We probe the capacity of HCC to further impair tolerogenic liver NK cells *via* NKG2D downregulation and the potential for cytokine-mediated rescue as an immunotherapeutic strategy in this setting.

# MATERIALS AND METHODS

## Research Ethics Approval

Blood and tissue sampling was approved by the University College London-Royal Free Hospital Research Ethics Committee, ref nos. 11/WA/0077 (liver explants/resections), 11/H0720/4 (liver perfusates). Blood sampling from healthy donors was approved by the South East Coast Research Ethics Committee, ref no. 11/LO/0421.

## Patient Cohort

Study participants with HCC had the following underlying liver diseases: five hepatitis B virus monoinfection, one hepatitis B/HIV coinfection, two hepatitis C virus monoinfection, one non-alcoholic steatohepatitis, and one autoimmune hepatitis.

## PBMC Isolation and Storage

PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare). Cells were either used immediately or counted using trypan blue (Sigma) and transferred to freezing medium (FBS) (Sigma) with 10% dimethylsulfoxide (DMSO) (Sigma) for cryopreservation, initially at −80°C before transfer to liquid nitrogen for long-term storage.

## Intrahepatic Lymphocyte Isolation From Liver Tissue

Single cell suspensions from surgical resection of liver and tumor tissues were generated by enzymatic digestion and mechanical dissociation as previously described (20). In brief, tissues removed from the resected specimen immediately following liver surgery were cut into small pieces and incubated for 30 min at 37°C in HBSS buffer containing 0.0001% DNAse (Roche) and 0.01% collagenase (ThermoFisher). Samples were transferred to C-tubes (Miltenyi Biotec) and processed by gentleMACS (Miltenyi Biotec) using the liver program. Tissue and supernatants were filtered by 70-μm filter and centrifuged at 500 rpm to knockdown large hepatocyte clumps. The supernatant was centrifuged and the pellet resuspended in 30% percoll (GE Healthcare) before further centrifugation at 2,000 rpm for 10 min. The pellet was resuspended in HBSS and layered onto Ficoll-Hypaque as for peripheral blood separation. The lymphocyte layer was removed and cells counted by ADAM counter (NanoEntek) and used immediately.

## Intrahepatic Lymphocyte Isolation From Liver Perfusates

Organ transport and perfusion fluid collected at time of liver transplant was centrifuged in 50 ml falcon tubes (Sarstedt) at 1,800 rpm for 15 min and the supernatant discarded. The tubes were vortexed to disrupt the pellet and the cells pooled and resuspended in RPMI before density gradient centrifugation over Ficoll Hypaque as above.

## Flow Cytometric Staining

All samples were treated with Fc receptor blocking reagent (Miltenyi Biotec) before staining. Surface staining was performed in 96-well plates (Sarstedt) in staining buffer of 50% PBS, 50%

Brilliant Violet staining buffer (BD). Fixable live/dead stain (Life Technologies) was added to the staining buffer. Antibody staining was conducted for 15 min at 37°C in the dark before washing with PBS. Samples for surface staining only were fixed in Cytofix (BD). Samples for intracellular staining were fixed in cytofix/cytoperm (BD) for 20 min at 4°C in the dark before staining with intracellular antibodies in saponin buffer [PBS + 1% FBS (Sigma) + 0.1% saponin (Sigma)] for 30 min at 4°C in the dark. Samples were then washed once in saponin buffer and once in PBS. Intranuclear staining was performed using FoxP3 staining buffer (BD). Surface staining was as above, then cells were fixed in buffer A for 10 min at room temperature followed by buffer A with 1:50 buffer B for 30 min at room temperature. Samples were washed in PBS and intranuclear staining performed in PBS for 30 min at 4°C in the dark. Samples were acquired on Fortessa X20 (BD) and data analyzed in FlowJo X (TreeStar).

## Image Analysis and Quantitation of NKG2D Internalization

Magnetic bead isolated human liver NK cells were cocultured with PLC/PRF/5 cells as described below. Cells were surface stained with anti CD3 PE-CF594, anti CD56 PE-Cy7, permeablized and stained intracellularly with anti NKG2D APC and then DAPI nuclear stain. Events were acquired on the Imagestream$^X$ (Amnis) and data transferred to IDEAS software (Amnis) for analysis. NKG2D internalization was quantified using the built-in "Internalization Wizard" to erode an object mask by four pixels.

## NK Cell Isolation

Untouched NK cells were isolated from PBMC or intrahepatic lymphocytes using magnetic beads for negative selection, according to the manufacturer's instructions (Miltenyi Biotec), achieving >95% purity and viability.

## NK Cell—PLC/PRF/5 Cocultures

PLC/PRF/5 cells were plated at a density of 50,000 cells/well in 48-well plates (Costar) and incubated in 0.5 ml CRPMI + 8% FBS for 3 days to adhere to the bottom of the well and grow to semi-confluence. Media was changed and isolated NK cells were added at 200,000 per well, centrifuged at 300 rpm for 3 min and incubated at 37°C for 12 h. Recombinant human IL-15 was added to a final concentration of 1 ng/ml (R&D). Blocking reagents for the appropriate conditions were recombinant human NKG2D final concentration 1 μg/ml (Sino Biological), anti-MICA final concentration 1 μg/ml (R&D), anti-TGFβ receptor final concentration 2 μg/ml (R&D), 0.4 μm transwell inserts (Falcon), or 3-day PLC/PRF/5 media mixed 1:1 with fresh CRPMI + 10% FBS were used for contact experiments. Supernatants containing NK cells were removed and washed with PBS containing EDTA (Sigma) and FBS. For some experiments, removed NK cells were resuspended in fresh medium and incubated for 18 h in the presence of IL-2 20 IU/ml (Miltenyi Biotec), IL-12 12.5 pg/ml (R&D), IL-18 5 ng/ml (MBL), IL-15 1 ng/ml (R&D), IFNα 10 U/ml (PBL interferon) or medium alone.

## HCC Tissue Slices

For some experiments, *ex vivo* human HCC tissue was used instead of cell lines. HCC tissue provided by the Tissue Access for Patient Benefit service at the Royal Free Hospital was sampled using a 5 mm punch biopsy (Stiefel) to prepare cores of uniform diameter, from which 1 mm slices were cut. Thin 5 mm circles of HCC tissue were carefully placed in wells of round bottom 96-well microtiter plates and allowed to rest for 4 h in CRPMI + 8% FBS. Magnetic bead isolated NK cells were stained with Cell Trace Violet (Life Technologies) as per the manufacturer's protocol before addition to the HCC slices as above.

## CD107a Assays

NK cells were mixed with K562 cells at 1:1 effector:target ratio in 200 μl CRPMI + 10% FBS in 96 well, round bottom plates (Sarstedt). Brefeldin to a final concentration of 1 μg/ml (Sigma), anti-CD107a V450 were added and the plate centrifuged at 300 rpm for 3 min before incubation at 37°C for 3 h and staining for flow cytometry as above.

## Cytokine Stimulation

PBMC, intrahepatic, and intratumor lymphocytes were stimulated with 5 ng/ml IL-12 and 50 ng/ml IL-18 for 12 h in the presence of 1 μg/ml brefeldin, before intracellular cytokine staining as above.

## Statistical Analysis

The Mann–Whitney *U* test was used for comparisons of two unpaired groups where $n = 6$ or greater, unpaired *t* test with Welch's correction was used for comparisons of smaller groups. The Wilcoxon signed rank test was used for comparisons of two paired groups. Spearman rank test was used for correlations of continuous variables. These tests were performed in GraphPad Prism version 6. MANOVA was performed in SPSS (IBM) version 24. $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: $^*p \leq 0.05$; $^{**}p \leq 0.005$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

# RESULTS

## Liver Tumors Contain a High Proportion of NK Cells Including a Tissue-Resident Subset

In order to determine whether NK cells make up a substantial proportion of the immune infiltrate of liver tumors, we compared their direct *ex vivo* frequency with those of CD8 T cells ($CD3^+CD56^-CD8^+$), CD8 negative T cells ($CD3^+CD56^-CD8^-$), and $CD56^+$ T cells ($CD3^+CD56^+$) in blood, healthy liver margins, and HCC or CRC liver metastases. Cells were identified by multiparameter flow cytometry of single cell suspensions derived from liver and tumor tissue (gated as shown in **Figure 1A**). In both primary liver tumors (HCC) and secondary liver tumors arising from CRC metastases NK cells were prevalent, accounting for around 25% of the $CD45^+$ lymphocytes, with a frequency intermediate between blood and uninvolved liver. We term NK cells derived from liver tissue or from liver perfusion fluid "intrahepatic NK cells," and NK cells derived from tumor tissue as "tumor-infiltrating NK cells." In both settings, NK cell and CD8 T cell proportions were similar, with mean proportions of 23.7 and 19.6% for NK and CD8 T cells in HCC, and 27.2 and 21.0% for NK and CD8 T cells in CRC metastases (**Figure 1B**). In a

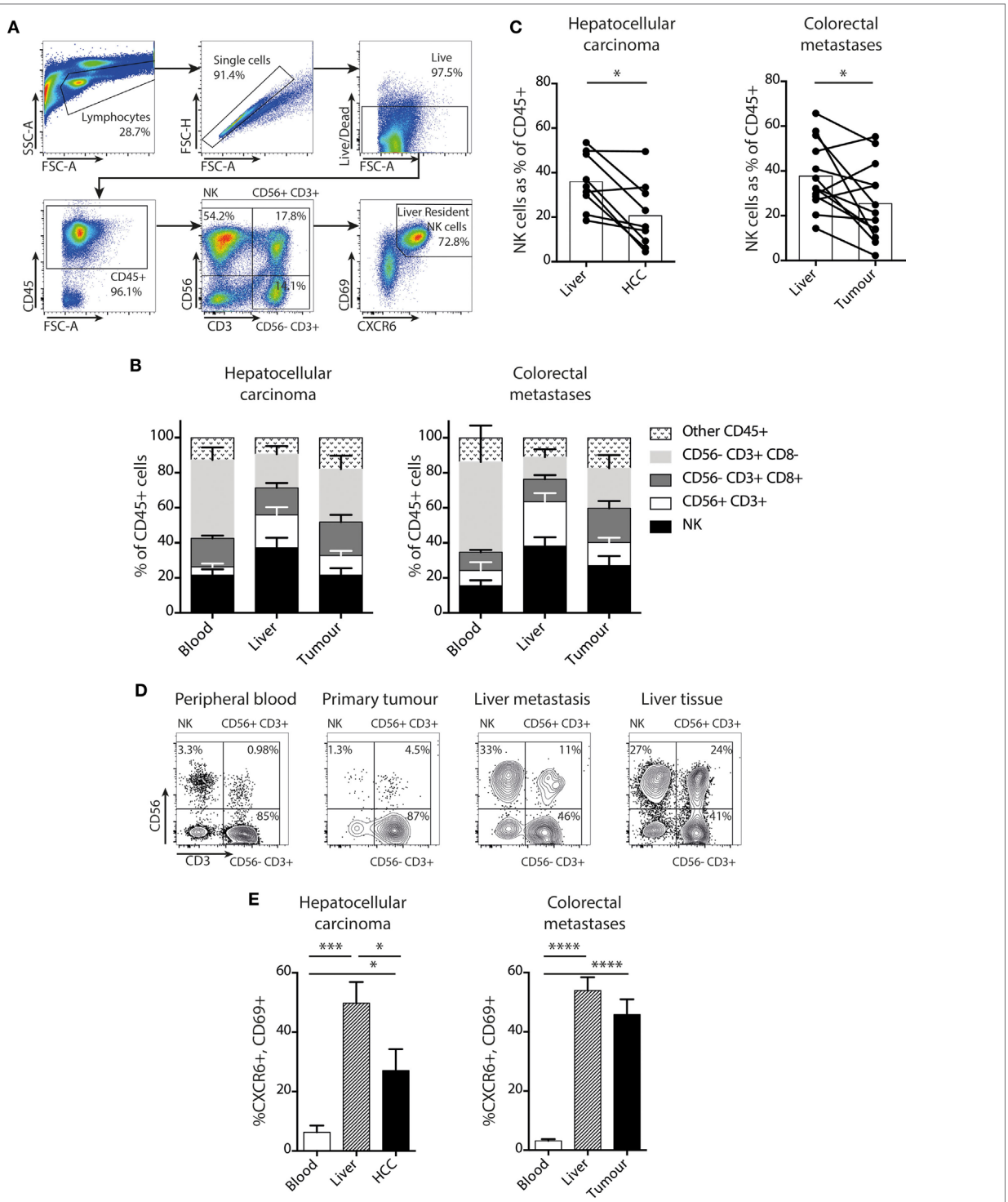


**FIGURE 1** | NK cells, including a liver-resident subset, are prevalent in liver tumors. **(A)** Gating strategy for identification of intrahepatic or tumor-infiltrating NK cells by flow cytometry, showing identification of CXCR6$^+$CD69$^+$ liver-resident NK cells (CD45$^+$CD56$^+$CD3$^-$CD69$^+$CXCR6+). **(B)** Proportion of NK cells, CD56$^+$CD3$^+$ cells, CD56$^-$CD8$^+$ T cells, and CD56$^-$CD8$^-$ T cells in blood, liver, and tumor tissue in patients with hepatocellular carcinoma (HCC) ($n$ = 6) and CRC ($n$ = 10). Bars show mean and SEM, $p$ values determined by MANOVA. **(C)** Total NK cells (CD56$^+$CD3$^-$) as a proportion of CD45$^+$ lymphocytes in HCC ($n$ = 9) and CRC ($n$ = 13) paired liver and tumor. Bars indicate mean of each group. **(D)** Flow cytometry dot plots showing CD45$^+$ lymphocytes divided into NK, CD56$^+$ T cells, and CD56$^-$ T cells for lymphocytes derived from peripheral blood, primary colonic adenocarcinoma, liver metastasis, and unaffected liver tissue in one individual. **(E)** CXCR6$^+$CD69$^+$ liver-resident NK cells as a proportion of total NK cells in blood, liver, and tumor from HCC ($n$ = 10) and CRC ($n$ = 13) patients by flow cytometry. Groups were compared using Mann–Whitney $U$ test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: *$p \leq 0.05$; **$p \leq 0.005$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

paired analysis, NK cells in tumors comprised a slightly lower proportion of the CD45$^+$ lymphocyte pool than in unaffected liver tissue (**Figure 1C**). However, NK cells were detectable in all liver tumor infiltrates and constituted up to a maximum of 49% in HCC and 55% in CRC metastases. In one example of blood, primary colonic adenocarcinoma, liver metastasis, and liver tissue from a single patient, the composition of the CD45$^+$ lymphocyte pool in the liver metastasis was more similar to that of the unaffected liver than to the peripheral blood or the primary tumor (**Figure 1D**). These data suggested that both primary and secondary liver tumors acquire an NK-enriched lymphocyte pool, a prototypic feature of the liver immune landscape.

We next asked whether a recently defined subset of liver-resident NK cells was able to infiltrate liver tumors. These tissue-resident NK cells are marked by surface co-expression of CXCR6 and CD69 and characterized by low cytotoxicity and pro-inflammatory cytokine capacity (20). We found that both HCC and CRC metastases contained a high proportion of NK cells with the liver-resident CXCR6$^+$CD69$^+$ phenotype, compared to the very low frequencies present in the blood (**Figure 1E**). Tumor-infiltrating NK cells in HCC contained up to 79% with a liver-resident phenotype, although the mean of 10 HCC was significantly less than in the uninvolved distant liver margins (28 versus 49%, respectively). In the 13 patients with CRC metastases examined, the proportion of NK cells with a liver-resident phenotype was comparable to the healthy distant liver margin (mean 46 versus 54%, respectively, **Figure 1E**). Thus both HCC and CRC metastases had an NK cell composition that was more reflective of the surrounding liver tissue than peripheral blood, with a similar proportion of liver resident and liver-infiltrating "non-resident" (CXCR6$^-$CD69$^-$) NK cells to the unaffected liver (**Figure 1E**). Because liver-resident NK cells have distinct phenotypic and functional features, all subsequent comparisons of NK cells within tumors and surrounding liver were analyzed for both liver-resident and liver-infiltrating subsets.

## Intratumoral NK Cells Have Downregulated NKG2D With Selective Impaired Proliferation of the NKG2D$^{lo}$ Fraction

We have previously reported that NK cells maintain paradoxically high expression of NKG2D within the liver, even in the presence of NKG2D ligands on neighboring T cells in the context of HBV-related inflammation (26). To further investigate this, we compared NKG2D expression on the CXCR6$^+$CD69 liver-resident subset with non-resident, liver-infiltrating NK cells (**Figure 2A**) in HCC, CRC metastases, and matched uninvolved liver tissue. In every case, the concentration of NKG2D was higher on the CXCR6$^+$ liver-resident NK cells than their liver-infiltrating counterparts (**Figure 2B**).

However, comparison of NK cells within tumors with those in matched liver revealed downregulation of NKG2D on those within HCC (**Figure 2C**) and CRC metastases (**Figure 2D**). On the non-resident NK cells from liver containing CRC metastases the NKG2D expression was already low, with a further subtle but consistent decrease within metastases (**Figure 2D**). Instead, NKG2D downregulation on global intratumoral NK cells was largely attributable to decreases on the liver-resident subset (**Figures 2C,D**). We postulated this downmodulation of NKG2D expression may result from engagement by NKG2D-L on liver tumors, vasculature or infiltrates, as described in other settings (13–15).

Next, we examined NK cell turnover using *ex vivo* staining for the proliferation marker Ki67. Overall NK cell proliferation was maintained within liver tumors compared to surrounding liver tissue and blood (**Figures 3A,B**). NK cell Ki67 was slightly higher in the tumor-infiltrating (non-resident) fraction than in the distant liver margin (**Figure 3C**), possibly indicating an initial proliferation on first encountering tumor cells. We then investigated whether the NK cell NKG2D downregulation we observed within tumors affected their proliferative capacity by comparing Ki67 expression on NKG2D$^{hi}$ versus NKG2D$^{lo}$ NK cells, gating on those with the highest and lowest 25% of NKG2D expression, respectively (**Figure 3D**). Intratumoral NK cells that had downregulated NKG2D had significantly lower proliferation than their NKG2D$^{hi}$ counterparts (**Figure 3E**).

## Intratumoral NK Cells Have Impaired Cytokine Production and Target Cell Cytotoxicity

To further investigate the impact of the liver tumor environment on NK cell antitumor efficacy, we examined their cytotoxicity and cytokine-producing capacity. As previously described (20), the key cytotoxic mediator granzyme B was observed to be markedly reduced in liver-resident (CXCR6$^+$CD69+) NK cells and was instead mainly confined to the recirculating non-resident fraction (**Figure 4A**). Consistent with their enrichment with the liver-resident subset, both intrahepatic and tumor NK cells tended to have reduced granzyme B compared to circulating NK cells (**Figures 4A,B**). Of note, NK cell cytotoxic potential was further reduced for the resident fraction within tumors compared to distant liver margins (**Figure 4B**).

NK cells isolated from matched liver tumors, distant margins, and blood were also compared for their capacity to produce IFNγ, another antitumor effector mechanism, following stimulation with IL-12/18 (**Figure 4C**). A significantly lower proportion of NK cells within tumors and liver were able to produce IFNγ than in the blood (**Figure 4D**); in four out of six cases, production was even lower within the tumor than the uninvolved liver (**Figure 4E**).

Taken together, these data indicate that local NK cells have NKG2D downregulation with impaired cytolytic and non-cytolytic potential against liver tumors.

## NK Cell Coculture With an HCC Cell Line Recapitulates NKG2D Downregulation and Functional Inhibition in a Contact-Dependent Manner

To investigate whether HCC itself, instead of its tumor milieu, is capable of imposing the changes observed on *ex vivo* tumor

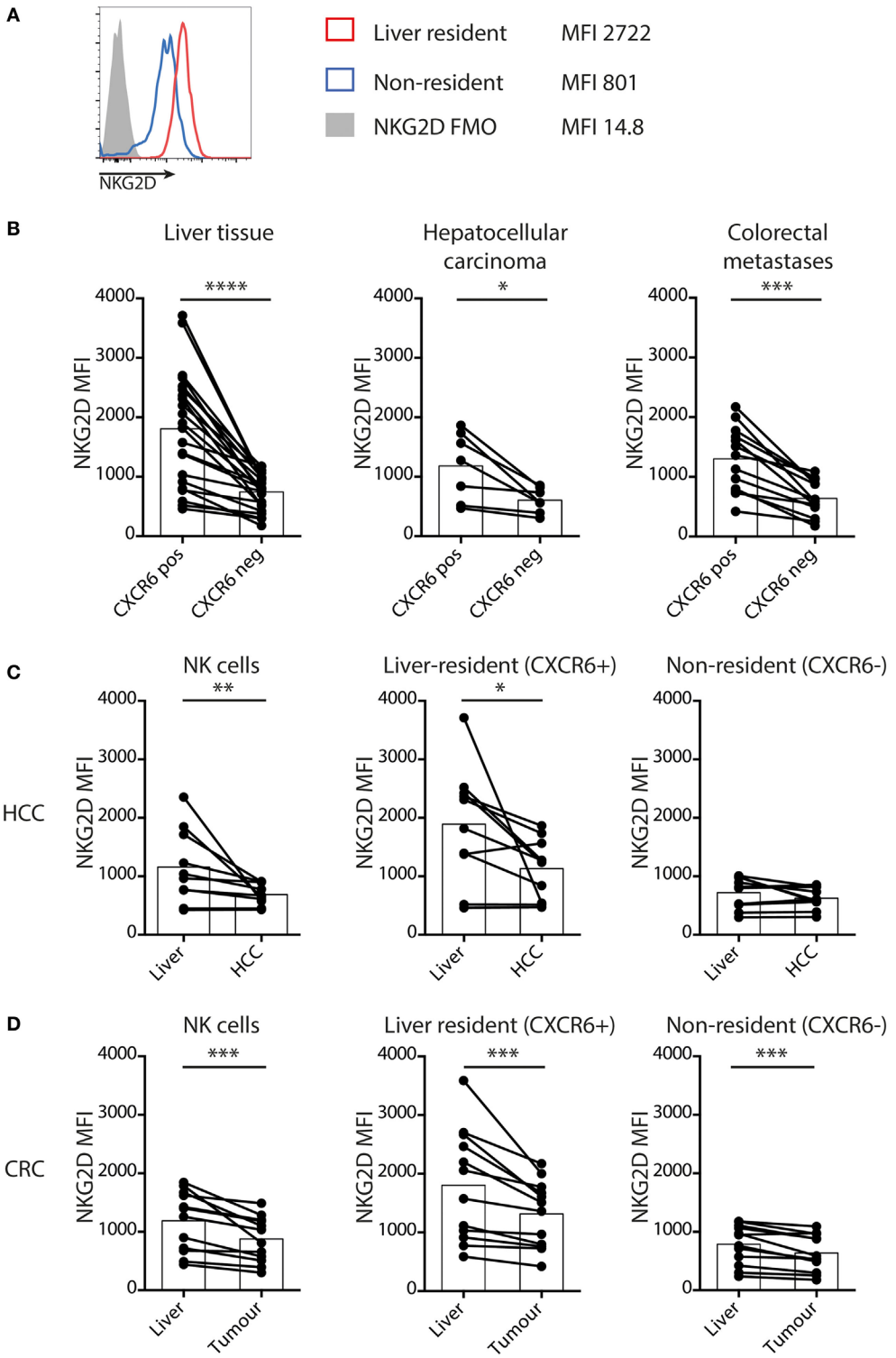


**FIGURE 2** | Liver-resident NK cells have increased NKG2D that is downregulated in tumors. **(A)** Representative histograms to show NKG2D expression by liver-resident and non-resident NK cells. **(B)** NKG2D expression by mean fluorescence intensity (MFI) on tissue-resident (CXCR6$^+$CD69+) and non-resident (CXCR6$^-$CD69$^-$) liver ($n$ = 23), hepatocellular carcinoma (HCC) ($n$ = 10), and CRC ($n$ = 13) NK cells quantified by flow cytometry. Bars indicate mean of each group. **(C)** NKG2D expression by MFI on total NK cells, liver-resident (CXCR6$^+$CD69+) and non-resident (CXCR6$^-$CD69$^-$) NK cells in HCC and paired liver ($n$ = 10) quantified by flow cytometry. Bars indicate mean of each group. **(D)** NKG2D expression by MFI on total NK cells, liver resident (CXCR6$^+$CD69+) and non-resident (CXCR6$^-$CD69$^-$) NK cells in CRC and paired liver ($n$ = 13) quantified by flow cytometry. Bars indicate mean of each group. Groups were compared using Wilcoxon matched-pairs signed rank test. $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: *$p \leq 0.05$; **$p \leq 0.005$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

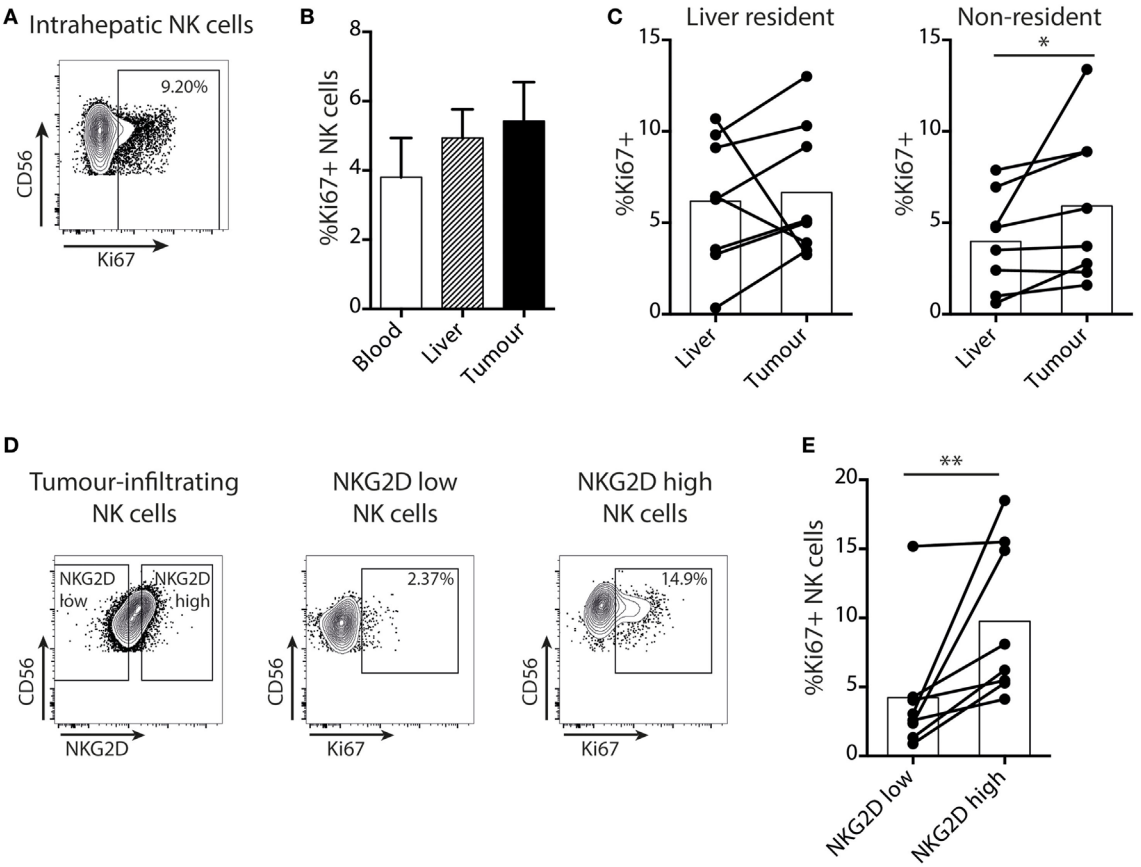


**FIGURE 3** | Proliferation of intratumoral NK cells is selectively impaired in the NKG2D$^{lo}$ fraction. **(A)** Representative example of intranuclear Ki67 staining on intrahepatic NK cells by flow cytometry. **(B)** Summary data for Ki67 expression by flow cytometry on total NK cells from paired blood, liver, and tumor. Bars shown mean and SEM. **(C)** Ki67 expression by liver-resident and non-resident NK cells from paired liver and tumor ($n = 8$). Bars indicate mean of each group. **(D)** Example of gating of highest and lowest 25% of tumor-infiltrating NK cells by NKG2D expression, and Ki67 expression in these two groups. **(E)** Ki67 expression on the highest and lowest 25% of tumor-infiltrating NK cells by NKG2D expression ($n = 8$). Bars indicate mean of each group. Ex indicates tissue explant. Groups were compared using Mann–Whitney *U* test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: $^{*}p \leq 0.05$; $^{**}p \leq 0.005$.

NK cells, we used a coculture system. To mimic the interaction between NK cells and HCC we used PLC/PRF/5 cells, an adherent line derived from HCC that expresses multiple ligands for NK cell receptors, including HLA class I and the NKG2D ligand MICA (Figure S1 in Supplementary Material). Overall, tumor-infiltrating NK cells resembled hepatic NK cells much more closely than peripheral NK cells, so liver NK cells were used to examine the functional impairment imposed on tumor-infiltrating NK cells. Magnetic beads were used to isolate liver NK cells by negative selection and these were then cocultured overnight with or without the HCC cell line. Intrahepatic NK cells downregulated NKG2D on their cell surface on coculture with PLC/PRF/5, an effect not seen consistently with peripheral NK cells (**Figure 5A**). This downregulation was consistently seen on both liver-resident and non-resident fractions. However, as previously observed directly *ex vivo*, the degree of downmodulation was more striking on the liver-resident subset that had higher baseline NKG2D staining (**Figure 5B**).

We next questioned whether NKG2D downregulation required cell contact or was mediated by a soluble factor produced by HCC. We observed that NKG2D downregulation did not occur when intrahepatic NK cells were cultured in 50% PLC/PRF/5 culture supernatant, suggesting it was not mediated by a soluble factor (**Figure 5C**). In addition, this HCC line was no longer able to induce NKG2D downregulation once it was separated from NK cells by a semipermeable membrane in a transwell system (**Figure 5C**), confirming the requirement for contact. To investigate the fate of cell surface NKG2D, we used imaging cytometry to visualize and quantitate the cellular localization of NKG2D molecules. Whereas CD56 showed a ring-like cell membrane-associated distribution, NKG2D was visualized within the NK cell cytoplasm following engagement with PLC/PRF/5 (representative example and summary data quantitating internalization following NK cell culture overnight with or without PLC/PRF/5, **Figures 5D,E**). Thus, the interaction of NKG2D-L on PLC/PRF/5 with NKG2D on intrahepatic NK cells drove NKG2D internalization, accounting for the downregulation observed.

Next, we tested the impact of HCC on intrahepatic NK cell cytotoxicity using a two-stage challenge model (**Figure 6A**). After coculture with PLC/PRF/5 cells overnight, NK cells

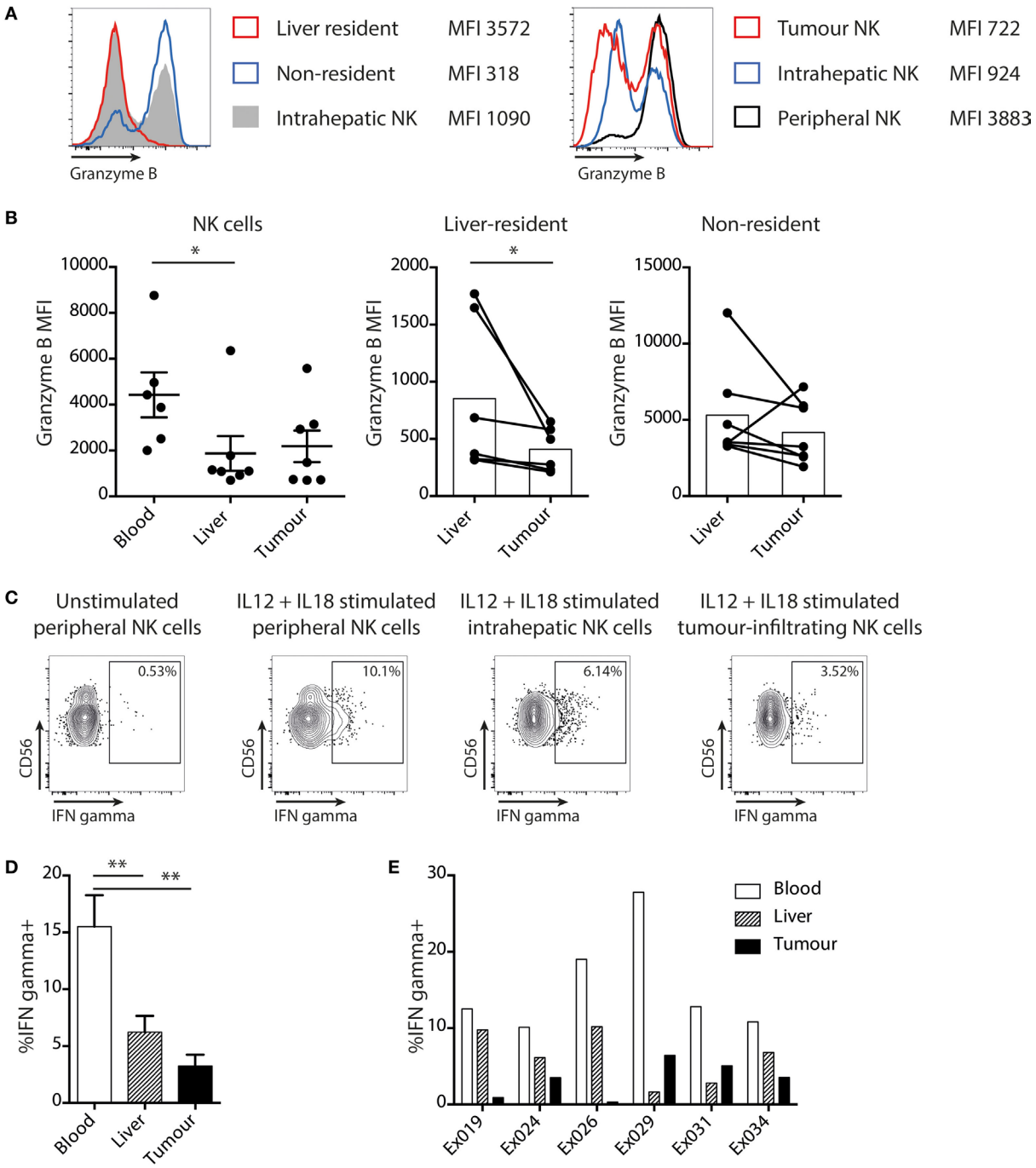


**FIGURE 4** | Liver tumor NK cells are functionally impaired. **(A)** Representative histograms of granzyme B staining on total intrahepatic NK cells, liver resident and non-resident subsets of NK cells, and on matched peripheral, liver, and tumor NK cells. **(B)** Granzyme B expression (mean fluorescence intensity) on global (mean and SEM shown), liver-resident and non-resident NK cells in matched peripheral blood, liver, and tumor. Bars indicate mean of each group. **(C)** Representative example of intracellular cytokine staining for IFNγ in unstimulated NK cells and NK cells stimulated with IL12 and IL18. **(D)** Summary data showing IFNγ production by total NK cells from blood, liver, and tumor (bars shown mean and SEM) and **(E)** for individual matched blood and tissue samples. Ex indicates tissue explant. Groups were compared using Mann–Whitney *U* test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: $^{*}p \leq 0.05$; $^{**}p \leq 0.005$.

were washed off and challenged with K562 cells at 1:1 ratio to assess residual cytotoxicity. Cytotoxic potential, as measured by CD107a expression, was consistently reduced in both peripheral and intrahepatic NK cells following exposure to PLC/PRF/5 (representative example **Figure 6B**, summary data **Figure 6C**). This effect was seen in both resident and non-resident NK cell

populations, although liver-resident NK cells had a lower level of baseline degranulation (**Figure 6D**). The HCC-driven impairment in NK cell cytotoxicity was abrogated using PLC/PRF/5 supernatants or upon separation by a transwell insert (**Figure 6E**), again implying a cell contact-dependent mechanism. IFNγ production was also significantly impaired after coculture with PLC/PRF/5 HCC cells (**Figure 6F**), with the bulk of the decline seen in non-resident NK cells (Figure S2 in Supplementary Material).

Using recombinant soluble NKG2D or anti-MICA to block the NKG2D-mediated NK cell-PLC/PRF/5 interaction, there was only a non-significant trend toward recovery of NKG2D expression by the NK cells and no effect on cytotoxic function (Figures S3A,B in Supplementary Material). Similarly, there was no restoration of NKG2D expression or NK cell cytotoxicity with anti-TGFβ receptor blockade (Figure S3A,B in Supplementary Material).

In one case, we obtained sufficient material from an HCC to test the impact of the autologous tumor on intrahepatic NK cells. Intrahepatic NK cells rested overnight were capable of some degranulation when exposed to K562 targets. By contrast, NK cells extracted from the same liver sample but exposed to

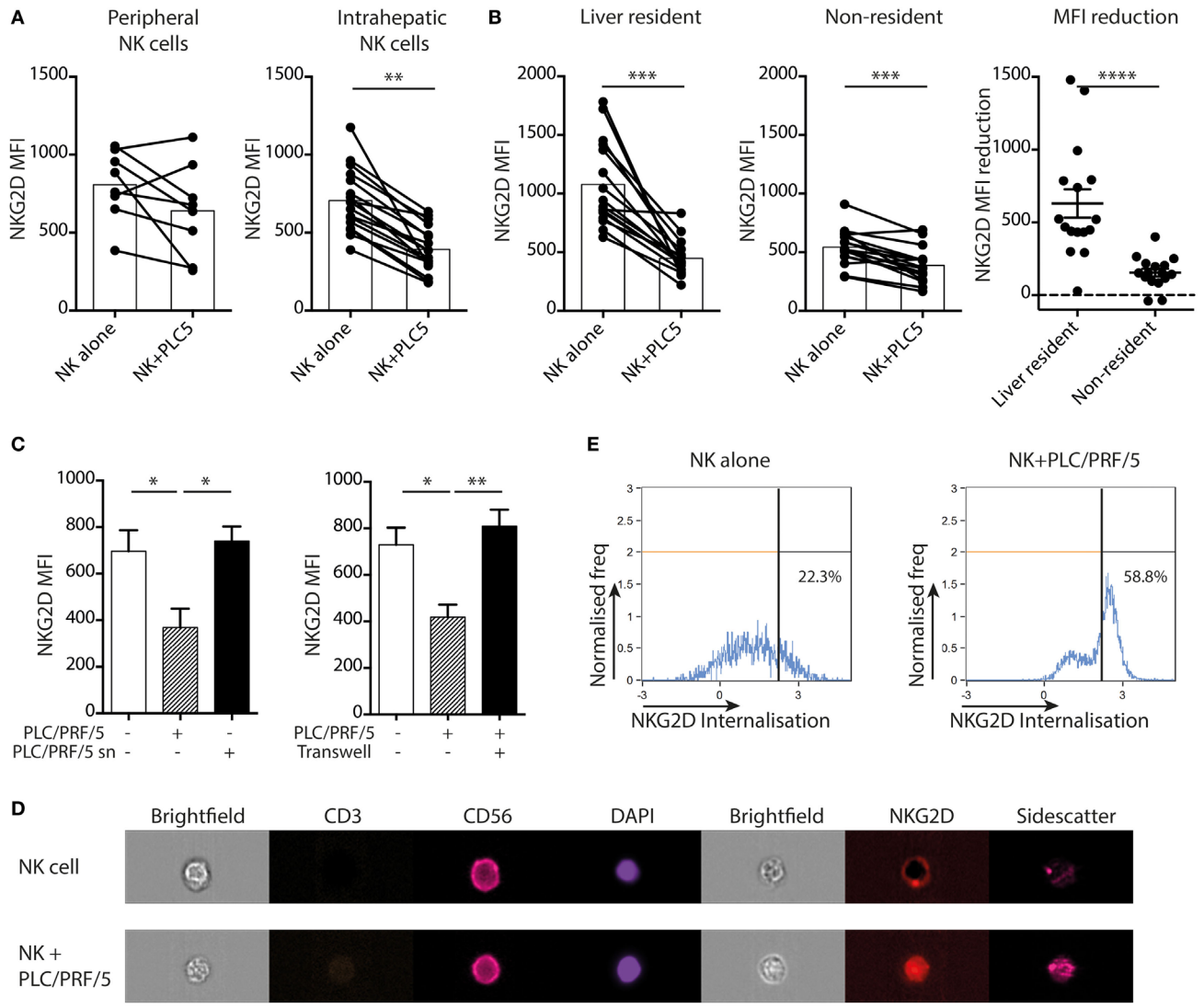


**FIGURE 5** | PLC/PRF/5 cells induce NKG2D downregulation on intrahepatic NK cells in a cell-contact-dependent manner. **(A)** NKG2D expression [mean fluorescence intensity (MFI)] by peripheral ($n = 8$) and intrahepatic ($n = 17$) NK cells following 18 h coculture with or without PLC/PRF/5 cells. Bars indicate mean of each group. **(B)** NKG2D expression (MFI) by liver-resident and non-resident intrahepatic NK cells following 12 h coculture with or without PLC/PRF/5 cells ($n = 16$), bars indicate mean of each group, and comparison of amount of reduction in NKG2D MFI for liver-resident and non-resident NK cells after PLC/PRF/5 coculture, mean and SEM shown. **(C)** NKG2D expression by intrahepatic NK cells following 12 h coculture alone or with PLC/PRF/5 cells or with their culture supernatant (sn) diluted 1:1 with fresh media ($n = 4$) or separated by a transwell ($n = 4$). **(D)** Representative example showing NKG2D internalization in NK cells following 12 h coculture with PLC/PRF/5 cells by imaging cytometry. **(E)** Summary data showing NKG2D internalization score (computed by IDEAS software, described in Section "Materials and Methods") following 12 h coculture with or without PLC/PRF/5 cells. Groups were compared using Mann–Whitney *U* test (unpaired) and Wilcoxon matched-pairs signed rank test except **(C)**, unpaired *t*-test with Welch's correction. $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: *$p \leq 0.05$; **$p \leq 0.005$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

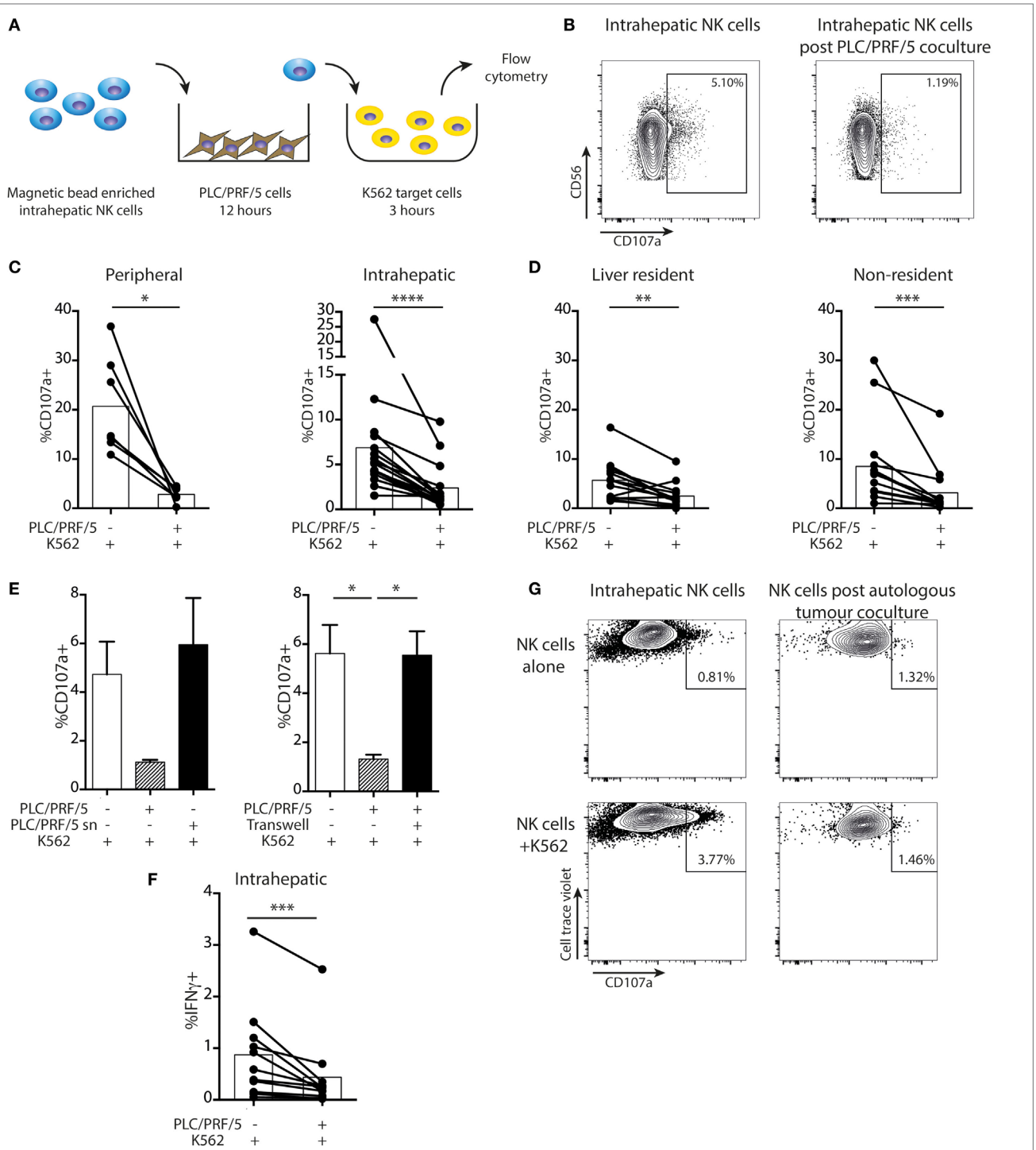


**FIGURE 6** | Intrahepatic NK cells are functionally impaired upon exposure to PLC/PRF/5 cells. **(A)** Diagram showing experimental design of PLC/PRF/5 coculture followed by K562 challenge. **(B)** Representative example of CD107a staining measured by flow cytometry on intrahepatic NK cells following 12 h coculture with PLC/PRF/5 cells (or alone) followed by challenge with K562 cells. **(C)** Summary data of CD107a expression by peripheral ($n = 7$) and intrahepatic NK cells ($n = 16$) following K562 challenge after PLC/PRF/5 coculture. **(D)** Summary data of CD107a expression on liver resident and non-resident intrahepatic NK cells ($n = 14$) following K562 challenge after PLC/PRF/5 coculture. **(E)** CD107a staining following K562 challenge after coculture with PLC/PRF/5 cells with culture supernatant diluted 1:1 with fresh media or separated by a transwell. **(F)** Intracellular IFNγ staining in intrahepatic NK cells ($n = 11$) following K562 challenge after PLC/PRF/5 coculture. Bars indicate mean of each group. **(G)** CD107a expression on intrahepatic NK cells following 12 h coculture with or without autologous hepatocellular carcinoma followed by challenge with K562 cells. Cells were gated on cell trace violet-positive NK cells (used to distinguish added intrahepatic NK cells from NK cells present in the autologous tumor). Groups were compared using Mann–Whitney *U* test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses) except **(E)**, unpaired *t*-test with Welch's correction. $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: $^{*}p \leq 0.05$; $^{**}p \leq 0.005$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

autologous tumor overnight showed no cytotoxicity toward K562 challenge (**Figure 6G**).

## IL-15 Restores NK Cell Function Following HCC-Driven Inhibition

Next, we sought to investigate mechanisms by which intratumoral NK cell function might be restored. NK cells were removed from overnight PLC/PRF/5 coculture and rested in fresh medium overnight in the presence of different cytokines, followed by challenge with K562 cells as before (experimental approach in **Figure 7A**). Degranulation was improved by overnight treatment with IL-15 but not IL-2, IL-12, or IL-18, compared to medium alone, before K562 challenge (**Figure 7B**). In the same experiments, IFNγ production was partially recovered by all the cytokines tested, but IL-15 showed the most pronounced boosting (**Figure 7C**). Using NK cells extracted from five different livers, we observed that NK cell degranulation was significantly impaired following incubation with PLC/PRF/5, even after a further 18 h resting in media, but was consistently restored to baseline levels after incubation with IL-15 (**Figure 7D**). NKG2D expression was increased following IL-15 stimulation, but recovery of PLC/PRF/5-induced NKG2D downregulation was modest (Figure S4A in Supplementary Material). NK cell IFNγ was also consistently boosted to even higher levels than those seen without exposure to PLC/PRF/5 after overnight incubation with IL-15 rather than media alone (**Figure 7E**). Both liver-resident and non-resident NK cells recovered function in a similar way to the global population following IL-15, although there was a suggestion that the liver-resident NK cells recovered cytotoxic function more while non-resident NK cells recovered more cytokine production (Figure S4 in Supplementary Material).

Finally, we tested the capacity of IL-15 to rescue NK cells after *in vivo* exposure to HCC. Intrahepatic and intratumoral lymphocytes from the same liver were incubated with IL-15 or media alone overnight and then challenged with K562. NK cells isolated from the liver showed a clear degranulation response to K562 cells that was strikingly augmented by IL15 (**Figure 7F**). By contrast, NK cells extracted from the HCC were unable to respond to K562 cells (CD107a comparable to baseline) unless they had been pre-stimulated with IL-15. IL-15 boosted degranulation of tumor-infiltrating NK cells to a higher percentage than the basal level seen upon K562 challenge of NK cells from the unaffected distant liver margin (**Figure 7F**).

# DISCUSSION

Here, we use direct *ex vivo* analysis of freshly isolated human tumor and liver lymphocytes to show that HCC and CRC metastases are populated by high frequencies of NK cells with liver-resident and liver-infiltrating phenotypes. We find that NKG2D expression is strikingly enriched on the CXCR6$^+$ liver-resident NK cell subset but is consistently downregulated on both these and their non-resident counterparts in the setting of liver tumors, with impaired proliferation of the NKG2D$^{lo}$ fraction. Intratumoral NK cells have low cytolytic and non-cytolytic potential, even more so than the tissue-resident subset of the uninvolved liver. These features are recapitulated when liver NK cells have direct contact with an HCC cell line and are amenable to reversal by IL-15.

T cells are generally considered to make up the majority of the lymphocytic infiltrate in liver tumors, although only a small fraction of these are likely to be tumor-specific (3). However, our data reveal that NK cells are more prevalent in liver tumors than generally recognized, tending to outnumber CD8 T cells in both HCC and CRC metastases. This is in contrast to primary colorectal tumors, where few NK cells are found (27), with this difference being underscored by our analysis of primary colon and secondary liver metastases resected simultaneously from the same patient. These data suggest that tumors can co-opt the predominant immune infiltrates of the organ they metastasize to, rather than imposing an infiltrate dictated by the primary cancer. We show for the first time that HCC and CRC metastases contain the recently defined CXCR6$^+$ human liver-resident NK cell subset as well as NK cells with a liver-infiltrating (non-resident) phenotype. Previous work by our group and colleagues has shown that these cells are NK cells as evidenced by their expression of CD56 and Eomes (20, 28) and are transcriptionally distinct from ILC1 cells (21). It is possible that NK cells residing in the liver move into tumors or that non-resident NK cells acquire a resident phenotype on encountering relevant signals within the tumor.

Unlike T cells, NK cells are capable of providing tumor surveillance and tumor immunity without specificity for tumor antigen or neoantigen, thereby offering a large pool of potential effectors (5). The relevance of assessing NK cell responses in liver tumor immunity has been supported by clinical studies finding associations between increased peripheral or tumor-infiltrating NK cell frequencies and better survival with HCC (29) and CRC metastases (30), respectively. A similar large clinical series would be required to assess whether the variable proportion of tissue-resident NK cells infiltrating liver tumors correlates with prognosis, as recently reported for lung-resident T cells (23, 24). One potential advantage conferred by liver-resident NK cells is the strikingly high expression of NKG2D we observed, which should facilitate sensing of NKG2D-L on tumors. Our *ex vivo* data show that NKG2D expression remains significantly higher on the CXCR6$^+$ tissue-resident fraction of NK cells within HCC and CRC metastases, even though it is lower than on their counterparts in unaffected distant liver margins. *In vitro* exposure to an HCC cell line recapitulated the *ex vivo* finding that the tumor-induced downregulation and internalization of NKG2D is largely attributable to reductions on the tissue-resident fraction of NK cells. The selective reduction of NK cell Ki67 expression on the NKG2D$^{lo}$ fraction suggests that NKG2D downregulation impedes their proliferative renewal within the tumor milieu.

By comparing intratumoral and matched unaffected liver NK cell function according to tissue-residency, we were able to determine that the majority of the functional impairment found within tumors is attributable to the hypo-functionality of liver-resident NK cells. Thus the overall reduction in NK cell granzyme B within liver tumors is primarily driven by the low levels in the liver-resident fraction, with the non-resident cells maintaining much higher expression. IFNγ production by

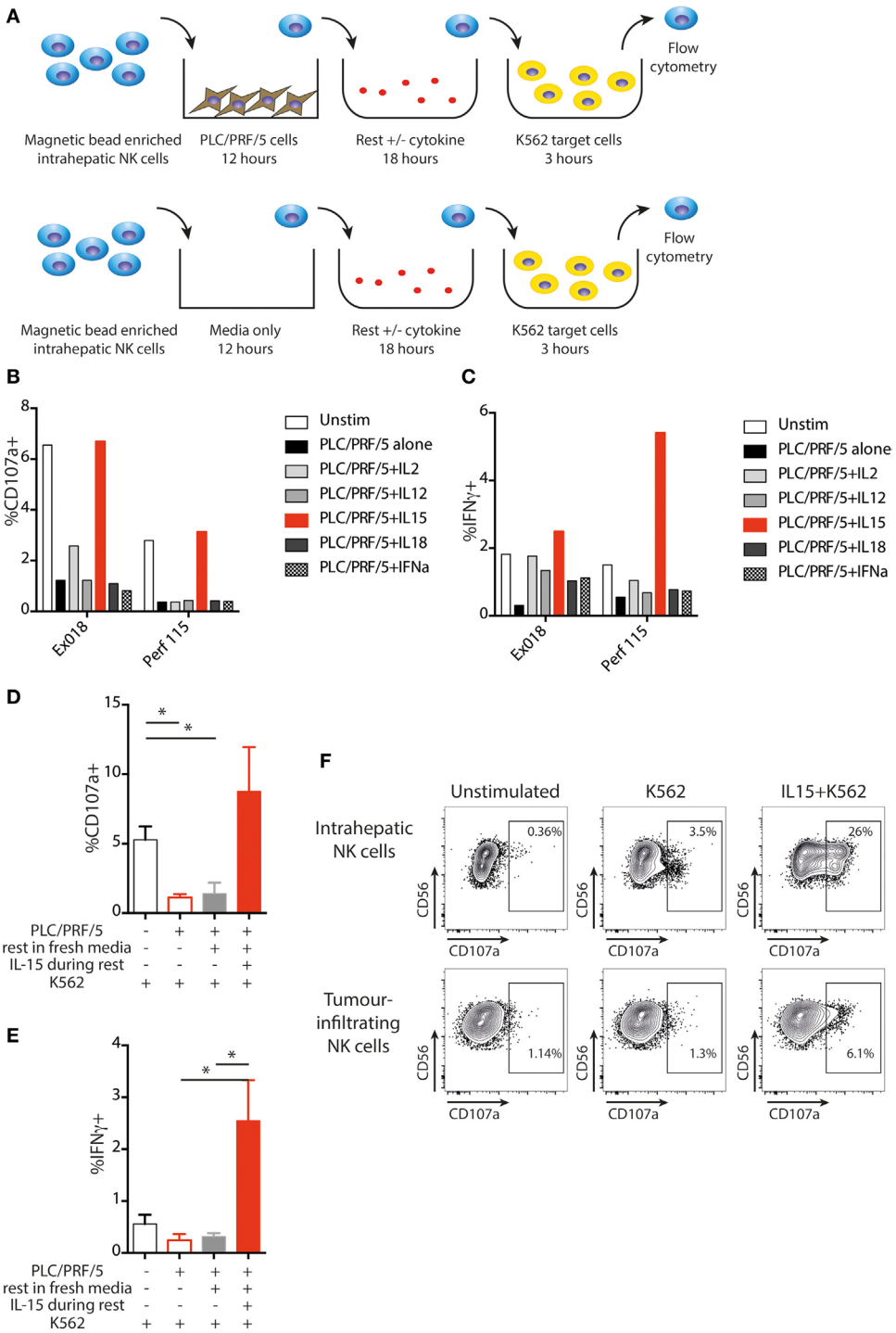


**FIGURE 7** | IL-15 restores NK cell function following PLC/PRF/5 coculture. **(A)** Diagram showing experimental design of PLC/PRF/5 coculture followed by rest in fresh media ± cytokine before K562 challenge. **(B)** CD107a staining following K562 challenge after PLC/PRF/5 coculture, then rest overnight in fresh media ± the indicated cytokine using intrahepatic NK cells isolated from a liver explant (Ex) and from a liver perfusate (Perf). **(C)** Intracellular IFNγ staining following K562 challenge after PLC/PRF/5 coculture, then rest overnight in fresh media ± the indicated cytokine using intrahepatic NK cells isolated from a liver explant (Ex) and from a liver perfusate (Perf). **(D)** Summary data showing CD107a staining following K562 challenge of intrahepatic NK cells unstimulated, after PLC/PRF/5 coculture, after PLC/PRF/5 coculture then rest in fresh media and after PLC/PRF/5 coculture then rest in IL-15 containing media ($n = 5$ liver samples). **(E)** Summary data showing intracellular IFNγ staining following K562 challenge of intrahepatic NK cells unstimulated, after PLC/PRF/5 coculture, after PLC/PRF/5 coculture then rest in fresh media and after PLC/PRF/5 coculture then rest in IL-15 containing media ($n = 5$ liver samples). **(F)** Example of CD107a staining on paired intrahepatic and tumor-infiltrating NK cells following K562 challenge after overnight rest with IL-15. Groups were compared using unpaired $t$ test with Welch's correction. $p \leq 0.05$ was considered to be significant for all tests. Figures are labeled: $^*p \leq 0.05$.

peripheral NK cells from HCC patients in response to IL-12 and IL-18 was in keeping with that of another HCC cohort (31) and by tumor-infiltrating NKs was similar to that seen in NK cells infiltrating breast tumors (32). We did not investigate the expression of IL12 or IL18 receptor but others have shown that IL12Rβ1 expression falls and IL12Rβ2 expression is increased in $CXCR6^+$ NK cells in the liver, suggesting subtle tuning of cytokine responses (33). Taken together, our data show that both cytolytic and non-cytolytic (IFNγ) antitumor effector functions are further impaired within liver tumors compared to NK cells from uninvolved liver. Such functional paralysis is likely due to a combination of factors, with previous studies implicating regulatory T cells (31) and myeloid-derived suppressor cells (34) infiltrating HCC. Our experiments implicate a direct contribution from HCC cells, that can drive NKG2D internalization and recapitulate the defects in production of cytotoxic and cytokine mediators in a contact-dependent manner, without the need for accessory cells.

*In vitro* blockade did not confirm a clear role for NKG2D downregulation or TGFβ in the HCC-mediated functional inhibition of NK cells we observed. Likewise, overnight rest in fresh media did not restore tumor NK cell degranulation or IFNγ production, suggesting that functional impairment was not due to a reversible metabolic restriction or competition for nutrients in coculture. By contrast, IL-15 was able to recover cytotoxic capacity and also conferred the most efficient boosting of IFNγ production. This enhancement in degranulation and IFNγ production was consistent, recovering function even when coculture had rendered the NK cells almost totally anergic. The use of IL-15 as an immunotherapeutic approach in HCC is supported by data from mouse models. An NK cell line transfected with IL-15 signals in an autocrine fashion to control HCC in mouse models (35). K562 cells transfected with membrane bound IL-15 and 4-1BB ligand can expand and activate NK cells for use in HCC immunotherapy (36). IL-15 is being used to maintain *ex vivo* activated NK cells for infusion in the context of hematological malignancy and solid tumors in clinical trials (NCT01875601, NCT01385423) (37). Similar approaches with modified IL-15/IL-15Rα complexes (IL-15 superantigen) are being developed for use in clinical trials (38). However, administration of IL-15 and associated molecules can cause significant toxicity (39), which appears to be related to systemic production of IFNγ by NK cells (40). To overcome this, IL-15 could be targeted to cancer cells by fusion with specific antibodies, shown to enhance antitumor activity in animal models (41). Our example of *ex vivo* tumor-infiltrating NK cells being re-activated by IL-15 suggests that direct delivery could be used in liver tumors to activate local NK cells *in vivo* while minimizing systemic toxicity.

In conclusion, human liver tumors have high frequencies of NK cells, a large fraction of which have the features of a recently described liver-resident subset. We find that tissue-resident NK cells have increased expression of NKG2D compared to their non-resident counterparts but remain susceptible to downregulation of this key receptor in tumors. The functional paralysis of intratumoral NK cells partly reflects features of NK cells residing in the tolerogenic liver environment, with additional inhibition imposed by the tumor. IL-15 is able to boost functionality of intrahepatic NK cells following exposure to HCC, underscoring the therapeutic potential of this cytokine to harness the potent antitumor potential of this large component of liver tumor infiltrates.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of University College London-Royal Free Hospital Research Ethics Committee and the South East Coast Research Ethics Committee. The protocol was approved by the University College London-Royal Free Hospital Research Ethics Committee and the South East Coast Research Ethics Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

## AUTHOR CONTRIBUTIONS

NE and MM prepared the manuscript and designed the study. NE, LS, KS, LP, AB, DO, NS, and W-CH performed experiments. NE analyzed the data. GF and BD provided tissue samples. All authors reviewed the manuscript.

## ACKNOWLEDGMENTS

This work was funded by a Wellcome Clinical Training Fellowship (102772/Z/13/Z) to NE and a Wellcome Senior Investigator Award (101849/Z/13/Z) and MRC grant (MR/M020126/1) to MM. We are very grateful to all patients and clinical staff who helped with sample provision, in particular Amir Gander at the Tissue Access for Patient Benefit (TAPb) service at The Royal Free Hospital. We thank Massimo Pinzani and Giuseppe Mazza for provision of the PLC/PRF/5 cell line.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01009/full#supplementary-material.

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**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: 17 May 2018
doi: 10.3389/fimmu.2018.01052

# Critical Contribution of NK Group 2 Member D Expressed on Invariant Natural Killer T Cells in Concanavalin A-Induced Liver Hepatitis in Mice

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Natural killer group 2D (NKG2D) is a well-characterized activating receptor expressed on many immune cells, including invariant natural killer T (iNKT) cells. These cells were shown to be responsible of liver injury in the model of concanavalin A (Con A)-induced hepatitis, considered to be an experimental model of human autoimmune hepatitis. In this study, we investigated whether NKG2D plays a role in the hepatitis induced by iNKT cell-mediated immune response to Con A. By using killer cell lectin-like receptor subfamily K, member 1 deficient (*Klrk1*$^{-/-}$) mice, we found that the absence of NKG2D reduced the hepatic injury upon Con A administration. This was not due to an intrinsic functional defect of NKG2D-deficient iNKT cells as mice missing NKG2D have normal distribution and function of iNKT cells. Furthermore, increased resistance to Con A-induced hepatitis was confirmed using neutralizing anti-NKG2D antibodies. The reduced pathogenic effect of Con A in the absence of NKG2D correlates with a reduction in pathogenic cytokine production and FAS-Ligand (FAS-L) expression by iNKT cells. We also found that Con A administration led to an increase in the retinoic acid early inducible (RAE-1) surface expression on wild-type hepatocytes. Finally, we found that Con A has no direct action on FAS-L expression or cytokine production by iNKT cells and thus propose that NKG2D-L expression on stressed hepatocytes promote cytotoxic activity of iNKT cells *via* its interaction with NKG2D contributing to hepatic injury. In conclusion, our results highlight NKG2D as an essential receptor required for the activation of iNKT cells in Con A-induced hepatitis and indicate that it represents a potential drug target for prevention of autoimmune hepatitis.

**Keywords: cytokines, FAS-L, RAE-1, NK-receptors, iNKT**

**Abbreviations:** IFN, interferon; IL, interleukin; iNKT, cell invariant natural killer T cell; *Klrk1,* killer cell lectin-like receptor subfamily K, member 1; NKG2D, NK group 2 member D; Con A, Concanavalin A; α-GalCer, α-galactosylceramide; Poly(I:C), Polyinosinic: polycytidylic acid; D-GalN, D-galactosamine.

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***Edited by:***
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***Reviewed by:***
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*Kazuya Iwabuchi, Kitasato University School of Medicine, Japan*

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

***Received:*** *27 February 2018*
***Accepted:*** *27 April 2018*
***Published:*** *17 May 2018*

***Citation:***
*Al Dulaimi D, Klibi J, Olivo Pimentel V, Parietti V, Allez M, Toubert A and Benlagha K (2018) Critical Contribution of NK Group 2 Member D Expressed on Invariant Natural Killer T Cells in Concanavalin A-Induced Liver Hepatitis in Mice. Front. Immunol. 9:1052. doi: 10.3389/fimmu.2018.01052*

## INTRODUCTION

Liver diseases, including autoimmune hepatitis, viral hepatitis, alcoholic liver disease, and primary biliary cirrhosis, afflict >10% of the world population; however, the immunopathogenesis remains largely undefined which limits the efficacy of clinical treatments of these diseases. Concanavalin A (Con A)-induced hepatitis is a well-established experimental murine model (1), which rapidly induces severe immune-mediated hepatitis due to the specific activation of invariant natural killer (iNKT) cells (2).

Invariant natural killer T cell functional subsets are determined by their cytokine profile and their transcriptional programs. Thus, "Th-1 like," "Th2-like," and "Th-17 like" iNKT cell subsets have been defined (3). The "Th-1 like" iNKT cells expressing the transcription factor T-bet produce large amounts of interferon (IFN)-γ [and interleukin (IL)-4 at lower levels] and form the majority of hepatic and splenic iNKT cells. "Th2-like" iNKT cells are enriched in the lungs and produce IL-4, IL-9, IL-10, and IL-13. "Th-17-like" iNKT cells produce IL-17, IL-21, and IL-22, are present in the lymph nodes and skin, and require the transcription factor retinoic acid receptor-related orphan receptor-γt (ROR-γt) for their development.

All iNKT cell subset, are positively selected by the MHC-I-like molecule CD1d (4), as indicated by complete absence of iNKT cells in CD1d-deficient mice (5). They express TCRs that consist of an invariant Vα14-Jα18 TCRαβ chain (human Vα24Jα18) paired with a limited number of TCRβ chains, Vβ8, Vβ7, orVβ2 (Vβ11 in humans). The TCRs expressed on mature iNKT cells recognize CD1d-presented glycolipids such as α-galactosylceramide (α-GalCer), a potent activator of both mouse and human iNKT cells (6). The acquisition of the phenotype and function of cells is regulated through three developmental stage defined by the progressive acquisition of CD44 and NK1.1, starting by stage 1 ($CD44^{-}NK1.1^{-}$) to stage 2 ($CD44^{+}NK1.1^{-}$), and ultimately to stage 3 ($CD44^{+}NK1.1^{+}$) iNKT cells (7). During the final stage of iNKT development, cells rapidly express NK lineage receptors such as NK1.1, NK group 2 member D (NKG2D), 2B4, CD94/NKG2A and Ly49 receptors (5). They also acquire NK function as stage 3 $NK1.1^{+}$ iNKT cells produce large amounts of cytokines such as IFN-γ, IL-4, TNF-α (8), allowing them once activated to initiate effector functions and modulate the immune response of other immune cells in microbial infections, autoimmune, allergic diseases, and cancer (9, 10). The iNKT cell subset involved in Con A-induced hepatitis are mature iNKT1 cells (mainly stage 3 iNKT cells) because the type of cytokine they produce where shown to be pathogenic in this model (11–13) and because of their preferential accumulation in the liver where they constitute up to 30% of all T cells and 90% of iNKT cells (5).

In the Con A-induced hepatitis model, in addition to cytokine production (IFN-γ, IL-4, TNF-α), it has been also shown that activated iNKT cells also upregulate FAS-L on their surface and induce hepatocyte apoptosis through the FAS/FAS-L pathway which appears to be an important mechanism for liver damage, as iNKT cells from FAS-mutant *gld*/*gld* mice fail to induce hepatitis (2). However, the mechanisms leading to the induction of FAS-L on the surface of iNKT are partly known (13).

NK group 2 member D is a type II transmembrane-anchored glycoprotein, which has been shown to be an activating or costimulatory receptor expressed on many immune cells such as NK cells, activated CD8 T lymphocytes, and iNKT cells (14–16). In mice, NKG2D-ligands include the retinoic acid early-inducible 1 family of proteins [retinoic acid early inducible 1 (RAE-1)], H60, and MULT1 (17–19). The ligands of NKG2D are known to be "stress-inducible" molecules, induced by cellular transformation, viral infection (20), and/or DNA damage (21). Furthermore, NKG2D serves a fundamental role in the surveillance against microbial infection and cancer (22), but an abnormal activation could also be deleterious by causing autoimmune responses. Indeed, the involvement of NKG2D and its ligands has been revealed in many autoimmune diseases, such as rheumatoid arthritis, celiac disease, and autoimmune diabetes (23–25). The physiological role of NKG2D expressed on the invariant Vα14 iNKT cells in hepatitis is yet to be determined.

In this study, we found that the absence of NKG2D reduced disease severity upon Con A administration that is not due to an altered iNKT cell development in these mice. The contribution of NKG2D in the disease severity is mediated by its interaction with NKG2D-ligands expressed on hepatocytes leading to increased cytokine production and FAS-L expression in iNKT cells and increased cytotoxic potential. Overall, our results indicate that NKG2D promotes the effector function of iNKT cells in this model of liver disease and thus represent a potential drug target for prevention of autoimmune hepatitis.

## MATERIALS AND METHODS

### Mice

NK group 2 member D-deficient mice on the B6 genetic background have been described elsewhere (26) and were purchased from The Jackson Laboratory. A scheme of heterozygote breeding pairs was chosen to generate littermates of mice of all three *Klrk1* genotypes (+/+, +/−, and −/−). *CD1D*$^{-/-}$ or *Tcra-J*$^{tmITg}$ (referred as $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice, respectively) on B6 background have been described elsewhere (27, 28). All mice were bred and maintained under specific pathogen-free conditions at our animal facility in compliance with institutional guidelines. Experimental studies were performed in accordance with the Institutional Animal Care and Use Guidelines. The study was approved by the ethics committee "Comité d'Ethique Paris-Nord; C2EA-121," affiliated to the "Comité National de réflexion Ethique en Expérimentation Animale (CNREEA) et au Ministère de l'Enseignement Supérieur et de la Recherche."

### Monoclonal Antibodies (mAbs) and Flow Cytometry

Anti-B220 (clone: RA3-6B2) BV510-, PE-, and FITC-conjugated; anti-TCRβ (H57-597) FITC and AF-700 conjugated, anti-Ly49F (HBF-719) PE-conjugated, anti-HSA (M1/69) BV510- and FITC-conjugated, anti-Ly49G2 (4D11) FITC-conjugated, anti-Ly49C/I (5E6) FITC-conjugated, anti-Ly49A (A1) V450-conjugated, and anti-Ly49H (3D10) PECF594-conjugated mAbs were purchased from BD Biosciences. Anti-CD8 (53-6.7) FITC-conjugated,

anti-CD62L (MEL-14) PE-conjugated, anti-NKG2A (16A11) APC-conjugated, anti-Ly49D (4E5) FITC-conjugated, anti-RAE-1d (d1.23) PE-conjugated, anti-IL-4 (11B11) BV421-conjugated, anti-IFN-γ (XMG1.2) FITC-conjugated, and anti-TNF-α (MP6-XT22) BV510-conjugated mAbs were purchased from Biolegend. Anti-PD-1 (J43) FITC-conjugated, anti-CD122 (5H4) PE-conjugated, anti-CD4 (RM4-5) PerCP Cy5.5-, PECy7- and PECy5-conjugated; anti-NK1.1 (PK136) PECy7-, PerCPCy5.5- and APC- conjugated; anti-CD69 (H1.2F3) FITC-conjugated, anti-2B4 (eBio244F4) PECy7-conjugated, and anti-CD94 (c18d3) efluor450-conjugated mAbs were purchased from eBioscience. CD1d-α-GalCer tetramers (tet) were produced with streptavidin-APC or -BV421 (BD Biosciences) and used for staining as described previously (29). For intracellular staining, cells were fixed with PBS-2% parafomaldehyde solution, washed, and then permeabilized with PBS-0.2% saponin solution (all from Sigma-Aldrich). Flow cytometry was performed with BD Fortessa instrument and FACSDiva v6.1.2 software (BD Biosciences).

## Mononuclear Cell Preparation

Thymus, pooled PLNs (comprising axillary, sub-axillary, maxillary, inguinal, and popliteal lymph nodes) and spleen were isolated, mechanically disrupted, and filtered through a 40-µm stainless steel mesh to obtain single-cell suspensions. Livers were dissected (gall bladder removed) and passed through a 40-µm-pore cell strainer. Cells were washed twice and suspended in PBS-FCS 5%, overlaid in Lymphocyte Separation Medium (Eurobio) and centrifuged at 2,000 rpm for 25 min at room temperature. Hepatic mononuclear cells were collected from the interface and washed.

## RNA Extraction

Each liver tissue was disrupted on ice with a tissueRuptor (Qiagen) in buffer RLT added with β-mercaptoethanol. Total mRNA was extracted and purified using RNeasy Fibrous Tissue mini kit (Qiagen), according to the manufacturer's instructions.

## *In Vivo* iNKT Cell Stimulation With Free α-GalCer

α-Galactosylceramide (KRN 7000) was a gift from the KIRIN company and was dissolved in PBS at a concentration of 220 µg/ml. Twelve-week-old mice were injected i.p. with 0.01, 0.02, 0.04, or 0.2 µg of α-GalCer in a final volume of 200 µl of PBS or vehicle as controls. After 2 h, splenocytes and hepatic mononuclear cells were prepared and incubated for 2 h with Brefeldin A (Sigma-Aldrich) at 5 µg/ml for intracellular staining.

## *In Vitro* Activation of iNKT Cells

For measurements of intracellular cytokines, cells were stimulated with 50 ng/ml phorbol-12-myristate-13-acetate (Sigma-Aldrich), 1 µM ionomycin (Cell Signaling Technologies), or 10 µg/ml of Con A (30), in the presence of 5 µg/ml brefeldin A (Sigma-Aldrich) for 4 h. For measurement of cytokine released in the supernatant, we performed ELISA as described previously (31).

## Induction of Con A-Induced Hepatitis

Concanavalin A (Sigma) was dissolved in pyrogen-free PBS and i.v. injected to mice through the tail vain at a dose of 15 or 25 mg/kg corresponding to no lethal and lethal dose, respectively (2). Sera from individual mice were obtained from 2 to 24 h after Con A injection. Serum alanine aminotransferase (ALT) activity was determined using "Sigma ALT-detection kit" according to manufacturer's protocol.

## Histological Examination and Fluorescence Microscopy

For histological examination, hematoxylin/eosin staining of paraffin-embedded liver sections was performed as described previously (2). RAE-1 was detected in immersion fixed frozen sections of mouse liver using Goat Anti-Mouse RAE-1 Pan Specific Antigen Affinity purified Polyclonal Antibody (Clone AF1136) at 5 µg/ml overnight at 4°C. DAB Cell & Tissue Staining Kit (brown; Catalog # CTS008) was used and counterstained with hematoxylin (blue).

For fluorescence microscopy, liver cells suspensions from mouse non-treaded or treated with Con A were plated on poly (L-lysine)-coated coverslips (Sigma-Aldrich). Cells were then fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% SDS or Triton X-100 for 10 min, followed by blocking with 10% FBS for 20 min. The fixed cells were stained with anti-mouse RAE-1 Pan Specific Alexa Fluor 647-conjugated mAb (Clone #186107), or Rat IgG2A Alexa Fluor 647-conjugared mAbs as isotype control (Clone # 54447), diluted in PBS containing 1 mg/ml BSA. Nuclei were stained with DAPI (Molecular Probes, Invitrogen). Coverslips were mounted with Vectashield (Vector laboratories) and analyzed with a fluorescence microscope (Carl Zeiss LSM-510) and LSM Image Examiner software (Carl Zeiss).

## Statistics

Statistical analyses were performed using PRISM 6 software (GraphPad Software, Inc., CA, USA). Results are expressed as the means ± SD. The statistical significance of differences between experimental groups was calculated by was assessed by the non-parametric Mann–Whitney *U* or the test a one-way ANOVA with a Tukey's post test. *P* values correspond to the following annotation: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

# RESULTS

## Normal Development of iNKT Cells in the Absence of NKG2D

To study the role of NKG2D in Con A-induced hepatitis, we used *Klrk1*$^{-/-}$ mice deficient in NKG2D protein described in a previous study (26). We first assessed the frequency and numbers of iNKT cells determined by using CD1d-tetramers that allow identifying iNKT cells based on their TCR specificity (29). We found in the thymus, spleen, PLNs, and liver, comparable frequencies and absolute numbers of iNKT cells in *Klrk1*$^{-/-}$, *Klrk1*$^{+/+}$, and *Klrk1*$^{+/-}$ littermate controls (**Figure 1A**). Frequencies of CD4$^{+}$ and CD4$^{-}$CD8$^{-}$ double negative (DN) iNKT cell subsets

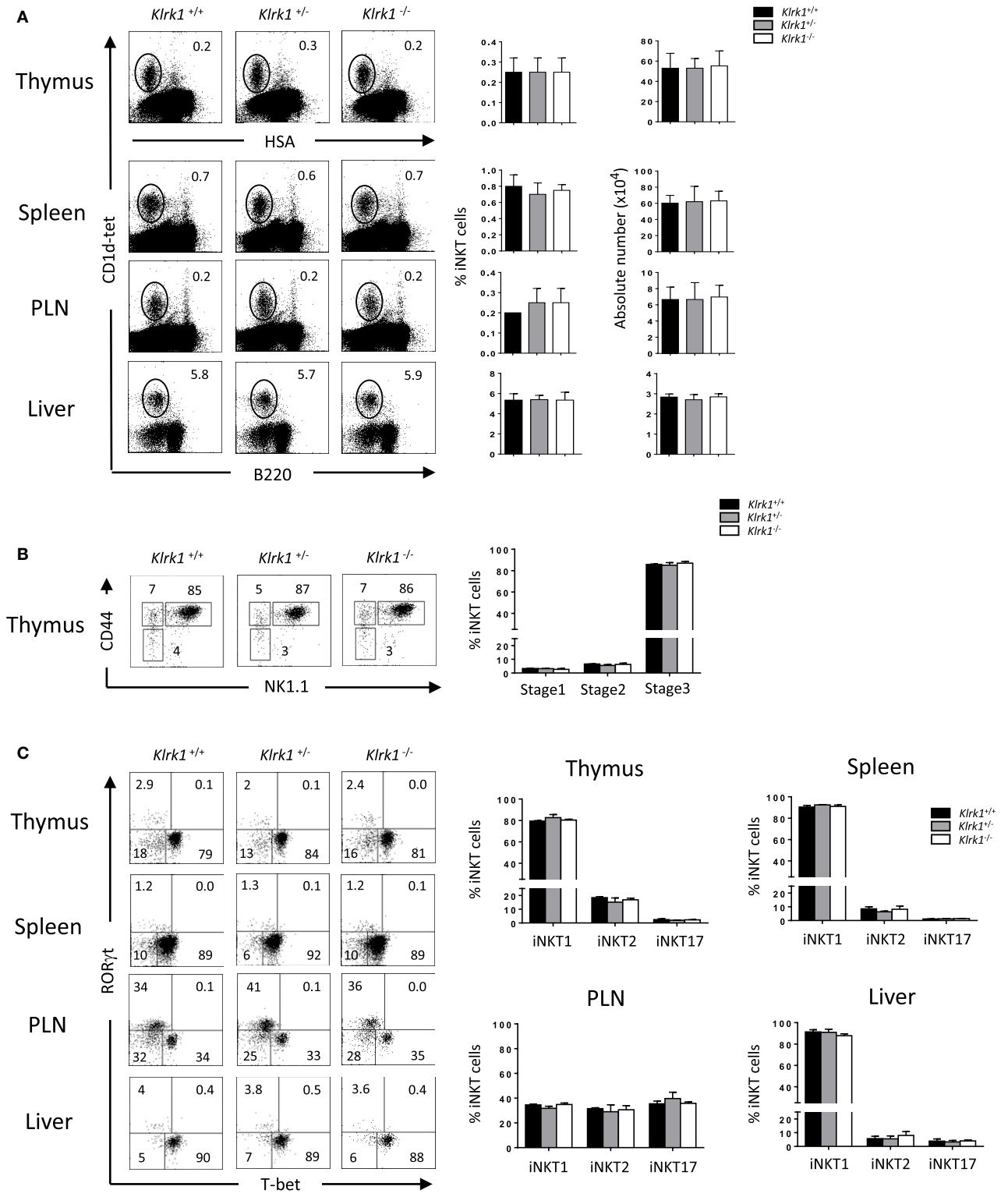


**FIGURE 1** | Normal development of invariant natural killer T cell (iNKT) cells in the absence of NK group 2 member D **(A)**. Representative Flow cytometry analysis of iNKT cells in the thymus, spleen, PLN, and liver. iNKT cells are defined as tet$^+$HSA$^{low}$ in the thymus or tet$^+$B220$^-$ in the periphery. Histograms show frequency of iNKT cells. **(B)** Representative dot plot of NK1.1/CD44 gated on thymic tet$^+$HSA$^{low}$ cells. Histogram plots show the frequency of iNKT cells at stage 1 (CD44$^-$NK1.1$^-$), stage 2 (CD44$^+$NK1.1$^-$), and stage 3 (CD44$^+$NK1.1$^+$) of iNKT cell development. **(C)** Representative staining of iNKT1 (T-bet$^+$), iNKT2 (RORγt$^-$/T-bet$^-$), and iNKT17 (RORγt$^+$) subsets among iNKT cells at the indicated organs. Frequencies of these different subsets are represented in histograms. Data are from five experiments where three mice aged 5–6 week were used per experiment and presented as mean ± SD. Numbers represent percentages.

in *Klrk1*$^{-/-}$ mice were not different from those of *Klrk1*$^{+/+}$ and *Klrk1*$^{+/-}$ littermate controls in all organ tested.

It has been shown that T cell-surface costimulatory molecules, such as CD28 and ICOS, play a role in iNKT cell development through the induction of T-bet (32). We next sought to assess if the absence of NKG2D, a costimulatory activating receptor, could impair the maturation of iNKT cells. We found comparable frequencies and absolute numbers of early precursor stage 1, stage 2, and stage 3 iNKT cells, the latter being the fully mature and the most abundant iNKT cells in the adult thymus, in *Klrk1*$^{-/-}$, and *Klrk1*$^{+/-}$, compared to *Klrk1*$^{+/+}$ mice (**Figure 1B**, and data not shown).

Because NKG2D is expressed on the majority of iNKT1 cells, we hypothesized that it might play a role in their terminal maturation or maintenance. We, thus, used T-bet and RORγt expression to evaluate the composition of iNKT subsets in the absence of NKG2D. As shown in **Figure 1C**, iNKT1 (T-bet$^{+}$), iNKT17 (RORγt$^{+}$), and iNKT2 (T-bet$^{-}$RORγt$^{-}$) iNKT cell frequencies and numbers are similar in the thymus, spleen, PLNs, and liver of *Klrk1*$^{-/-}$ mice compared to *Klrk1*$^{+/-}$ or *Klrk1*$^{+/+}$ littermate control.

We also assessed proportions and numbers of other effector cells that express NKG2D, such as NK and CD8$^{+}$ cells in the thymus and spleen and found that the proportions and absolute numbers of CD8$^{+}$ and NK cells remain unchanged in NKG2D-deficient mice compared to wild-type mice (Figure S1 in Supplementary Material) confirming previous results (26).

Taken together, these results show that NKG2D is dispensable for the early developmental stages and terminal maturation of thymic and peripheral iNKT cells. They also indicate that the generation all iNKT cells subsets, the one expressing (iNKT1) or non-expressing (iNKT2 and iNKT17) NKG2D, is not altered in the absence of NKG2D.

## The Expression of iNKT Cell Phenotypic Makers Is Preserved in the Absence of NKG2D

To address the relevance of NKG2D on shaping the NK-cell receptor repertoire on iNKT cells, we analyzed the expression of inhibitory and activating receptor on NK1.1$^{+}$ iNKT cells in the absence of NKG2D expression. Among the inhibitory receptor tested we observe normal (Ly49F, Ly49G2, and Ly49A) or minor (CD94/NKG2A/C/E) differences of the expression of these receptors in iNKT cells from *Klrk1*$^{-/-}$ mice (**Figure 2**, and Figure S2A in Supplementary Material) compared to *Klrk1*$^{+/+}$ mice. The minor difference for CD94/NKG2A/C/E expression is organ specific

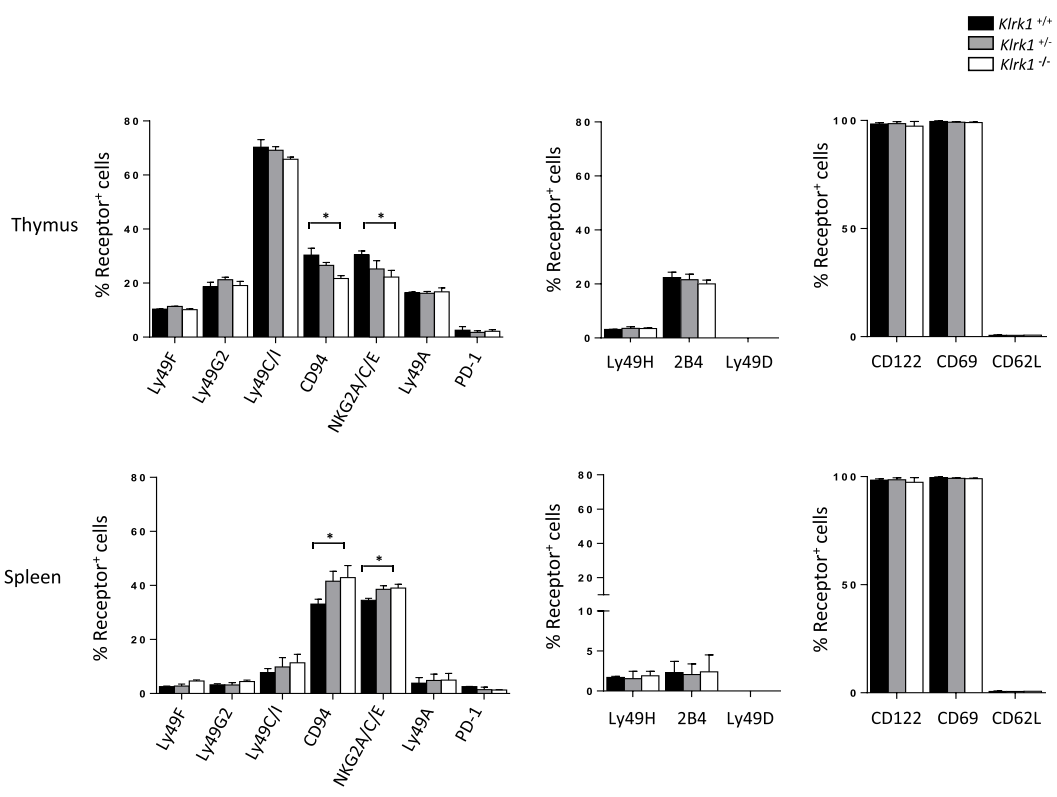


**FIGURE 2** | The expression of invariant natural killer T cell (iNKT) cell phenotypic makers is preserved in the absence of NK group 2 member D. Frequencies of inhibitory (left) and activating (middle) NK-receptors, and other activating receptors (right) among NK1.1$^{+}$ iNKT cells in the thymus and spleen. iNKT cells are defined as tet$^{+}$HSA$^{low}$ in the thymus or tet$^{+}$B220$^{-}$ in the spleen. Data are from five experiments where three mice aged 5- to 6-week old were used per experiment and presented as mean ± SD. Numbers represent percentages. Significance is represented by an asterisk and was evaluated with non-parametric Mann–Whitney *U* test.

because, while slightly upregulated in the thymus, the opposite effect is observed in the spleen (**Figure 2**; Figure S2A in Supplementary Material) and liver (Figure S2B in Supplementary Material). We also found that basal expression of the inhibitory costimulatory receptor PD-1 was not altered in NKG2D-deficient thymic and splenic iNKT cells (**Figure 2**; Figure S2A in Supplementary Material).

On the other hand, frequencies of iNKT cells expressing activating receptors tested (Ly49H, 2B4, and Ly49D) were similar in the spleen and thymus of all three genotypes (**Figure 2**; Figure S2C in Supplementary Material, upper panel). We also found that the frequency of iNKT cells expressing memory T cell ($CD44^{high}CD62L^{low}$) and activation (CD122, CD69) markers was similar between genotypes in both the thymus and spleen (**Figure 2**; Figure S2C in Supplementary Material, lower panel).

Taken together, these data indicate that NKG2D expression is dispensable for phenotypic maturation of iNKT cells.

## NK1.1$^+$ iNKT Cells Generated in the Absence of NKG2D Keep Their Potential to Produce Cytokine Upon Stimulation

To test if the function of iNKT cells is altered in the absence of NKG2D, we first assessed their cytokine production *in vitro* after PMA/ionomycin stimulation. As show in the histograms of **Figure 3A** and in representative dot plots in **Figure 3A** in Supplementary Material, the frequencies of IFN-γ-, TNF-α-, and IL-4-positive cells among NK1.1$^+$ iNKT cells from *Klrk1*$^{-/-}$, *Klrk1*$^{+/-}$, and *Klrk1*$^{+/+}$ littermate controls were not different in both organs of each genotype.

We next sought to assess the consequences of NKG2D deficiency on iNKT cells function *in vivo* with the rationale that NKG2D activation on iNKT cells can act synergistically with IL-12 to promote a Th-1-skewed response (33). To do so, injected intraperitoneally optimal dose of α-GalCer (0.2 μg as determined in Figure S3B in Supplementary Material) and found that frequencies of cytokine positive iNKT cells were not statistically different in the spleen and liver of α-GalCer-treated or vehicle-treated *Klrk1*$^{-/-}$, *Klrk1*$^{+/-}$, and *Klrk1*$^{+/+}$ littermate controls (**Figure 3B**; Figure S3C in Supplementary Material). Because a strong TCR stimulation through optimal dose of α-GalCer might have masked a possible contribution of NKG2D, we used a suboptimal dose of α-GalCer (0.02 μg) and found that cytokine production by iNKT cells is also not altered in NKG2D-deficient iNKT cells (data not shown).

We also measured mean fluorescence intensity (MFI) of cytokine expressed by iNKT, assessed by intracellular staining,

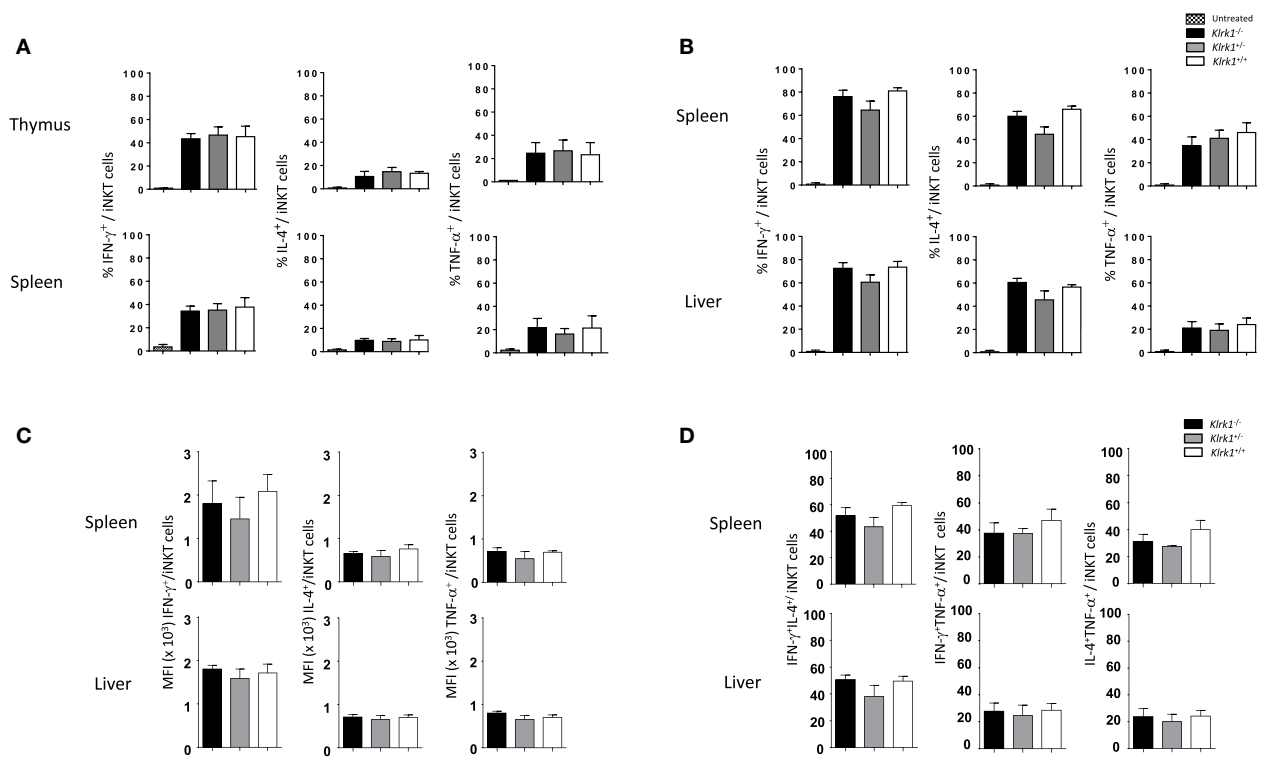


**FIGURE 3** | Unchanged cytokine production capabilities of invariant natural killer T cell (iNKT) cells in the absence of NK group 2 member D. **(A)** Frequencies of interferon (IFN)-γ, interleukin (IL)-4, and TNF-α production by iNKT cells *in vitro,* assessed by intracellular staining, in the thymus and spleen upon PMA/ionomycin stimulation for 4 h. **(B)** Frequencies of IFN-γ, IL-4, and TNF-α production by iNKT cells, assessed by intracellular staining, in the spleen and liver *in vivo* 2 h after i.p. injection of α-galactosylceramide. **(C)** Mean florescence intensity (MFI) of IFN-γ, IL-4, and TNF-α expression by iNKT cells from the experiment in **(B)**. **(D)** Frequencies of IFN-γ$^+$IL-4$^+$, IFN-γ$^+$TNF-α$^+$, and IL-4$^+$TNF-α$^+$ producing iNKT cells from the experiment in **(B)**. iNKT cells are defined as tet$^+$HSA$^{low}$ in the thymus or tet$^+$B220$^-$ in the spleen or liver. Data are from three experiments where three mice aged 5- to 6-week old were used per experiment and presented as mean ± SD.

upon α-GalCer injection and found that MFI of IFN-γ, IL-4, and TNF-α are comparable in NKG2D-deficient mice and wild-type mice (**Figure 3C**). We also compared the frequencies of iNKT cells producing IFN-$\gamma^+$IL-$4^+$, IFN$\gamma^+$TNF-$\alpha^+$, and IL-$4^+$TNF-$\alpha^+$ in wild-type and NKG2D-deficient mice and found similar distribution (**Figure 3D**). These results indicate that NKG2D-deficient iNKT cells do not differ in their capabilities to produce cytokine on a per cell basis and do not lose their polyfunctionality.

Altogether, these *in vitro* and *in vivo* results show that iNKT cells that develop in the absence of NKG2D keep their potential to produce cytokines.

## Reduced Hepatic Injury and Increased Survival in the Absence of NKG2D-Mediated Signals in Con A-Induced Hepatitis

Several experimental results demonstrated that iNKT cells are key effector cells in Con A-mediated liver damage (2, 13). To explore the possible contribution of NKG2D expressed on iNKT cells to the pathogenesis of Con A-induced hepatitis, we i.v. injected non-lethal doses (15 mg/kg) of Con A into NKG2D-deficient mice and measured serum ALT levels after Con A injection. In preliminary experiments, elevated serum levels of ALT were first observed 4–5 h after injection and peaked at 8–12 h after injection (**Figure 4A**). We thus measured serum ALT levels 10 h after Con A injection and as shown in **Figure 4B**, serum ALT levels were markedly reduced in *Klrk1*$^{-/-}$ as compared to *Klrk1*$^{+/+}$ mice. CD1d$^{-/-}$ or Jα18$^{-/-}$ mice, both missing iNKT cells and resistant to Con A-induced hepatitis, were used as controls. Histological findings of degenerative changes in the liver were clearly correlated with the serum ALT levels (**Figure 4C**). In fact, histological examination showed focal and mild injury in *Klrk1*$^{-/-}$ mice whereas in *Klrk1*$^{+/+}$ mice a diffuse and massive degenerative change was observed as previously reported (2). We next evaluated whether reduced liver injury in *Klrk1*$^{-/-}$ mice would have a positive impact on mice survival after injection of lethal doses of Con A (30 mg/kg). As shown in **Figure 4D**, whereas all wild-type mice died between 15 to 20 h, 20% of *Klrk1*$^{-/-}$ mice died within this time frame, and 60% of them were still alive at 40 h, clearly demonstrating the less sensitivity of *Klrk1*$^{-/-}$ mice to Con A-induced hepatitis. The pretreatment of *Klrk1*$^{+/+}$ mice with neutralizing anti-NKG2D antibodies reduced ALT levels (**Figure 4B**) and increased the survival rates to the same level as in genetically modified *Klrk1*$^{-/-}$ mice (**Figure 4D**), indicating that this beneficial effect observed in *Klrk1*$^{-/-}$ mice could not be attributed to a developmentally related alteration in the function of iNKT cells.

Overall, these results indicated that NKG2D expressed on iNKT cells contribute substantially to the Con A-induced hepatitis.

## Reduction of Cytokine Production and Fas-L Expression in the Absence of NKG2D in ConA-Induced Hepatitis

Different cytokines, such as TNF-α, IFN-γ and IL-4, that are released upon application of Con A have been suggested to mediate hepatic inflammation and parenchymal necrosis (11–13). We examined intracellular cytokine production by liver iNKT cells 2 h after injection of non-lethal doses of Con A. We found that the frequency of IFN-γ producing liver NK1.1$^+$ NKT cells from *Klrk1*$^{-/-}$ mice dropped to 35±4% SD compared to 50±4% SD observed in wild-type mice (**Figure 5A**). A more drastic drop for NK1.1$^+$ NKT cells producing TNF-α was observed as only 16±4% SD of cells from *Klrk1*$^{-/-}$ mice produced TNF-α compared to 50±4% SD observed in wild-type mice. A reduction in IL-4 cytokine production was also observed and the frequency of NK1.1$^+$ iNKT cells producing IL-4 dropped from 30±4% SD to 10±4% SD in cells from *Klrk1*$^{+/+}$ versus *Klrk1*$^{-/-}$, respectively. Such fluctuation in cytokine production was not observed in spleen iNKT cells (Figure S4A in Supplementary Material).

Different studies have shown that FAS/FAS-L-mediated cytotoxicity by iNKT cells is critically important for hepatic injury in the Con A model (2, 13). As shown in **Figure 5B**, hepatic iNKT cells rapidly upregulate cell-surface FAS-L expression upon Con A administration. In the absence of NKG2D in *Klrk1*$^{-/-}$ mice, this upregulation is three time lower than the one observed in *Klrk1*$^{+/+}$ mice (MFI of 1,800 ± SD vs 600 ± SD in iNKT cells from *Klrk1*$^{+/+}$ and *Klrk1*$^{-/-}$ mice, respectively). Such upregulation was not observed in spleen iNKT cells (Figure S4B in Supplementary Material), although they produced cytokine in response to Con A (Figure S4A in Supplementary Material).

Overall, our results indicate that NKG2D deficiency reduced cytokine production and FAS-L expression by liver iNKT cells upon Con A injection. Because these produced cytokines and FAS-L are key mediators of the disease, these findings are likely to explain the reduced liver injury and increased survival we observed in *Klrk1*$^{-/-}$ mice.

## Crosstalk Between iNKT Cells and Hepatocytes Occur *via* NKG2D–NKG2D-L Interaction in Con A-Induced Hepatitis

So far our results indicate that NKG2D is important to induce cytokine production and FAS-L expression by iNKT cells upon Con A injection. To assess if Con A could induce directly these biological changes in iNKT cells, we incubated liver mononuclear cells in the presence of Con A and measured cytokine production and FAS-L expression. Four hours after Con A incubation, we found that NK1.1$^+$ iNKT cells produce lower amount of cytokines compared to what observed after PMA/ionomycin stimulation (around five times less IFN-γ and IL-4, and 10 times less TNF-α), indicating the inefficacy of Con A to stimulate iNKT cells (**Figure 6A**). Our results are confirmed by measuring cytokine production in the supernatant by ELISA as we barely detect cytokine produced in the presence of Con A compared to PMA/ionomycin (**Figure 6B**). We were also not able to detect FAS-L expression on the surface of iNKT cells after 4 or 18 h incubation in the presence of Con A, while we observed CD25 expression indicating activation of these cells (**Figure 6C**). The limited cytokine production and absence of FAS-L expression are also observed in spleen iNKT cells (Figure S5 in Supplementary Material). These *in vitro* results suggest strongly that Con A could not promote directly the

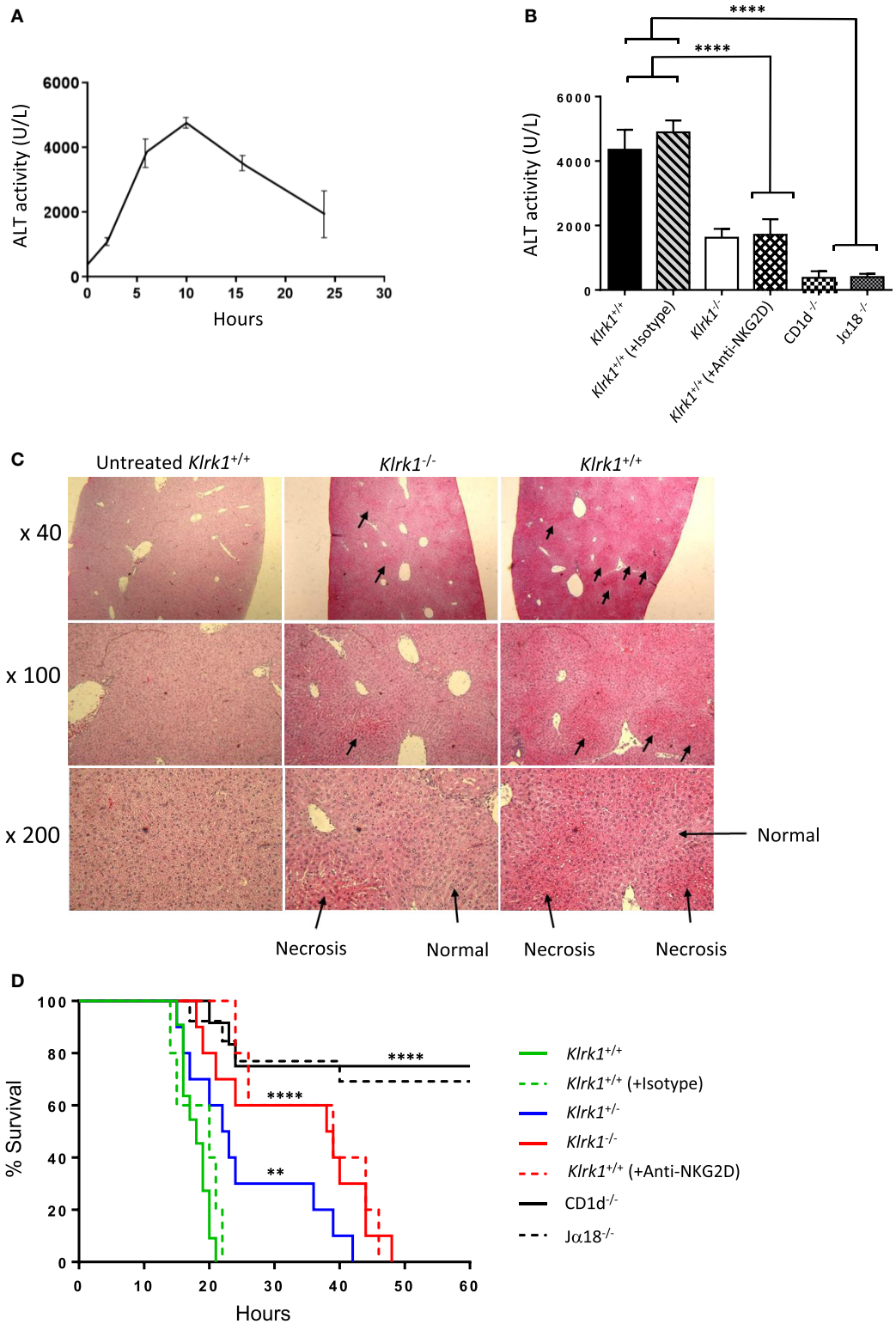


**FIGURE 4** | Reduced hepatic injury and increased survival in the absence of NK group 2 member D (NKG2D)-mediated signals in concanavalin A (Con A)-induced hepatitis **(A)**. Kinetics of serum transaminase (ALT) levels in B6 mice after Con A administration (15 mg/kg). **(B)** ALT levels 10 h after Con A administration (15 mg/kg) in mutant, wild-type, and wild-type mice injected or not with anti-NKG2D neutralizing antibodies. **(C)** Light micrographs of the liver 10 h after administration of Con A (15 mg/kg) with hematoxylin and eosin stating (40×, 100×, 200×) are shown. **(D)** Survival curve of mice injected with lethal dose (25 mg/kg) of Con A are shown. Results are from four experiments where five mice of each genotype were used per experiment. Significance compared to wild type is represented by asterisks and was evaluated by a one-way ANOVA with a Tukey's post test.

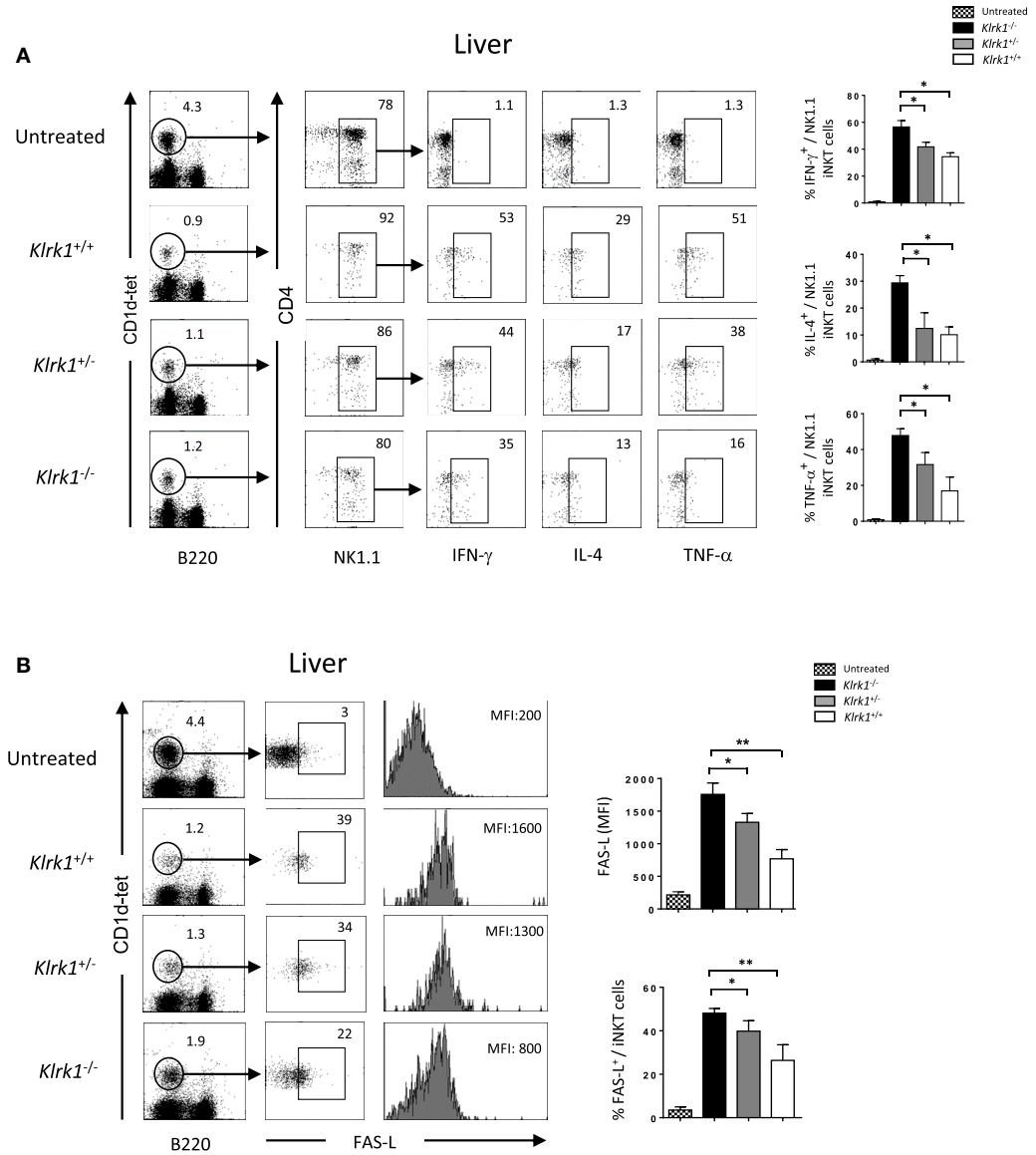


**FIGURE 5** | Reduction of cytokine production and FAS-L expression by liver invariant natural killer T cell (iNKT) cells in the absence of NK group 2 member D upon concanavalin A (Con A) administration, Representative intracellular staining of interferon (IFN)-γ, interleukin (IL)-4, and TNF-α production **(A)**, or FAS-L expression **(B)** by liver NK1.1$^+$ iNKT after 2 h of i.v. administration of Con A (15 mg/kg). iNKT cells are defined as tet$^+$B220$^-$. The frequencies of positive cells and mean florescence intensity (MFI) of FAS-L are shown in the histograms. Results are from three experiments where three mice of each genotype were used per experiment and presented as mean ± SD. Numbers represent percentages. Significance is represented by an asterisk and was evaluated with non-parametric Mann–Whitney *U* test.

pathogenicity of iNKT cells *in vivo* upon Con A injection. We thus reasoned that NKG2D action will be mediated by its interaction with NKG2D-ligands expressed on stressed hepatocytes. We thus investigated the expression of RAE-1, one of the major ligand of NKG2D, in liver cells. We found that RAE-1 transcripts are upregulated upon Con A administration (**Figure 7A**). At the protein level, we found by FACS staining that RAE-1 is expressed at basal level on hepatocytes and that its expression is upregulated upon Con A injection (**Figure 7B**). By florescence microscopy, we were able to confirm these results by visualizing RAE-1-expressing hepatic cells upon Con A administration compared to basal level in non-treated mice (**Figure 7C**). Finally, histological examination *in situ* showed a dose-dependent increase in RAE-1 expression on hepatocyte section upon administration of a non-lethal and lethal dose of Con A (**Figure 7D**). The higher expression of RAE-1 observed at lethal dose of Con A correlate with the higher degenerative changes observed by H&E staining.

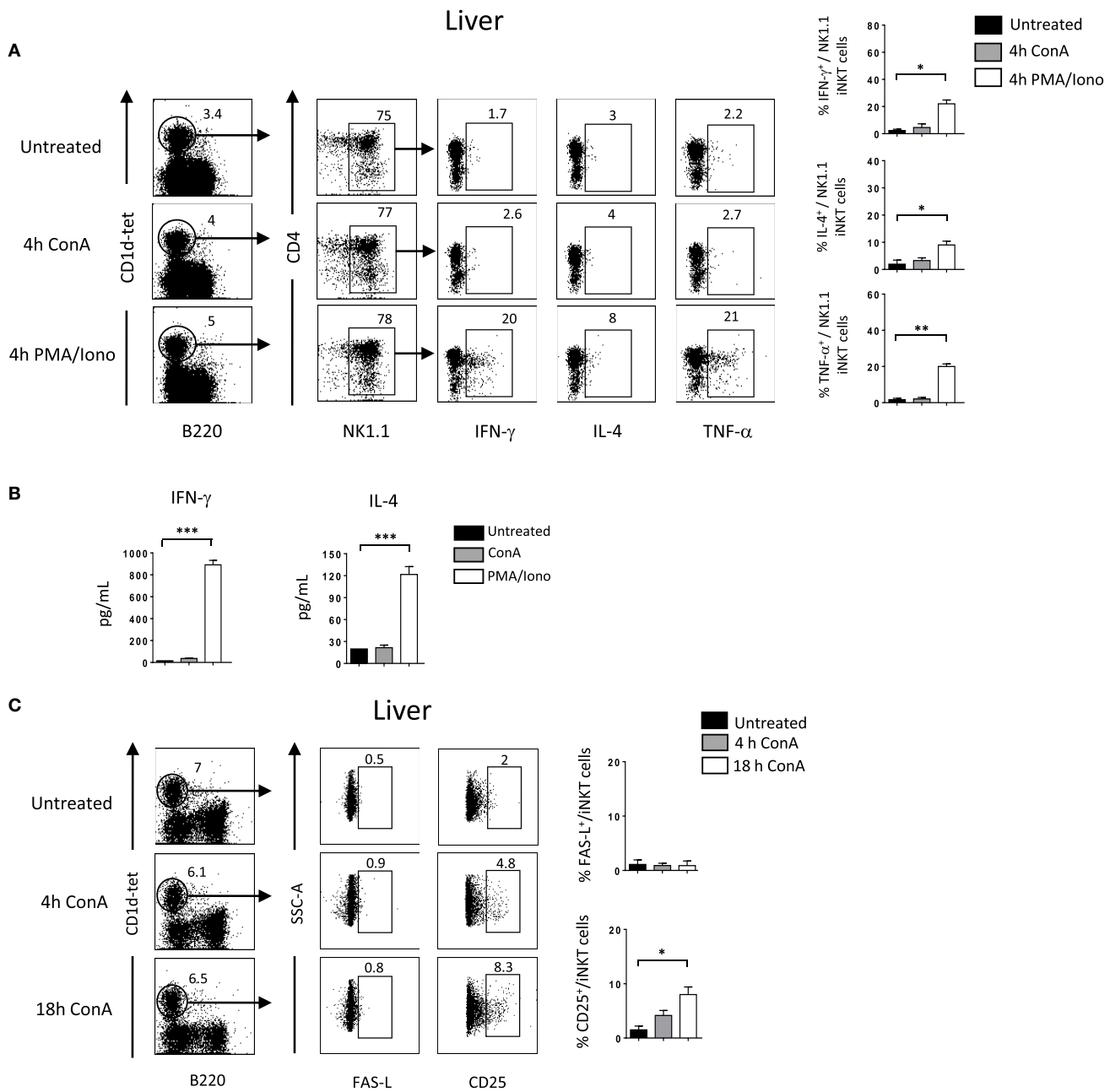


**FIGURE 6** | Cytokine production and FAS-L expression by liver invariant natural killer T cell (iNKT) cells is not induced directly *in vitro* by Concanavalin A (Con A) **(A)**. Representative intracellular staining of interferon (IFN)-γ, interleukin (IL)-4, and TNF-α production expression by liver NK1.1$^+$ iNKT after 4 h incubation in the presence of Con A (10 μg/ml) or PMA/ionomycin. iNKT cells are defined as tet$^+$B220$^-$. The frequencies of positive cells are shown in the histograms. **(B)** Cytokine level in the supernatant by liver mononuclear cells after O/N incubation with Con A or PMA/ionomycin at the previously indicated concentration. **(C)** Representative cell-surface staining of FAS-L expression by liver NK1.1$^+$ iNKT after 4 or 18 h incubation in the presence of Con A (10 μg/ml). Results are from three to four experiments where three mice of each genotype were used per experiment and presented as mean ± SD. Significance is represented by an asterisk and was evaluated with non-parametric Mann–Whitney *U* test.

Overall, these results confirm the hypothesis that the cytotoxic activity of iNKT mediated by NKG2D upon Con A administration is promoted by its interaction with NKG2D-L expression on stressed hepatocytes and contribute to hepatic injury in this murine model.

## DISCUSSION

Invariant natural killer T cell cells preferentially distribute to the liver and have been shown to trigger hepatic injury following Con A injection (2). To elucidate the role of NKG2D in this model, we

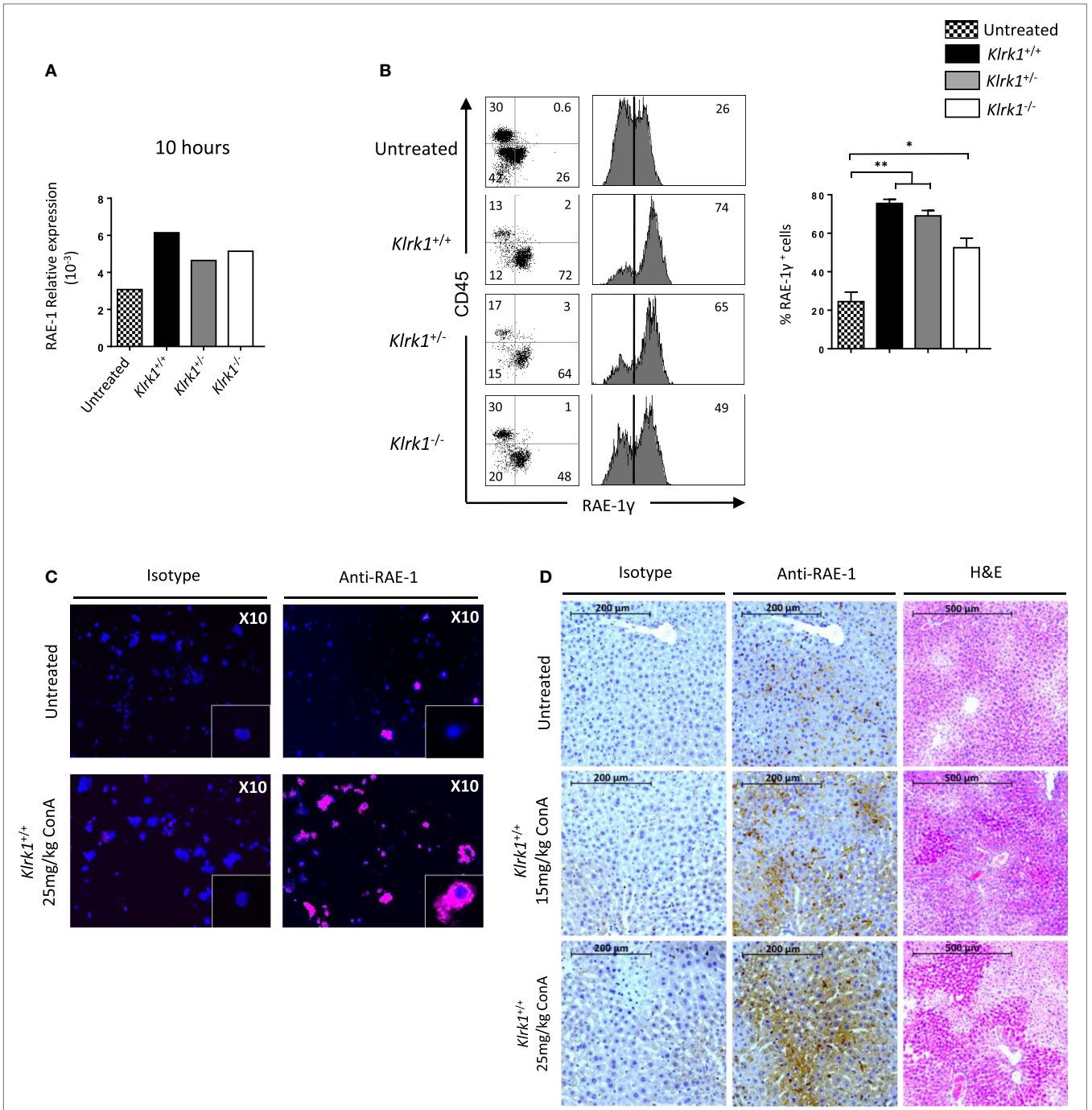


**FIGURE 7** | Retinoic acid early inducible 1 (RAE-1) expression is upregulated upon concanavalin A (Con A) administration *in vivo* **(A)**. Relative expression of RAE-1 transcript in liver tissue in non-injected, or 10 h after Con A administration (25 mg/kg). **(B)** Representative histogram staining of RAE-1 expression at the cell-surface of hepatocytes in non-injected or after 8 h after Con A administration (25 mg/kg). **(C)** RAE-1 expression assessed in hepatocytes by fluorescent microscopy in non-injected or 3 h after Con A administration (25 mg/kg). **(D)** Histological examination of RAE-1 expression on hepatic sections from mice non-injected or 8 h after Con A administration (15 or 25 mg/kg). Note that increased doses of Con A correlate with increased RAE-1 expression and liver degenerative changes as assessed by H&E staining. Results are from four experiments where three mice of the indicated genotype were used per experiment and presented as mean ± SD. Significance is represented by an asterisk and was evaluated with non-parametric Mann–Whitney *U* test.

used deficient mice for this receptor and report that liver injury was reduced significantly in the absence of the NKG2D, correlating with increased survival. Our results show that NKG2D plays a role in the disease development by augmenting the cytotoxic potential of iNKT cells through induction of FAS-L on their surface after Con A injection. In addition, we found that NKG2D

is also necessary to upregulate the production of inflammatory cytokines, such as IFN-γ, IL-4, and TNF-α by iNKT cells. Mechanistically, we show that activation of iNKT cells by Con A involves the upregulation NKG2D-L on hepatocyte and their interaction with NKG2D on iNKT cells.

The ontogenetic signals that lead to the development and functional changes of iNKT cells are poorly understood. Even though mature thymic, splenic, and hepatic iNKT1 cells are phenotypically similar, they express NK-cell receptors at very different levels, besides behaving as different functional populations. These differences might be due to distinct agonistic ligands that provide different signals depending on the anatomical location and microenvironment. There is evidence that NKG2D-ligands can be expressed to a limited extent on healthy cells in the thymus, liver, spleen, lungs, intestine, and other organs of mice and humans (34, 35). We could, therefore, hypothesize that NKG2D/NKG2D-ligand interactions in the thymus and peripheral tissues might deliver signals to iNKT cells that would exert an influence on their development and functional differentiation. Our study explored the role of NKG2D in the development and function of iNKT cells in the absence of NKG2D by using *Klrk1*$^{-/-}$ mice and show that NKG2D do not affect the maturation or the production of different iNKT cell subsets (iNKT1, iNKT2, and iNKT17) including the one expressing NKG2D (iNKT1). We found, however, that NKG2D slightly shapes the repertoire of NK-cell receptor on iNKT cells, especially of CD94, whose expression was decreased in thymic iNKT cells and, conversely, increased in splenic and liver iNKT cells. These differences could be due to tissue-specific regulation on the acquisition or selection of the NK receptor repertoire. The fact that thymic iNKT cells express NK-receptors at higher frequencies points to this direction. CD94 is a co-receptor that is expressed as a heterodimer along with NKG2A, to form an inhibitory receptor, or with NKG2C or NKG2E, forming an activating receptor. However, there is evidence that thymic, splenic, and hepatic iNKT cells preferentially express NKG2A/CD94 in B6 mice (36, 37). With this regard, we found that the expression of NKG2A parallel the one of CD94 as assessed with anti- NKG2A/C/E antibodies. A previous study provided evidence of a moderate alteration of NK-cell differentiation in NKG2D-deficient mice (26). Another study provided evidence for an important regulatory role of NKG2D in the development of NK cells (38). This latter finding points to different molecular mechanisms of development between NK and iNKT cells. For instance, an explanation could be that NK cells express NKG2D in early stages of development (38), whereas this receptor is not expressed early on common iNKT cell precursors but relatively late during differentiation of iNKT1 cells.

Our findings reveal that iNKT cells that developed in the absence of NKG2D have no intrinsic functional defect. In fact, our results reveal that iNKT cells from *Klrk1*$^{-/-}$ mice keep their Th-1-like profile upon stimulation and are as efficient as iNKT cells from wild-type mice to rapidly produce large amounts of cytokines response *in vitro* in response to PMA/ionomycin and *in vivo* to an optimal dose of a specific antigen. Since 1/NK-receptors can regulate TCR signaling threshold to antigen, 2/NKG2D increases IFNγ production of NK cells stimulated with suboptimal doses of IL-12, 3/the activation of iNKT cells by α-GalCer is IL-12-mediated (9, 39), it is possible that the threshold of activation of iNKT cells could be impaired in NKG2D deficiency. We excluded this possibility as we used suboptimal concentrations of α-GalCer but were not able to shed any difference in the response of iNKT cells in the absence of NKG2D.

Having excluded an impact of NKG2D on iNKT cell development, there is a possibility that some functions of NKG2D-deficient iNKT cells could be compromised in the context of disease. NKG2D is a potent costimulatory receptor of TCR-dependent activation of cytotoxic CD8$^+$ T cells, γδ T cells, and NKT cells (22). It has been shown that the expression of NKG2D is required for disease induction in murine models of primary Hepatitis B virus infection (40, 41) and liver inflammation in atherosclerosis (42). In our present study, we used a model of Con A-induced liver injury to assess the role of NKG2D-expressing iNKT cells in a pathologic setting. In this model, iNKT cells are pathogenic through their cytotoxic FAS-L mediated action and their cytokine production that act directly or indirectly on hepatocytes. We found that upon Con A injection in the absence of NKG2D cytokine production by iNKT cells is reduced and FAS-L expression is downregulated (by three) compared to what observed in the presence of NKG2D. The lower response of iNKT cells is accompanied by a reduced liver injury and an increased survival and point out on the role of NKG2D in Con A-induced hepatitis. Our results could not be interpreted as result of a developmental and/or functional defect of iNKT cells in the absence of NKG2D as experiments performed with anti-NKG2D neutralizing antibodies showed a reduced activation of iNKT cells and a resistance of Con A-induced hepatitis similar to the one observed in genetically modified *Klrk1*$^{-/-}$ mice.

Our study provides a mechanism by which NKG2D play a role in Con A-induced hepatitis. The model we propose implicate hepatocytes that are stimulated by Con A and express NKG2D-L including RAE-1 (Figure S6 in Supplementary Material). As indicated by previous studies, engagement of NKG2D with its ligands leads to phosphorylation of the adaptor protein DAP12 (DAP 10 in humans) and recruitment of phoshpoinositide-3-kinase, triggering a downstream signaling with the subsequent cytotoxic response and IFN-γ production (43). For iNKT cells, the cytotoxic response is mediated by FAS-L expression upon NKG2D–NKG2D-L interaction. Hepatic cells are then killed upon FAS-L and FAS interaction, the latter expressed constitutively. IL-4 produced by iNKT cells also contributes to potentiate FAS-L expression (13). IFN-γ and TNF-α could act directly on hepatocyte to cause liver injury (12, 30, 44). The absence of NKG2D in *Klrk1*$^{-/-}$ mice shut off the aforementioned cascade of event reducing FAS-L expression and cytokine production leading to reduced iNKT cell cytotoxic potential and liver injury. The lesser expression of RAE-1 we observed upon Con A injection in *Klrk1*$^{-/-}$ compared to *Klrk1*$^{+/+}$ is likely the consequence of a lesser inflammatory environment induced by iNKT cells in the absence of NKG2D expression. In this model, a direct action of Con A on iNKT cells is limited if not absent as assessed by *in vitro* stimulation experiment showing limited amount of cytokine produced and absence of FAS-L upregulation.

Although our results show the involvement of NKG2D in the development of Con A-induced hepatitis, the participation of

NKG2D/RAE-1 (and other ligands) appeared to be partial and the survival was not equivalent to that of NKT cell-deficiency. This indicates the existence other potential of cytocidal molecules/mechanisms expressed by NKT cells. With this regard, a recent report showed that TNF superfamily receptor OX40 triggers iNKT cells pyroptosis and liver injury (45). Interestingly, Con A injection failed to induce liver injury and iNKT cell depletion, demonstrating an important role for OX40/NKT cells in liver injury in this model.

It is worth mentioning that the role of iNKT cells and NKG2D in the liver is not unambiguous. A recent study showed that iNKT cells are required to protect the liver from $CCL_4$-induced fibrosis treated with IL-30 (46). Reminiscent to our study, iNKT cells require NKG2D surface expression to inhibit liver fibrosis after IL-30 treatment by selectively removing $CCL_4$-activated hepatic stellae cells through an NKG2D/RAE-1interaction. On the other hand, a recent study showed that NKG2D, known to have antitumorigenic functions, promotes tumor growth in a model of hepatocellular carcinoma (47). Also, NKG2D promoted natural killer cell-mediated fulminant hepatitis in mice (48). In this polyinosinic:polycytidylic acid/D-galactosamine-induced model of hepatitis, it was shown that simultaneous knockdown of multiple ligands of NKG2D alleviated the disease.

Overall, our result provides the first examination of the role NKG2D in the development and function of NKG2D expressed on iNKT cells. We found no intrinsic defect in the functional capabilities of iNKT cells developed in the absence of NKG2D, even though we observed a minor alteration of the expression of CD94/NKG2A. We also report a critical role for NKG2D in controlling iNKT cell response in a model of Con A-induced hepatitis and provided new insight in the comprehension of the mechanism of action of iNKT cell in this hepatitis model providing novel strategies for clinical applications.

## AUTHOR CONTRIBUTIONS

DD and VOP designed, performed, and analyzed the experiments; JK designed, performed, analyzed, guided experiments, and addressed editor/referees' comments; KB designed, analyzed the experiments, wrote the paper, and supervised the study; VP performed experiments; MA and AT analyzed the experiments and provided essential knowledge.

## ACKNOWLEDGMENTS

The authors would like to thank IUH FACS facility, particularly Niclas Setterblad, Christelle Doliger and Sophie Duchez. The authors are grateful to Nadia Guerra for sharing information, Nicolas Dulphy for helpful discussion, and all Benlagha's team members. The authors are especially indebted to the NIH Tetramer Facility for providing CD1d-tetramres.

## FUNDING

This work was supported by INSERM, idex-SLI, idex-MelaTNK (KB and JK), Université Paris Diderot, Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation (DA).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01052/full#supplementary-material.

**FIGURE S1** | Frequency and numbers of $CD8^+$ and NK cells in the absence of NK group 2 member D. Representative Flow cytometry analysis of CD8/TCRβ and NK1.1/TCRβ staining in the thymus and spleen. Histograms show frequencies of $CD8^+$ cells ($CD8^+/TCR\beta^+$) and NK cells ($NK1.1^+/TCR\beta^-$) as defined by the gates. Data are from five experiments where three mice aged 5- to 6-week were used per experiment and presented as mean ± SD. Numbers represent percentages.

**FIGURE S2** | Phenotypic characterization of invariant natural killer T cell (iNKT) cells in the absence of NK group 2 member D. **(A)**. Representative staining of inhibitory NK-receptors among $NK1.1^+$ iNKT cells in the thymus and spleen. **(B)** Representative staining of CD94 and NKG2A/C/E inhibitory NK-receptors among $NK1.1^+$ iNKT cells in the liver. Histograms show frequencies. **(C)** Representative staining of activating NK-receptors (upper panel) and other activating receptors (lower panel) among $NK1.1^+$ iNKT cells in the thymus and spleen. Numbers represent percentages. Data are representative of five experiments where three mice aged 5- to 6-week old were used per experiment and presented in histograms as mean ± SD. Significance is represented by an asterisk and was evaluated with non-parametric Mann–Whitney *U* test.

**FIGURE S3** | Cytokine production capabilities of invariant natural killer T cell (iNKT) cells in the absence of NK group 2 member D. **(A)** Representative staining of interferon (IFN)-γ, interleukin (IL)-4, and TNF-α production by iNKT cells *in vitro,* assessed by intracellular staining, in the thymus and spleen upon PMA/ionomycin stimulation for 4 h. **(B)** IFN-γ production by spleen iNKT cells assessed by intracellular staining in response to increased doses of α-galactosylceramide (α-GalCer) injected i.p. in normal B6 mice. **(C)** Representative staining of IFN-γ, IL-4, and TNF-α production by iNKT cells in the spleen and liver *in vivo* 2 h after i.p. injection of α-GalCer. Data are from three experiments where three mice aged 5- to 6-week old were used per experiment.

**FIGURE S4** | Unchanged cytokine production and Fas-L expression by spleen invariant natural killer T cell (iNKT) cells in the absence of NK group 2 member D upon concanavalin A (Con A) administration. Representative intracellular staining of interferon (IFN)-γ, interleukin IL-4, and TNF-α production **(A)**, or FAS-L expression **(B)** by spleen $NK1.1^+$ iNKT after 2 h of i.v. Con A administration (15 mg/kg). The frequencies of positive cells and in addition mean florescence intensity (MFI) for FAS-L are shown in the histograms. Results are from three experiments where three mice of each genotype were used per experiment and presented as mean ± SD. Numbers represent percentages. Significance was evaluated with non-parametric Mann–Whitney *U* test.

**FIGURE S5** | Cytokine production and Fas-L expression by liver invariant natural killer T cell (iNKT) cells is not induced directly *in vitro* by concanavalin A (Con A). **(A)** Representative intracellular staining of interferon (IFN)-γ, interleukin IL-4, and TNF-α production expression by spleen $NK1.1^+$ iNKT after 4 h incubation in the presence of Con A (10 μg/ml) or PMA/ionomycin. The frequencies of positive cells are shown in the histograms. **(B)** Cytokine level in the supernatant by spleen cells after O/N incubation with Con A or PMA/ionomycin at the previously indicated concentration. **(C)** Representative cell-surface staining of FAS-L expression by spleen $NK1.1^+$ iNKT after 4 or 18 h incubation in the presence of Con A (10 μg/ml). Results are from three to four experiments where three mice of each genotype were used per experiment and presented as mean ± SD.

**FIGURE S6** | Model of the role of NK group 2 member D (NKG2D) expressed on invariant natural killer T cell (iNKT) cells in concanavalin A (Con A)-induced hepatitis. In wild-type mice, upon Con A administration: hepatocytes upregulate NKG2D-L cell-surface expression including retinoic acid early inducible 1 (RAE-1) (1); NKG2D-L interact with NKG2D constitutively expressed by liver iNKT cells (2); NKG2D signal iNKT cells to produce cytokines (3), and to express FAS-L (4); liver damage is caused by iNKT cell FAS–FAS-L mediated killing of hepatocytes and directly or indirectly by the cytokine produced by these cells (5). The absence of NKG2D in *Klrk1*$^{-/-}$ mice shut off the aforementioned cascade of event reducing FAS-L expression and cytokine production leading to reduced iNKT cell cytotoxicity and liver injury and consequently increasing mice survival upon Con A administration.

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**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: 30 May 2018
doi: 10.3389/fimmu.2018.01219

# The Role of Natural Killer Group 2, Member D in Chronic Inflammation and Autoimmunity

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Current medicine and medical science puts great effort into elucidating the basis of chronicity and finding appropriate treatments for inflammatory diseases; however, the mechanisms driving aberrant immune responses are mostly unknown and deserve further study. Of particular interest is the identification of checkpoints that regulate the function and differentiation of pro-inflammatory cells during pathogenesis, along with means of their modulation for therapeutic purposes. Natural killer group 2, member D (NKG2D) is a potent activator of the immune system, known as a sensor for "induced-self" ligands, i.e., cellular danger signals that, in the context of chronic inflammation and autoimmunity, can be presented by cells being exposed to an inflammatory cytokine milieu, endoplasmic reticulum stress, or cell death. Engagement by such ligands can be translated by NKG2D into activation or co-stimulation of NK cells and different subsets of T cells, respectively, thus contributing to the regulation of the inflammatory response. In this review, we discuss the current knowledge on the contribution of the NKG2D–NKG2DL signaling axis during intestinal inflammation, type 1 diabetes, multiple sclerosis, and rheumatoid arthritis, where the role of NKG2D has been associated either by aberrant expression of the receptor and its ligands and/or by functional data in corresponding mouse models.

**Keywords: autoimmunity, natural killer group 2, member D, natural killer group 2, member D ligand, intestinal inflammation, rheumatoid arthritis, multiple sclerosis, type 1 diabetes**

OPEN ACCESS

***Edited by:***
*Nadia Guerra, Imperial College London, United Kingdom*

***Reviewed by:***
*Mary A. Markiewicz, University of Kansas Medical Center, United States*
*Daniel M. Altmann, Imperial College London, United Kingdom*

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***Specialty section:***
*This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology*

***Received:*** *28 February 2018*
***Accepted:*** *15 May 2018*
***Published:*** *30 May 2018*

***Citation:***
*Babic M and Romagnani C (2018) The Role of Natural Killer Group 2, Member D in Chronic Inflammation and Autoimmunity. Front. Immunol. 9:1219. doi: 10.3389/fimmu.2018.01219*

## INTRODUCTION

Natural killer group 2, member D (NKG2D; encoded by the gene *Klrk1*) is a molecular sensor of stressed cells and a potent activator of the immune system, and is largely expressed by NK cells as well as $CD8^+$ and $\gamma\delta^+$ T cells (1). It binds to a variety of well-defined danger molecules, such as retinoic acid inducible early (RAE) 1α–ε, H60a–c and murine UL-16-binding protein-like transcript (MULT)-1 in mice, or the MHC class-I-related chain (MIC)A/B and the UL-16 binding proteins (ULBP)1–6 in humans (2). The transcripts of NKG2D ligands are found in many tissues under healthy conditions but the cell surface expression of the corresponding proteins is kept under control by post-transcriptional regulation (3). Cellular stress, notably DNA damage, toll-like receptor signaling, and specific cytokine exposure can induce NKG2D ligand surface expression, as shown on tumor or virally infected cells (2). Ligand recognition by NKG2D can be integrated into a DNAX-activating protein (DAP)12- or DAP10-dependent signal, as in activated mouse NK cells, or into a DAP10-PI3K-mediated killing signal in human NK cells as well as a co-stimulation signal

in human and mouse $CD8^+$ T cells (4–6). Based on this, NKG2D plays an important role in immune surveillance by mediating direct recognition and clearance of infected and transformed cells expressing cognate ligands (1, 7, 8). However, upregulation of NKG2D ligands has also been reported in tissue samples from patients with chronic inflammatory and autoimmune disorders as well as in *in vivo* experimental models thereof, and $NKG2D^+$ cells have been implicated in their pathogenesis. Interestingly, in addition to NK, $CD8^+$ T, and $\gamma\delta^+$ T cells, increased frequency of rarely occurring $NKG2D^+CD4^+$ T cells has been observed in many different human pathologies (9–12) and in inflammatory models in mice (13, 14).

Here, we discuss the role of NKG2D-expressing cells as well as of NKG2D ligands during selected chronic inflammatory diseases, where the aberrant expression of the receptor and its ligands or data in corresponding mouse models implicate the role of NKG2D in the development of the respective disease.

## CELIAC AND INFLAMMATORY BOWEL DISEASES (IBDs)

Celiac disease (CeD) is a malabsorption syndrome that is elicited by gluten intolerance in individuals with genetic susceptibility. The pathology is manifested in massive cell death in the epithelial compartment which is infiltrated by autoreactive cytotoxic lymphocytes (CTL). The disruption in the homeostasis of intraepithelial lymphocytes (IEL) can additionally lead to the development of lymphoid malignancies, often associated with the refractory celiac sprue (RCS) (15), caused either by the clonal expansion of T cells or lately characterized $sCD3^-iCD3^+$ innate IEL (16). Patients with active disease are characterized by high levels of IL-15 expressed by intestinal enterocytes and lamina propria (LP) mononuclear cells, which correlates with the degree of mucosal damage (17).

IL-15 seems to mediate priming of $CD8^+$ T cells and turns them into potent cytolytic cells (18), which kill epithelial cells based on the recognition of stress signals (19). It became evident that IL-15 contributes to the cytotoxic potential of CTL by increasing the expression of NKG2D and its adaptor DAP10 in $CD8^+$ T cells, and indeed, patients with active CeD display a 4- to 20-fold higher expression of NKG2D on intraepithelial CTL, when compared with healthy individuals (18). Importantly, the expression of NKG2D ligands, namely, MICA, is upregulated in intestinal epithelial cells (IEC) as well as in mononuclear cells of patients. NKG2D blocking inhibited lysis of $MIC^+$ or $ULBP^+$ IEC lines (or of MICA-transfected tumor cell lines) by IL-15-primed human effector CTL, suggesting that upregulation of NKG2D on CTL converts them into potent killers (18). Conversely, the role of NKG2D expression by other T cell subsets or by innate lymphoid cells (ILC) during CeD has not been investigated so far.

The crucial role of gluten in the pathogenesis of CeD is also partially linked to the regulation of the NKG2D pathway. Interestingly, upon transition of patients to a gluten-free-diet (GFD), the levels of both NKG2D and MICA dropped, reaching levels observed in healthy controls, but remained high in GFD-resistant RCS patients. Moreover, culturing intestinal samples with gliadin, resulted in the upregulation of MICA exclusively in patients on a GFD, but not in healthy controls. Upon testing of different gliadin-derived peptides, non-immunodominant p31–49 and immunodominant p57–89, it became clear that only p31 induced MICA expression (20). p31–49 induced IL-15 expression by LP dendritic cells and macrophages of CeD patients (21), and the p31-mediated upregulation of MICA could be blocked by α-IL-15 neutralizing antibodies (20), suggesting that this might be a mechanism of how the sensing of gluten and IL-15 is integrated to result in the expression of NKG2D and MICA and contribute to CeD pathogenesis. Despite these interesting observations, data reporting on NKG2D or MIC polymorphisms in association with susceptibility to CeD are either absent or conflicting (20, 22, 23) and the demonstration of a functional role of NKG2D in an *in vivo* CeD experimental model is still missing to date.

Inflammatory bowel disease represents a group of intestinal disorders that cause prolonged inflammation of the digestive tract and is prominently represented by Crohn's disease (CD) and ulcerative colitis (UC). IBD is characterized by dysregulated gut microbiota, and an aberrant immune response, typically dominated by Th1 and Th17 cells during CD and Th2 cells during UC (24, 25). Association of the NKG2D–NKG2DL axis in the pathogenesis of IBD was implicated after the discovery of significantly upregulated MICA expression in IEC from CD and to a lesser extent from UC patients when compared with IEC from area-matched healthy controls (9). However, characterization of $CD4^+$ T cells from the peripheral blood (PB) and LP of CD and UC patients or control individuals revealed an increase in the frequency of $NKG2D^+$ cells exclusively in CD patients. These cells displayed a Th1-like phenotype, reflected in perforin expression, secretion of IFN-γ upon stimulation and cytotoxicity toward MICA-expressing targets (9). $NKG2D^+CD4^+$ T cells from LP of CD patients expressed IL-15Rα and IL-15 provision increased NKG2D and DAP10 expression in $CD4^+NKG2D^+$ clones, similarly as described for $CD8^+$ T cells in CeD. Whether IL-15 is the main and only factor driving NKG2D expression on $CD4^+$ T cells remains to be determined. Following the discovery of Th17 cells and their role in mediation of intestinal inflammation, further profiling of $NKG2D^+CD4^+$ T cells from CD patients showed enrichment of IL-17-producing cells among $NKG2D^+CD4^+$ T cells when compared to the $NKG2D^-$ compartment. Co-stimulation of $CD4^+$ T cells *via* NKG2D resulted in expression of IL-17, IFN-γ, and TNF. IL-17A/IFN-γ and IL-17A/IL-22 co-producers were specifically contained within $NKG2D^+CD4^+$ T cells (26). T-cell receptor repertoire analysis showed that most $CD4^+$ T cell oligoclonal expansions found in PB and small intestine LP of CD patients are contained within the $NKG2D^+$ subset (27). Interestingly, the expansions found in LP and PB were different, suggesting that the ones in the LP are a consequence of a local expansion. Two separate studies demonstrated accumulation of $NKG2D^+CD4^+$ T cells in colon LP in a $CD4^+$ T cell transfer-induced colitis model and disease amelioration after treatment with blocking α-NKG2D antibodies (13, 28).

Using a model of dextran sulfate sodium (DSS)-induced colitis, Qian and colleagues reported perturbation of numbers and

frequency of NKG2D$^+$CD4$^+$ and NKG2D$^+$CD8$^+$ T cells in colon and spleen (29). Further dissection of splenic NKG2D$^+$CD4$^+$ T cells according to NK1.1 expression revealed two major subsets, namely, TGF-β$^+$FasL$^+$T-bet$^+$NK1.1$^-$ cells and IFN-γ$^+$IL-17$^+$IL-21$^+$granzymeB$^+$perforin$^+$T-bet$^-$RORγt$^+$NK1.1$^+$ cells. Transfer of NK1.1$^-$NKG2D$^+$CD4$^+$ T cells delayed the onset of DSS-induced colitis and the protective effect was dependent on TGF-β. Conversely, the transfer of NK1.1$^+$NKG2D$^+$CD4$^+$ T cells exacerbated the colitis outcome.

An interesting study by Hosomi and colleagues demonstrated an important role of the NKG2D–NKG2DL interaction in recognition of endoplasmic reticulum stress by immune cells and how this is converted into intestinal inflammation (30). Mice with specific deletion of *Xbp1*, a crucial gene involved in unfolded protein response in IEC (*Xbp1*$^{\Delta IEC}$) showed increased expression of *Ulbp1* (gene encoding MULT-1) in IEC, as well as spontaneous development of intestinal inflammation. This process seems to be mediated by direct binding of the transcription factor, named CCAAT-enhancer-binding protein homologous protein (CHOP), to the promotor region of *Ulbp1* in IEC. Spontaneous enteritis was ameliorated by treatment with an α-NKG2D-blocking antibody as well as by depletion of NK1.1$^+$ cells that include cytolytic NKG2D-expressing group 1 ILC. Despite increased frequency of intraepithelial NKG2D$^+$γδ$^+$ T cells observed in *Xbp1*$^{\Delta IEC}$ mice, γδ$^+$ or αβ$^+$ T cells play a redundant role in causing spontaneous intestinal inflammation in this model (30). Altogether, these reports show that upregulation of NKG2D ligands might represent a common response to intestinal epithelium stress, rendering IEC susceptible to NKG2D-mediated immune surveillance and regulation.

## TYPE 1 DIABETES (T1D)

During T1D, the body's own immune system attacks the β-cells in the pancreatic islets resulting in damage, reduced and subsequently abrogated insulin production (31). Genetically susceptible individuals carry the high-risk HLA DR4-DQ8 and DR3-DQ2 haplotype in more than 90% of cases. Although it is generally believed that the disease is mediated by self-reactive CD8$^+$ and CD4$^+$ T cells and macrophages, a role for regulatory T cells in the regulation of diabetogenic IFN-γ-producing NK cells in the pancreatic islets has been described (32, 33). In humans, genetic linkage studies showed positive association of the MICA allele 5 with T1D (34). The role of NKG2D in T1D pathogenesis has been assessed mainly using the non-obese diabetic (NOD) mice, with conflicting conclusions (35–37). An initial study reported *Raet1* transcripts (encoding for RAE1) in the β-cells of the pancreas of 4- to 6-week-old NOD mice and linked an increase in *Raet1* expression with age (36). Following studies could not confirm the expression of *Raet1* in pancreatic β-cells of NOD mice (37–39), while Trembath et al. rather observed expression of *H60a* in pancreas-infiltrating T cells (37). A pathogenic role for NKG2D was reported by Ogasawara et al. (36), who demonstrated that antibody-mediated blocking of NKG2D signaling led to reduced infiltration of autoreactive CD8$^+$ T cells into the pancreas of 16-week-old NOD mice and decreased diabetes incidence. Along this line, by using the C57BL/6J mice with transgenic expression of RAE1ε in islet β-cells of the pancreas (*Rae1*-Tg mice), it was shown that CTL were recruited to the pancreas of *Rae1*-Tg mice in an antigen-independent but NKG2D-dependent manner (40). Although transgenic expression of RAE1 led to spontaneous insulitis in old *Rae1*-Tg mice, no diabetes development could be observed in this model. Disease amelioration by antibody-mediated blocking of NKG2D signaling in NOD mice, as initially reported by the Lanier group (36), could not be reproduced in the report by Guerra et al. (35). In addition, crossing of NKG2D-deficient mice to the NOD background (NOD × *Klrk1*$^{-/-}$) did not result in any disease amelioration (35), questioning the role of NKG2D in this disease model.

One potential explanation of such discrepancies can be related to microflora differences, which appear to impact T1D incidence in NOD mice (41, 42). Interestingly, Trembath and colleagues reported that *Klrk1*$^{-/-}$ NOD mice have lower diabetes incidence when compared with littermate NOD mice housed in specific pathogen-free conditions; however, this effect was lost and even reversed upon treatment of NOD and *Klrk1*$^{-/-}$ NOD littermate mice with broad-spectrum antibiotics (37). Supporting this hypothesis, it was shown that treatment of C57BL/6N mice with vancomycin can reduce the expression of *Raet1* in small intestine epithelium, and this effect was correlated with the presence of *A. muciniphila* in the vancomycin-treated mice (43).

In light of these data, the role of NKG2D in T1D autoimmunity should be reconsidered by taking into account the differences in colonizing microbiota. In addition, the contribution of other NKG2D-expressing cell types to the regulation of T1D needs further evaluation.

## MULTIPLE SCLEROSIS (MS) AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

Multiple sclerosis, an inflammatory disease of the central nervous system (CNS), is characterized by the loss of oligodendrocytes, followed by a reduction in myelin production. While CD4$^+$ T cells predominate in acute lesions of MS patients and are better studied in the EAE, a mouse model of MS (44), CD8$^+$ T cells seem to play an important role in tissue damage and are observed more frequently in the chronic lesions of MS patients (45, 46). Patients display elevated levels of soluble MICB but not MICA in the sera (47) and the MICB*004 allele was associated with higher MS susceptibility (48), suggesting a role for NKG2D signaling in the development of MS. Similar to other inflammatory diseases, IL-15 was elevated in the serum of MS patients and could be expressed *ex vivo* in astrocytes and infiltrating macrophages from MS lesions (49). Astrocyte-derived IL-15 reinforced the cytotoxic program in CD4$^+$ and CD8$^+$ T cells, with increased expression of NKG2D, perforin, and granzyme (49, 50). MICA/B ligand engagement on human oligodendrocytes could induce their killing *in vitro* by IL-2 activated NK cells, γδ$^+$ T cells, or polyclonal CD8$^+$ T cells in an NKG2D-dependent fashion (50).

Multiple sclerosis patients are characterized by an enrichment in NKG2D$^+$CD4$^+$ but not CD8$^+$ T cells in cerebrospinal fluid and in inflammatory lesions (51), as compared to PB and to healthy

donors, which might be a result of a local expansion mediated by an inflamed milieu or an increased migratory capacity (49). In the same study, by using the EAE model, the authors could show that treatment of mice with a blocking α-NKG2D antibody after immunization, but before disease onset, resulted in a reduced disease score (51), which could partially be reproduced by using *Klrk1*$^{-/-}$ mice (35). The treatment did not seem to affect peripheral cell activation, but rather the infiltration of NKG2D$^{+}$CD4$^{+}$ and NKG2D$^{+}$CD8$^{+}$ T cells into CNS at the peak of the disease. Moreover, *Raet1* transcripts were detected in myeloid cells in the spinal cord of the EAE mice, with expression levels correlating with the disease score (52). It was suggested that NKG2D$^{+}$CD4$^{+}$ T cells might contribute to killing of oligodendrocytes. Similar to human, *in vitro* cultured and cytokine activated mouse oligodendrocytes expressing MULT-1 and RAE1 were susceptible to killing by α-CD3/α-CD28 activated CD4$^{+}$ T cells enriched for the expression of NKG2D to 8–12% (51). Although the killing mechanism remains unclear, it seems to be partially reduced by blocking of NKG2D, and independent of MHC class I or II peptide presentation.

While accumulation of NKG2D$^{+}$CD4$^{+}$ T cells into CNS of EAE and MS remains an interesting finding, the contribution of NKG2D on various cell types in mediating cell priming in the periphery, migration into the tissue and reactivation *in situ* needs to be further explored.

## RHEUMATOID ARTHRITIS (RA) AND MODELS OF JOINT INFLAMMATION

Rheumatoid arthritis is a chronic inflammatory disease that causes inflammation and destruction of the joints. The disease is characterized by high levels of TNF and IL-15 which are found in patient sera (10) and prominently mediated by joint-infiltrating autoreactive CD4$^{+}$ T cells that promote autoantibody production by plasma cells, along with macrophage and endothelial activation (53). It was shown that TNF and IL-15 could upregulate NKG2D *in vitro* on PB CD4$^{+}$ T cells or on NKG2D$^{-}$CD4$^{+}$ T cells from the inflamed synovia of RA patients. Indeed, around 18% of potentially autoreactive CD28$^{-}$CD4$^{+}$ T cells from the PB and synovial tissue of RA patients expressed NKG2D. Membrane bound MICA and MICB were abundantly expressed in the synoviocytes of RA patients and could trigger autologous autoreactive T cells in an NKG2D-dependent manner (54). Crosslinking of NKG2D on CD4$^{+}$CD28$^{-}$ T cells seemed to co-stimulate TCR-mediated secretion of IFN-γ and TNF as well as proliferation (10).

Single-nucleotide polymorphisms in both MICA and NKG2D have been associated with RA, suggesting MICA and NKG2D as RA susceptibility genes (55, 56). Moreover, a recent study evaluated the role of polymorphisms in *KLRK1* gene with efficacy of α-TNF therapy in RA patients (57), identifying two polymorphisms associated with better response and two polymorphisms associated with inefficient response to therapy. More functional studies that would dissect the direct effect of the polymorphisms to the TNF production and resistance to α-TNF therapy would be beneficial to understand the impact of this association.

Using a collagen-induced arthritis (CIA) model, Andersson and colleagues could demonstrate that the treatment of mice with a blocking α-NKG2D antibody reduced the clinical score, even when applied after the disease onset (14). Along this line, NKG2D-blocking preserved joint architecture and reduced infiltration of γδ$^{+}$ and CD4$^{+}$ T cells in mouse paws, while it did not affect CD8$^{+}$ T and NK cells. This study also reported that treatment with an α-NKG2D-blocking antibody reduced infiltration of IL-17$^{+}$CD4$^{+}$ but not of IL-17$^{+}$γδ$^{+}$ T cells in the paws. Interestingly, the per-cell expression of NKG2D on NK cells was slightly reduced during late CIA, possibly related to chronic exposure of these cells to the ligands. Similar to what was observed in human RA samples, NKG2D ligand expression did not affect the expression of NKG2D on γδ$^{+}$ and CD4$^{+}$ T cells, suggesting different regulation of NKG2D expression on innate and adaptive cells.

## CONCLUDING REMARKS

Current data support the role of inflammatory cytokine- and endoplasmic reticulum stress-induced expression of NKG2D ligands in several immune-mediated diseases, suggesting that the NKG2D–NKG2DL axis can represent an interesting target for the modulation of selective inflammatory disorders. Recently, the efficacy of a blocking α-NKG2D antibody was tested within phase I/II clinical trial that included 78 patients diagnosed with CD. No difference between the single dose treated and placebo receiving cohort was observed at week 4, but effects became visible at week 12 as manifested in a significant clinical response in patients responding to biological therapy or those with untreated disease (58). A phase I/II study with a blocking α-NKG2D antibody was also performed in a cohort of patients diagnosed with RA, however, with no published results to date. The field would benefit from functional studies performed *in vivo*, which would allow comparisons between complete NKG2D-deficient mice to mice with cell-specific NKG2D-deficiency (59), as well as from studies in mice with conditional deficiency of NKG2D ligands (60). Given the complexity of the regulation of NKG2D ligand expression and the possibly multifaceted role of microbiota, understanding the specific role of each NKG2D$^{+}$ population in the pathogenesis of the particular disease would enable better design of future therapeutic approaches.

## AUTHOR CONTRIBUTIONS

MB searched the literature and wrote the majority of the manuscript. CR contributed to writing and editing of the manuscript.

## ACKNOWLEDGMENTS

We thank Daniela Hernández-Torres for reading the manuscript. We apologize to our colleagues whose work is not cited due to space restrictions. This work was supported by the Leibniz ScienceCampus Chronic Inflammation (www.chronische-entzuendung.org) and by the German Research Foundation (DFG) grants RO3565/2-1 and RO3565/4-1 to CR and European Union Framework Program 7, Marie Curie Intraeuropean Fellowship 327643 to MB. CR is supported by the DFG Heisenberg Program (RO3565/1-1).

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**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.

The reviewer DA and handling Editor declared their shared affiliation.

frontiers in Immunology

ORIGINAL RESEARCH
published: 15 June 2018
doi: 10.3389/fimmu.2018.01282

# Functional Characterisation and Analysis of the Soluble NKG2D Ligand Repertoire Detected in Umbilical Cord Blood Plasma

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

**Received:** 29 January 2018
**Accepted:** 22 May 2018
**Published:** 15 June 2018

**Citation:**
Cox ST, Danby R, Hernandez D,
Laza-Briviesca R, Pearson H,
Madrigal JA and Saudemont A (2018)
Functional Characterisation and
Analysis of the Soluble NKG2D
Ligand Repertoire Detected in
Umbilical Cord Blood Plasma.
Front. Immunol. 9:1282.
doi: 10.3389/fimmu.2018.01282

We previously reported that cord blood plasma (CBP) contains significantly more soluble NKG2D ligands (sNKG2DLs), such as sMICB and sULBP1, than healthy adult plasma. Viral infection or malignant transformation upregulates expression of NKG2D ligand on affected cells, leading to NK group 2, member D (NKG2D)-mediated natural killer (NK) cell lysis. Conversely, sNKG2DL engagement of NKG2D decreases NK cell cytotoxicity leading to viral or tumour immune escape. We hypothesised that sNKG2DLs detected in CBP may represent an additional fetal–maternal tolerance mechanism. To further understand the role of sNKG2DL in pregnancy and individual contributions of the various ligand types, we carried out functional analysis using 181 CBP samples. To test the ability of CBP to suppress the function of NK cells *in vitro*, we measured expression of NKG2D, CD107a, and IFN-γ in NK cells from control donors after exposure to 181 individual CBP samples and characterised the sMICA, sMICB, and sULBP1 content of each one. Furthermore, to detect possible allelic differences between samples that may also affect function, we carried out umbilical cord blood typing for MHC class I-related chain A (MICA) and MHC class I-related chain B (MICB) coding and promoter allelic types. Strongest functional correlations related to increasing concentration of exosomal sULBP1, which was present in all CBP samples tested. In addition, common MICB alleles, such as MICB*005:02, resulted in increased concentration of sMICB. Interestingly, MICB*005:02 uniquely associated with eight different promoter types. Among promoter polymorphisms, P2 resulted in the highest expression of sMICB and P9 the least and was confirmed using *luciferase* reporter assays. Higher levels of sMICB associated with lower IFN-γ production, indicating that sMICB also suppressed NK cell function. We also examined the MICA functional dimorphism encoding methionine (met) or valine (val) at residue 129 associated with strong or weak NKG2D binding, respectively. Most sMICA associated with val/val, some with met/val but none with met/met and, counter-intuitively, the presence of sMICA in CBP increased NK cell cytotoxicity. We propose a model for fetal–maternal tolerance, whereby NK cell activity is limited by sULBP1 and sMICB in

CBP. The release of 129val sMICA with weak NKG2D signalling may reduce the overall net suppressive signal and break tolerance thus allowing fetal NK cells to overcome immunological threats *in utero*.

**Keywords: NK cell, pregnancy, tolerance, NKG2D, ligand, luciferase, MICA, ULBP1**

## INTRODUCTION

Natural killer (NK) cells play an important role in innate immunity, providing a first line of defence against pathogens and early detection and elimination of transformed cells. Complex interactions between NK cell receptors and potentially aberrant ligand expression on "self" tissues or cells take place continuously *via* NK cell immunosurveillance (1). Whether or not an NK cell becomes activated leading to target cell lysis depends on the overall balance of activating and inhibitory receptor stimulation (2).

Among the NK cell-activating receptors, the NK group 2, member D (NKG2D) receptor is perhaps the most studied but the mechanisms governing activation potential are still far from being fully understood. NKG2D interacts with ligands encoded by eight different genetic loci, including the highly polymorphic MHC class I-related chain A and B (MICA/B) and the unique long 16 binding proteins (ULBP1-6), which are also polymorphic (3–6). Apart from constitutive expression in the gut, NKG2D ligand (NKG2DL) expression is upregulated on infected and transformed cells. This enables NK cell cytotoxicity through engagement with the NKG2D activating receptor, demonstrated by studies investigating viral infection such as hepatitis B (7, 8) or cellular transformation leading to numerous types of cancer (9). Stress-induced upregulation of NKG2DL expression alone is sufficient to initiate NK cell activation and degranulation, while at the same time cytokines such as IFN-γ are released that can prime other immune cells. Viruses or tumours can avoid immune recognition by this mechanism by augmenting production of exosomal or shed soluble NKG2D ligands (sNKG2DLs) that are released into the local microenvironment. This counter-strategy successfully enables virally infected or rogue cells to escape NK cell immunosurveillance as sNKG2DL interaction with the NKG2D receptor on NK cells downregulates NKG2D expression. Thereby, the NK cell's ability to interact with ligands *via* NKG2D is reduced but more importantly, interaction with sNKG2DLs causes NK cells to become hyporesponsive to further stimulation as shown previously by ourselves (10) and others. The opposing mechanisms of soluble and membrane-bound NKG2DLs are illustrated in **Figure 1**.

Soluble sNKG2DLs are essentially immunosuppressive agents targeting NK cells and other cells expressing the NKG2D receptor, such as NKT cells, γδ T cells, and $CD8^+$ T cells. Such an intricate mechanism would, presumably, also have an important physiological role, such as homeostasis in immunoregulation but this has not been clearly demonstrated. These ligands, particularly MICA and MICB, are highly polymorphic but the biological significance of this diversity is still largely unknown. It is possible that MICA or MICB allelic differences between individuals may alter the potential immune response. Differences in the promoter region could alter transcriptional levels or amino acid substitutions in the molecule itself may alter protein folding or stability and influence interaction and binding with NKG2D. Thus, differences in MICA/B expression levels or their signalling through NKG2D may lead to variable NK cell function. Identification of NKG2DL polymorphisms, or alleles, that influence the immune response may enable prognosis of severity in the case of viral infection (11, 12) or cell transformation (13) or identify individuals that may benefit from immunotherapy treatment to counteract escape from NK cell immunosurveillance. In the transplantation setting, further understanding could enable better donor selection to reduce graft rejection or post-transplant complications.

We previously reported that the sNKG2DLs sMICA/B and sULBP1 are detectable in cord blood plasma (CBP) samples and responsible for decreasing adult donor NK cell cytotoxicity (10), as determined by K562 killing and chromium release assays. Therefore, it is possible that the physiological role of sNKG2DLs relates to allogeneic tolerance mechanisms in pregnancy and increasing our understanding may enable interpretation of pathologies that can arise through perturbation of NKG2DL expression *via* genetic or environmental means. This study extends our previous findings by identifying the sNKG2DLs responsible for functional inhibition of adult donor NK cell cytotoxicity. We also explore the correlation between the allelic variants and promoter polymorphisms of MICA/B genes found in umbilical cord blood (UCB), the levels of sNKG2DLs found in CBP and their potential functional consequences.

## MATERIALS AND METHODS

### Subjects and Samples

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of consenting healthy adult male and female volunteer donors by density-gradient centrifugation using Lympholyte®-H solution (Cedarlane, ON, Canada). UCB, containing acid citrate–phosphate–dextrose anticoagulant, was collected from the placenta of full-term healthy deliveries and used for DNA extraction and plasma preparation, as previously described (10). For this study, 181 different UCB units were utilised. DNA (Qiagen Blood Miniprep, Qiagen GmbH, Hilden, Germany) and plasma was isolated from each UCB unit. Four additional units were used for cord blood mononuclear cell (CBMC) isolation.

This study was carried out with the approval of the local Research Ethics Committee (reference HC71/IU). UCB units were obtained from the Anthony Nolan Cord Blood Bank with prior

**Abbreviations:** CBP, cord blood plasma; MICA, MHC class I-related chain A; MICB, MHC class I-related chain B; MP, maternal plasma; NKG2D, NK group 2, member D; UCB, umbilical cord blood; ULBP, UL-16 binding protein.

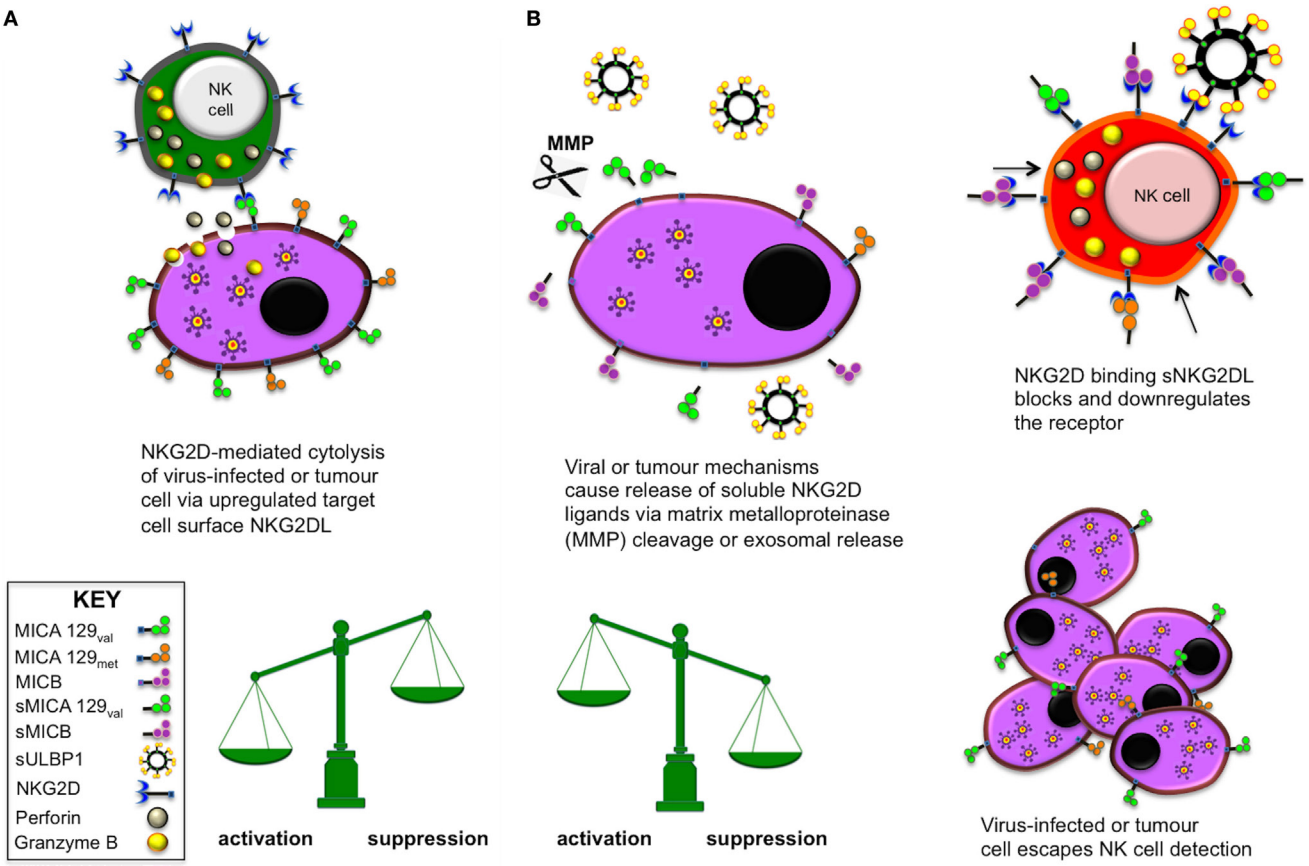


**FIGURE 1** | Natural killer (NK) cell activation and suppression *via* NK group 2, member D (NKG2D) engagement with membrane-bound or soluble NKG2D ligands (NKG2DLs), respectively. **(A)** When the activating receptor NKG2D on NK cells and various other lymphocytes interacts with stress-induced, upregulated NKG2DL on virus-infected or tumour cells, the target cell is eliminated by lytic actions of the effector cell. **(B)** Certain viruses and tumours are able to release soluble NKG2D ligands (sNKG2DLs) *via* MMP enzymatic cleavage or direct production of exosomal sNKG2DLs as molecular decoys. In this situation, the NKG2D activating receptor becomes blocked or is downregulated and the effector cell becomes anergic and unresponsive to further activation. This mechanism allows the tumour or virus to escape immune surveillance and proliferate.

written consent from pregnant mothers and ethical committee approval (Research Ethics Committee reference 10/H0405/27).

## Cell Culture

For assessment of NK cell function, donor control PBMCs or CBMCs were plated in RPMI (Lonza, Slough, UK) containing 10% heat-inactivated fetal calf serum (FCS) supplemented with 1% penicillin and streptomycin (complete media) and containing human IL-2. Test cultures using 50% CBP diluted with complete media or complete media only contained 200 IU IL-2/well in 96-well plates. All cultures were incubated at 37°C with 5% $CO_2$ for 48 h before PMA and ionomycin stimulation. Duplicate cultures were carried out for each experiment without PMA and ionomycin stimulation to assess levels of NKG2D and baseline CD107a. NKG2D expression on the relevant cells was determined using the unstimulated cultures and CD107a background levels in unstimulated cultures were subtracted from the values obtained for equivalent stimulated cells.

Experiments were carried out using four different, healthy PBMC donors, each tested with CBP samples from the 181 UCB units and data points represent donor means. For experiments using CBMCs, four CB units were used with seven different CBP samples. HCT116 (human colon carcinoma) used for luciferase assays were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). HCT116 cells were cultured in McCoy's 5A medium (Lonza, Slough, UK) supplemented with 2 mM L-glutamine and 10% FCS at 37°C with 5% $CO_2$.

## Soluble NKG2DL and IFN-γ Detection and Quantification Assays

Soluble MICA/B (DY1300/DY1599) and ULBP1 (DY1380) were detected in CBP and IFN-γ (DY285) was detected in PBMC stimulation supernatants using Duoset ELISA kits (R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

## Flow Cytometry

Briefly, cells were labelled with fluorochrome-conjugated antibodies in PBS with BSA (0.5%) for 10 min at 4°C. Antibodies (BD Biosciences, Oxford, UK) were as follows: CD3 (SK7), CD56

(B159), CD107a (HA4A3), and NKG2D (BAT221, Miltenyi Biotec, Bisley, UK). For quantitation of CD107a, cells were re-suspended in complete media containing 100 ng/ml PMA, 1 μg ionomycin and 0.1% 2-mercaptoethanol (stimulated) or complete media with 0.1% 2-mercaptoethanol (non-stimulated) for 2 h at 37°C. Fluorescence minus-one controls (where samples are stained sequentially with all antibodies except one) were used to set gates and analysis was performed using Fortessa flow cytometer (BD Biosciences, Oxford, UK) and FlowJo version 10.0.8 (Tree Star Inc., OR, USA). The gating strategy used for analysis of lymphocyte subtypes was performed as described in our previously study (10) and is shown in Figure S1 in Supplementary Material.

## Comparison of IFN-γ Production by PBMCs or Isolated NK Cells

To compare the relative amounts of IFN-γ production following PMA and ionomycin stimulation, PBMCs and NK cells from the same donor ($n = 4$) were isolated. PBMCs were prepared as described above and purified NK cells were obtained by negative selection using the NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. NK cell purity was determined by flow cytometry. PBMCs were plated at 200,000 cells/well and purified NK cells at 50,000 cells/well. Incubation with media and IL-2 was performed as described above, for 48 h. Prior to stimulation with PMA and ionomycin, cell enumeration and NK cell purity were determined for PBMC and isolated NK cell cultures. IFN-γ in the supernatants of the respective cultures was measured using ELISA as described above. PMA and ionomycin stimulation was performed for 2 h at 37°C. Results were calculated and expressed as IFN-γ (pg/ml)/10,000 NK cells.

## MICA/B Allelic and Promoter Polymorphism Analysis

Genotyping of MICA and MICB allelic and promoter type was performed on 181 DNA samples obtained from UCB as fully described previously (14).

## Generation of MICA/B Promoter Reporter Constructs

We cloned 10 MICA and 13 MICB 5′ proximal promoter fragments from sequences identified from International Histocompatibility Workshop (IHW) cell line DNA or specific populations (14, 15). MICA promoter fragments of 568 bp and 5′ MICB promoter fragments of 581 bp were amplified by polymerase chain reaction (PCR) from homozygous IHW cell line DNA or cloned using heterozygous IHW cell line or UCB DNA. The following primers were modified with restriction digest sequence tags and used for amplification (16). MICA-sense: 5′-ACTATCTACGAGCTCCGACGTCRCCACCCTCTCA-3′ (SacI underlined), MICA-antisense: 5′TGATAGATCGGTACCCAGGTGCTTCTGAGAGGCAGAGGT-3′ (KpnI underlined), MICB-sense: 5′-ACTATCTACGAGCTCCTACGTCGCCACCTTCTCAGCTG-3′,MICB-antisense:5′-TGATAGATCGGTACCCAAGTGCTTCTGAAAGGCAGAGGC-3′. PCR conditions were 95°C for 1 min followed by 30 cycles of 95°C for 1 min, 66°C for 1 min, and 75°C for 1 min and a final extension of 75°C for 5 min. Amplified products and pGL3-Basic were treated with Thermosensitive Alkaline Phosphatase (Promega, Southampton, UK), digested with SacI and KpnI, gel extracted and ligated using T4 DNA ligase. Clones were selected after JM109 transformation and sequencing of isolated plasmids to verify integrity and promoter type. Plasmid midi preps (Promega, Southampton, UK) were then prepared, according to the manufacturer's instructions, ready for *luciferase* experiments.

## Transient Transfection and *Luciferase* Reporter Assays

MHC class I-related chain A and MICB promoter activity was assessed with the constructs described above using dual *luciferase* reporter assays (Promega, Southampton, UK). HCT116 cells were seeded in 96-well plates at $1 \times 10^5$ cells/well and grown to 50% confluence in 24 h. 100 ng of promoter pGL3-basic plasmid and 4 ng of pRL-TK (*Renilla*) was transfected per well using Lipofectamine® 3000 transfection reagent (Thermo Fisher) according to the manufacturer's recommendations. Each assay included wells transfected with pGL3-control containing SV40 promoter and enhancer (positive control) for maximal luminescence and pGL3-basic, which has no promoter and enhancer region (negative control). Activities of test promoter constructs were normalised by co-transfection with pRL-TK plasmid to correct differences in well-to-well transfection efficiency and plasmid-associated background transcription levels were determined using the negative control plasmid. A mock control (no DNA in transfection mix) was also included for subtraction of general background levels. Each test condition was assayed in triplicate for four independent experiments using three different preparations of plasmid DNA. *Luciferase* and *Renilla* luminescence was measured using a Varioskan® Flash instrument (Thermo Fisher, MA, USA).

For experiments using proliferating HCT116 cells, cultures were harvested 24 h after transfection. For experiments using quiescent and heat-shocked HCT116 cells, transfection was carried out for 7 days until cells reached high confluence. Heat-shock treatment was performed by sealing plates with Parafilm and floating in a water bath for 1.5 h at 42.5°C, followed by 5.5 h recovery culture at 37°C as previously described (17).

Mock transfection luminescence was subtracted from all results. Sample and control ratios were calculated by dividing *luciferase* luminescence values by *Renilla* luminescence. Results were expressed as relative response ratios (RRR) and calculated as follows:

$$\mathrm{RRR} = \frac{\left(\text{experimental sample ratio}\right) - \left(\text{negative control ratio}\right)}{\left(\text{positive control ratio}\right) - \left(\text{negative control ratio}\right)}$$

## Sequencing for MICA/B 3′ Untranslated Region (UTR) Polymorphisms

The 3′ UTR of MICA and MICB genes were sequenced to identify polymorphisms that may affect transcriptional repression by microRNAs (miRNAs). A 692-bp fragment of the MICA 3′UTR

was amplified by PCR with the following primers: MICA3UFWD 5′-CCACAGGGATGCCACACAGCTC-3′ sense primer and MICA3UR 5′-CGTGCCTGGCCTGAGACT-3′ antisense primer as previously described (18). The MICB 3′UTR fragment of 1,209 bp was amplified using sense primer MICB3UF 5′-AACA CCCAGTTGGGACAGGA-3′ and antisense primer MICB3UR 5′-GGAGATTGCTTTGATGCTGG-3′ as previously described (19). Amplification primers were also used as sequencing primers at standard concentration (1.6 pmol/25 µl reaction). Sequencing was carried out using a 3730XL DNA analyser (Applied Biosystems, CA, USA).

## Statistical Analysis

Results are shown as mean with SEM and were evaluated using Graphpad Prism 6 (Graphpad Software, CA, USA). Unpaired datasets were compared using the nonparametric Mann–Whitney test. Where more than two groups were compared analysis was performed using the Kruskal–Wallis test with Dunn's *post hoc* test. Correlation and linear regression analysis significance was assessed using the nonparametric Spearman test and results are shown as *P*-values with Spearman *r* correlation value. A *P*-value ≤0.05 in two-sided tests was considered significant. Significance levels are indicated as $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, unless the exact *P*-value is given.

# RESULTS

## NKG2D Soluble Ligands ULBP1 and MICB Present in CBP, Decrease NK Cell Functional Potential *In Vitro*

We previously demonstrated that the sNKG2DLs present in CBP decreased functional capacity of NK cells (10) but as several ligands are often present together it was not clear if more than one type was required or whether one particular ligand was responsible. To determine effects of differing sMICA/B and sULBP1 concentrations on phenotypic and functional parameters of NK cells, we cultured healthy adult donor PBMCs with media containing 181 different preparations of CBP. After 48-h culture and PMA/ionomycin stimulation, cells were analysed for expression of CD107a, NKG2D, CD56, and CD3 by flow cytometry, and the concentration of IFN-γ released into culture supernatants was measured by ELISA. The results shown in **Figure 2** indicate that increasing concentration of sULBP1 in CBP significantly downregulated CD107a expression on both $CD56^{bright}$ and $CD56^{dim}$ NK cells and also on $CD3^{+}$ T cells in a dose-dependent manner. However, a significant correlation was not seen with $CD56^{+}CD3^{+}$ NKT cells, although this result may be affected by low numbers of these cells. By contrast, the concentration of neither sMICA nor sMICB affected expression of CD107a whatever cell type was considered, but higher concentrations of sMICB had a moderately suppressive effect on NKG2D receptor expression in both $CD56^{bright}$ and $CD56^{dim}$ NK cells. Linear regression and Spearman *r* analysis showing significant sNKG2DL-mediated down-modulation of CD107a, NKG2D or IFN-γ are shown in **Figures 2A–C**. Increasing sULBP1 concentration also decreased NKG2D on all cell types and decreased IFN-γ levels detected in supernatants of stimulated PBMCs.

By grouping results according to ranges of sULBP1 concentrations, we found that levels below 1 ng/ml resulted in the highest levels of IFN-γ secretion of around 48% relative to complete media only controls. Increasing concentration of sULBP1 decreased detectable levels of IFN-γ in a dose-dependent manner. However, very high levels of sULBP1, exceeding 3 ng/ml, did not reduce the detectable levels of IFN-γ any further, indicating maximal functional effects when sULBP1 concentration lies between 2 and 2.9 ng/ml (**Figure 2D**).

## The Expression Level of CD107a and NKG2D Correlates With the Production of IFN-γ

The results shown for IFN-γ production in **Figure 2** were obtained by ELISA and represent total production of IFN-γ by PBMCs following PMA and ionomycin stimulation. To determine whether there is a relationship between levels of NKG2D or CD107a and IFN-γ production, we plotted Spearman *r* correlations. The results shown in **Figures 3A,B** show highly significant correlations with both NKG2D and CD107a with IFN-γ. This result indicates that the suppression of NK cell function shown by reduced CD107a expression and NKG2D downregulation is directly related to the amount of IFN-γ produced by $CD56^{bright}$ and $CD56^{dim}$ NK cells, $CD56^{+}CD3^{+}$ NKT cells and $CD3^{+}$ T cells.

Next, we compared the amount of IFN-γ produced by total PBMCs or isolated NK cells incubated for 48 h in RPMI containing 200 IU followed by PMA and ionomycin stimulation. **Figure 3C** shows the mean ± SEM concentration of IFN-γ produced by NK cells alone was 54.17 ± 12.29 pg/ml/10,000 NK cells. This compares with 91.44 ± 10.85 pg/ml/10,000 NK cells obtained by cultures using PBMCs. This result indicates that most IFN-γ is produced by NK cells and we hypothesised that the remainder was most likely the product of T cell stimulation. Since CD107a is correlated with IFN-γ production, we determined the frequency of CD107a-positive cells in media only cultures from our previous experiments to determine the relative amounts for each cell type. The results, shown in **Figure 3C**, show the mean ± SEM frequencies of CD107a-expressing lymphocytes were 42.7 ± 3.7 for NK cells, 6.6 ± 0.6 for NKT cells, and 32.5 ± 2.0 for T cells. The remaining double-negative gated cells accounted for 18.3 ± 4.9% CD107a-positive cells (others), which possibly represents B cells (20).

## Incubation With CBP Also Reduces Functional Capacity of CBMCs

Since this study was performed using PBMCs from healthy adult volunteers, we wanted to confirm that the effect we observed also applied to equivalent cells obtained from cord blood (CB). Using four different cord blood units, we performed the same experiments to determine percentage of maximum expression levels of NKG2D, CD107a, and IFN-γ after 48 h incubation with CBP ($n = 7$). We compared the results from CBMCs (CB) with results from PBMCs (PB) using exactly the same CBP samples.

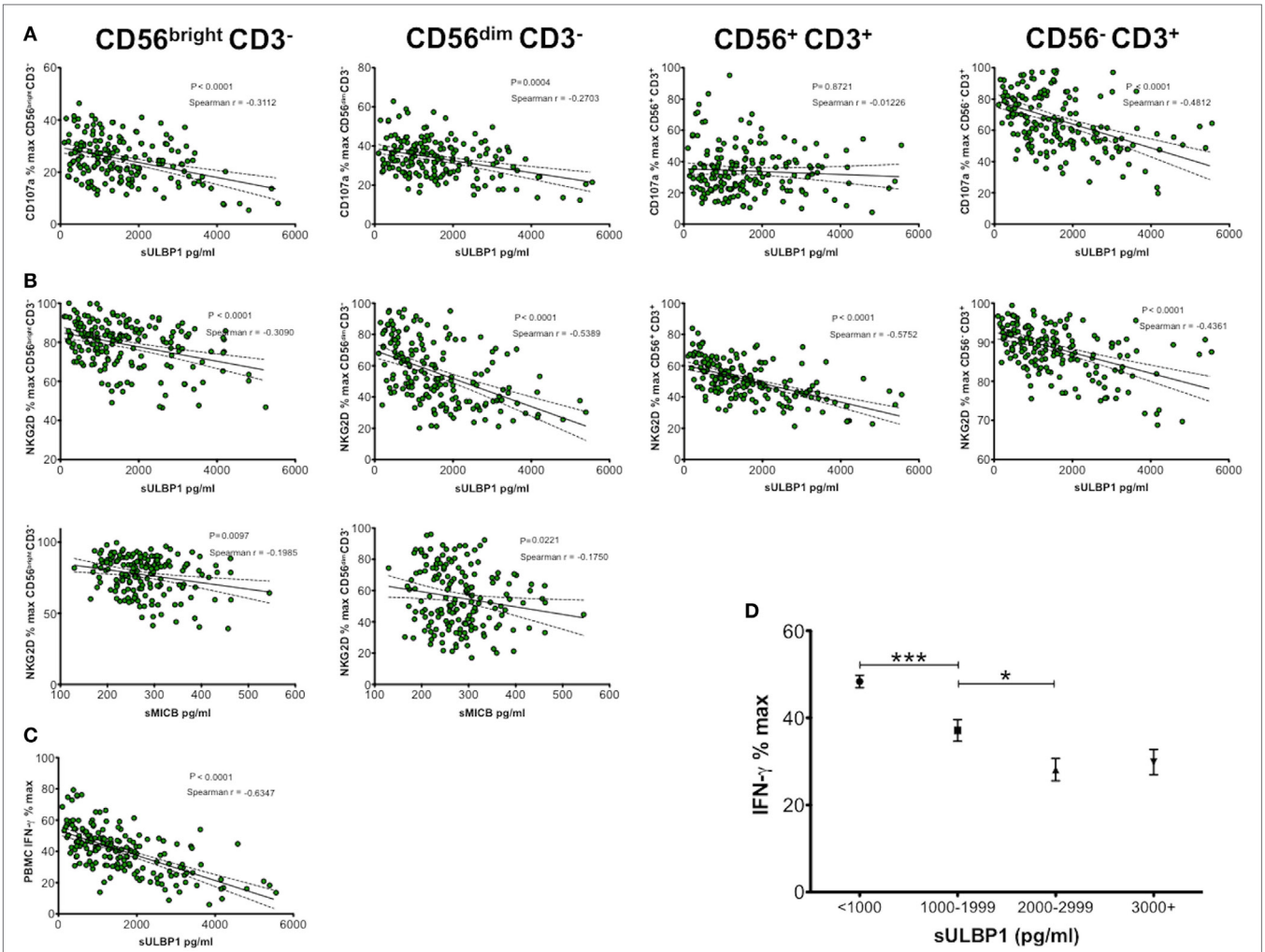


**FIGURE 2** | Soluble NKG2D ligand (sNKG2DL) effect on NK group 2, member D (NKG2D) and CD107a expression by $CD56^{bright}$ $CD3^{-}$, $CD56^{dim}$ $CD3^{-}$ natural killer (NK) cells, $CD56^{+}$ $CD3^{+}$ NKT cells, $CD56^{-}$ $CD3^{+}$ T cells, and IFN-γ production by peripheral blood mononuclear cells (PBMCs). **(A)** Correlation and linear regression analysis of sULBP1 concentration effect on CD107a expression. **(B)** Correlation and linear regression analysis of sULBP1 and sMICB concentration on NKG2D expression. **(C)** Correlation and linear regression analysis of sULBP1 effect on production of IFN-γ by PBMCs. PBMCs were incubated with 50% cord blood plasma (CBP) ($n$ = 181) or media only for 48 h and then stimulated with PMA and ionomycin. IFN-γ in culture supernatants and sNKG2DLs in CBP was measured by ELISA and expression of CD107a and NKG2D on the different cell types was measured by flow cytometry. $P$-values for Spearman $r$ correlations are indicated. **(D)** Increasing concentration of soluble ULBP1 decreases potential IFN-γ production by NK cells in a dose-dependent manner ($n$ = 181). Each experiment was repeated with four different PBMC donors and data points represent donor means. Statistical analysis **(D)** was performed using Mann–Whitney test ± SEM ($^{*}P \leq 0.05$ and $^{***}P < 0.001$).

We did not include analysis of $CD56^{+}CD3^{+}$ NKT cells, as their frequency is very low, if detected at all, in CB. The results shown in **Figure 3D** show slightly lower levels of NKG2D in CBMCs (not significant) for NK cells and T cells. CD107a expression was significantly lower for both $CD56^{bright}$ and $CD56^{dim}$ NK cells in CB cells but there was no significant difference when comparing T cells. Analysis of IFN-γ also revealed significantly lower levels in CB compared with PB mononuclear cells. Overall, it can be concluded that the same effect is observed with CBMCs as with PBMCs. The significantly lower CD107a expression and IFN-γ production by CBMCs may be due to phenotypic difference between the cell types and also because CB cells are already suppressed due to the presence of sNKG2DLs in the plasma.

## MICB Allele and/or Promoter Types Affect Levels of sMICB and Functional Activity

It is possible that the highly polymorphic nature of the MICA/B genes compared with ULBP1 (which is largely conserved) could explain why we did not see strong correlations between sMICA/B levels and immunosuppressive potential, as we did with sULBP1 (**Figure 2**). Using DNA extracted from UCB cells, we performed MICB allele (exons 2–6) and 5′UT promoter typing as previously described (14). The aim was to establish whether allelic differences in either the promoter or the coding regions of the MICB gene influence the amount or the immunosuppressive potential

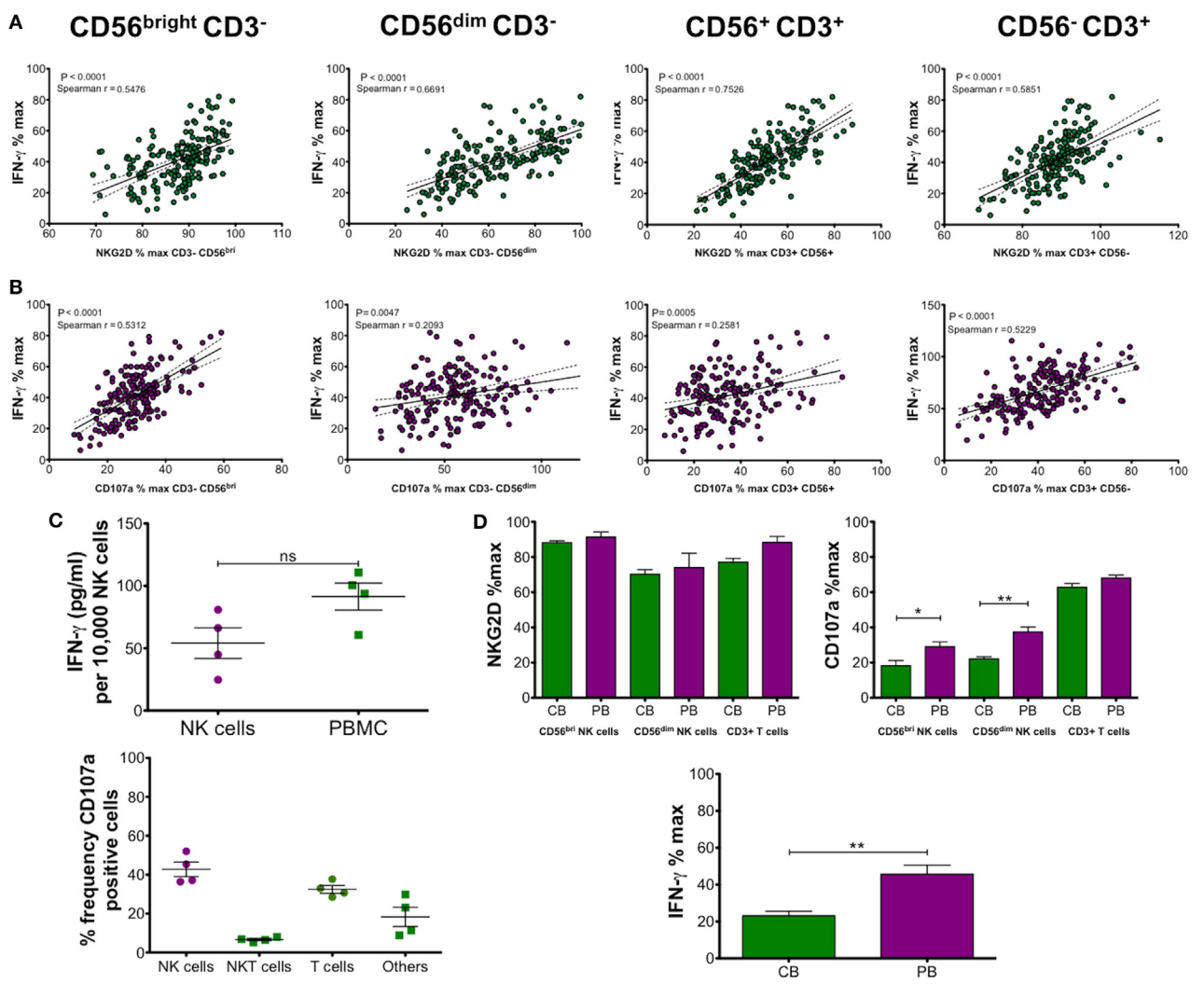


**FIGURE 3** | **(A,B)** Correlation and linear regression analysis of NK group 2, member D (NKG2D) and CD107a expression on CD56$^{bright}$ CD3$^-$, CD56$^{dim}$ CD3$^-$ natural killer (NK) cells, CD56$^+$ CD3$^+$ NKT cells, or CD56$^-$ CD3$^+$ T cells with IFN-γ production. Peripheral blood mononuclear cells (PBMCs) were incubated with 50% cord blood plasma (CBP) ($n$ = 181) or media only for 48 h and then stimulated with PMA and ionomycin. IFN-γ in culture supernatants was measured by ELISA and expression of CD107a and NKG2D on the different cell types was measured by flow cytometry. **(C)** IFN-γ production by isolated NK cells or PBMCs was measured after CBP incubation and stimulation with PMA and ionomycin. The upper graph shows a comparison of isolated NK cells with PBMCs after calculation of IFN-γ detected by ELISA (pg/ml)/10,000 NK cells. The lower graph shows relative frequencies of CD107a-positive CD56$^+$CD3$^-$ NK cells, CD56$^+$ CD3$^+$ NKT cells, and CD56$^-$ CD3$^+$ T cells detected by flow cytometry. **(D)** Comparison of cord blood (CB) and adult peripheral blood (PB) derived mononuclear cells after incubation with equivalent CBP for 48 h and PMA/ionomycin stimulation ($n$ = 7). NKG2D and CD107a expression relative to media only controls was measured by flow cytometry and IFN-γ detected by ELISA. Each experiment was repeated with four different PBMC donors or cord blood units and data points represent donor means. $P$-values for Spearman $r$ correlations are indicated **(A,B)**. Statistical significance **(C,D)** was determined by Mann–Whitney test ± SEM (*$P \leq 0.05$ and **$P < 0.01$).

of sMICB present in CBP using, where possible, UCB samples from homozygous individuals.

The graph in **Figure 4A** shows concentration of soluble MICB found in UCB from homozygous individuals for five common coding alleles of the MICB gene. The allele MICB*005:02 is highly frequent, with an allele frequency (AF) of around 60% in population studies (21, 22) and 64% in our cohort. Plasma samples from homozygote individuals with this allele show significantly higher levels of sMICB that those from individuals carrying either the *002 or *008 alleles.

We also examined the contribution of promoter types on the concentration of MICB in CBP. Promoter MICB-P2 was associated with the highest levels of sMICB and MICB-P9 with the lowest (**Figure 4A**). It is difficult to determine whether the higher levels of sMICB arise from structural differences in the MICB molecule or polymorphisms within the promoter region as the MICB allele*005:02 is uniquely associated with seven different MICB promoter types, including MICB-P2 (14, 15, 23), whereas all other MICB alleles are associated with only one or two promoter types (**Table 1**). The MICB promoter type MICB-P9 is associated with

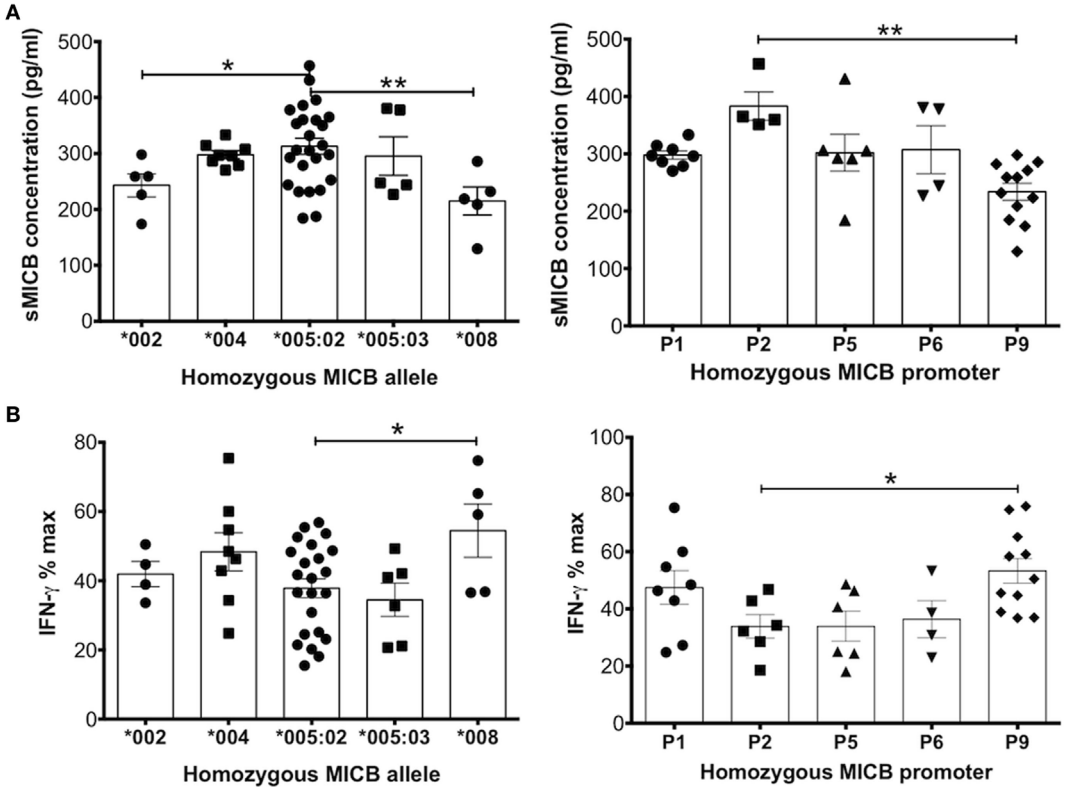


**FIGURE 4** | MHC class I-related chain B (MICB) allelic and promoter polymorphisms result in differential levels of soluble MICB found in cord blood plasma that influences the potential of these ligands to reduce IFN-γ production after peripheral blood mononuclear cell (PBMC) stimulation. **(A)** Levels of soluble MICB are significantly different depending on the MICB allele or the MICB proximal promoter type detected in cord blood DNA. **(B)** Similarly, polymorphic allelic differences or the proximal promoter type of MICB associate with higher or lower levels of IFN-γ. For both types of polymorphism, higher soluble MICB associates with lower IFN-γ. Data represent homozygous MICB typing obtained from a total of 181 cord blood DNA samples. Each experiment was repeated with four different PBMC donors and data points represent donor means Statistical analysis was performed using Kruskal–Wallis test with Dunn's *post hoc* test ± SEM (*$P \leq 0.05$ and **$P < 0.01$).

both MICB*002 and *008 and previously shown to result in reduced MICB transcription (16) owing to a two-nucleotide deletion at position -138-139 of the proximal promoter region. Since UCB typed as MICB*002/*008 with MICB-P9 promoter type shows significantly lower expression of sMICB in CBP, it is likely that promoter polymorphisms are responsible. Similarly, the promoter type MICB-P2 has a unique deletion of one nucleotide at position -126 and a G to T mutation at nucleotide position -66 and associated with significantly higher sMICB levels compared with other MICB promoter types. Therefore, to directly investigate whether promoter polymorphisms result in different levels of expression, we carried out *luciferase* assays using constructs based on most known MIC promoter polymorphisms, as described below.

NK group 2, member D-mediated activation of NK cells occurs *via* NKG2DL upregulation on the cell surface. However, soluble NKG2DLs such as MICA and MICB block and downregulate the receptor and renders the cell anergic or hyporesponsive to activation (24, 25). In line with this concept, higher levels of sMICB in CBP should impair function of NK cells and decrease production of IFN-γ. **Figure 4B** shows levels of IFN-γ produced in supernatants of stimulated PBMC cultures after incubation with CBP, stratified according to homozygous MICB allele or promoter type. As expected, samples typed as MICB*005:02, having the highest sMICB levels, resulted in significantly lower IFN-γ production compared with MICB*008, which had the lowest sMICB levels and the highest levels of IFN-γ. When considering the promoter types, MICB-P9 (linked with MICB*002/*008 alleles) also showed lower levels of sMICB and therefore significantly higher IFN-γ production. Overall, it could be concluded that increased levels of sMICB decreased NK cell functional potential, as determined by lower levels of IFN-γ. In addition, it is possible that MICB promoter region polymorphism abrogated transcription factor binding, causing differences in transcription levels.

## MICA Genotype Affects Levels of sMICA and Is Associated With Increasing NK Cell Functional Capacity

Unlike sMICB and sULBP1, which were detected in all CBP samples tested, sMICA was detected in only 67 of 181 samples or 37% with a mean concentration of 126.8 pg/ml. MICA allele

**TABLE 1** | MICA and MICB 5′UT promoter, allele and 3′UTR allelic associations.

| 5′UT promoter type(s) | Allele (exons 2–6) | 3′UTR type |
|---|---|---|
| **MICA** | | |
| P7 | 001 | ND |
| P3, P4, P7 | 002:01 | UTR2, UTR5 |
| P7, P8, P13 | 004:01 | UTR1 |
| P7 | 006 | ND |
| P7, P11 | 007:01 | UTR2 |
| P11 | 007:02 | ND |
| P1, P6, P7 | 008:01/04 | UTR1 |
| P7 | 009:01 | UTR1, UTR6 |
| P7 | 009:02 | UTR1 |
| P7, P9 | 010:01 | UTR1 |
| P4 | 011 | UTR7 |
| P5 | 012:01 | UTR4 |
| P7 | 015 | ND |
| P4 | 016 | UTR1 |
| P3 | 017 | UTR2 |
| P7 | 018:01 | UTR4 |
| P2 | 019 | UTR1 |
| P4 | 023 | ND |
| P7 | 027 | UTR1 |
| P7 | 033 | UTR1 |
| P10 | 045 | UTR2 |
| P10 | 059 | UTR2 |
| **MICB** | | |
| P9 | 002:01 | UTR1 |
| P11 | 003 | UTR2 |
| P1, P3 | 004:01 | UTR2 |
| P1, P4 | 005:01 | ND |
| P1, P2, P5, P6, P8, P9, P10, P12 | 005:02 | UTR1 |
| P6, P7 | 005:03 | UTR1 |
| P5 | 006 | ND |
| P9 | 008 | UTR1 |
| P11 | 009N | UTR2 |
| P6 | 013 | ND |
| P9 | 014 | UTR1 |
| P10 | 023 | ND |

*ND, not determined; UTR, untranslated region; MICB, MHC class I-related chain B; MICA, MHC class I-related chain A.*

and promoter genotyping was carried out on all UCB DNA samples (14). Most individuals are heterozygous for MICA alleles and homozygotes were mostly restricted to MICA*002 or MICA*008 in this study. Therefore, we analysed the data on the basis of methionine (met) or valine (val) amino acid presence at residue 129 of the MICA protein. This dimorphism has been shown to have functional consequences as the met protein variant has stronger binding affinity for NKG2D than val and leads to enhanced NKG2D-mediated NK cell activation (26–28). By categorising MICA genotypes as MICA-129met or MICA-129val, we found significantly higher levels of sMICA in plasma from UCB with the MICA-129val/val and 129val/met alleles, whereas almost no sMICA could be detected from MICA-129met/met UCB samples (**Figure 5A**). Overall, the results indicated that the presence of sMICA in CBP was restricted to UCB with genotypes encoding MICA-129val, which has weaker affinity for NKG2D. As seen with sMICB, we expected plasma samples positive for sMICA to decrease NK cell function; however, this was not the case as shown in **Figures 5B,C**. Cells exposed to plasma from UCB samples homozygous for MICA-129val showed significantly increased production of IFN-γ as well as higher levels of CD107a and NKG2D compared with those from MICA-129met/met or MICA-129val/met indicating enhanced NK cell function.

An interesting observation that may relate to shedding of soluble MICA molecules was made by stratifying samples on the basis of MICA transmembrane (TM) polymorphisms, designated A4, A5, A5.1, A6, and A9 based on the number of alanine repeats in the TM region. Type MICA-A5.1 is commonly found among MICA*008 alleles and has a nucleotide deletion leading to a premature stop codon that truncates the TM region and cytoplasmic tail. Shedding of sMICA is dependent on the disulphide isomerase Erp5 that causes a large conformational change, allowing metalloprotease cleavage (24). **Figure 6A** shows increasing levels of sMICA with increasing alanine repeats in heterozygotes, with the exception of MICA-A9. These results were confirmed by examining heterozygous allele combinations with the frequent MICA-A5.1 (**Figure 6B**). Due to insufficient homozygous samples, it was only possible to confirm that MICA-A6 associated sMICA has significantly higher concentration than MICA-A5 (**Figure 6C**). It is possible that the length of the TM region determines the efficiency of Erp5 and metalloprotease. For example, UCB typed as MICA-A4 had a mean concentration of $13.8 \pm 4.7$ pg/ml compared with $35.52 \pm 9.0$ pg/ml for A5 positive samples ($P = 0.04$). Maximal sMICA concentration was found with A6 ($79.51 \pm 8.2$ pg/ml) but A9 was significantly lower having $24.6 \pm 6.4$ pg/ml ($P < 0.0001$), perhaps indicating A9 is too long and destabilises the complex resulting in less cleavage and less sMICA. Further investigation revealed that most MICA-129val alleles had 5 or 6 alanine repeats and most MICA-129met alleles had 4 or 9 (**Table 2**).

## Transcriptional Analysis of MICA and MICB Promoters Confirms Allelic Differences Can Affect Levels of Gene Transcription

To clarify how different promoter polymorphisms affect levels of transcription we performed experiments using proximal promoter polymorphic regions of MICA/B genes in *luciferase* reporter assays to determine whether sMICA or sMICB concentration in CBP corresponds to transcriptional activity. This would enable further confirmation of a likely fetal origin of sMICA/B if the transcriptional activities of the promoters correspond to sMICA/B levels found in CBP and also help identify whether any other events are involved in their shedding and release. The transcription level profiles relative to the SV40 promoter in proliferating HCT116 cells for MICA/B promoter types are shown in **Figure 7**. In general, slightly higher levels of around 45% RRR are seen with MICA compared with approximately 35% with MICB. Although only moderate differences are seen in transcription levels between promoter types, there are some notable exceptions for both MICA and MICB. Transcriptional activity for MICA-P6 was significantly lower than the next lowest value seen for MICA-P2 ($P < 0.0001$) and more than 50% lower RRR than other MICA promoter types. The MICA promoter that is found in association with the vast majority of MICA coding alleles is MICA-P7 and had highest RRR of all promoter types

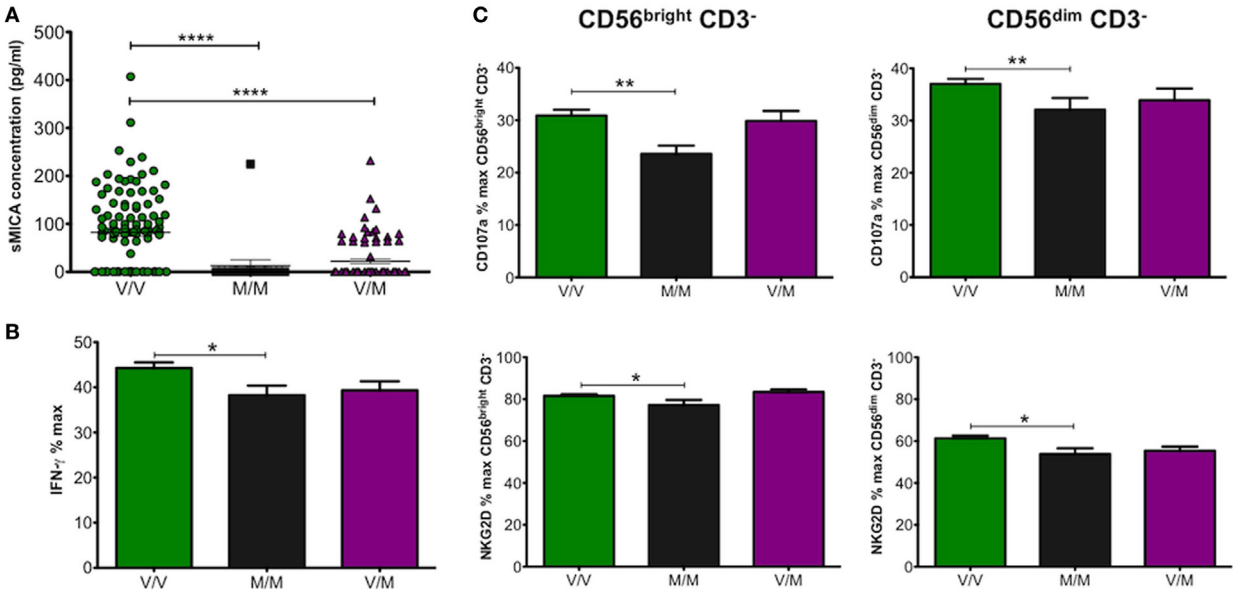


**FIGURE 5** | **(A)** Effect of allelic MHC class I-related chain A (MICA) differences encoding MICA-129val (V/V), MICA-129met (M/M), or heterozygotes (V/M) on concentration of sMICA (pg/ml) found in cord blood plasma (CBP) samples. Values for sMICA concentration were obtained by ELISA for each CBP sample. **(B)** Production of IFN-γ (%max) by peripheral blood mononuclear cells (PBMCs) incubated with CBP from umbilical cord blood encoding MICA allelic variants MICA-129val or -met. **(C)** CD107a and NK group 2, member D (NKG2D) levels detected on $CD56^{bright}$ and $CD56^{dim}$ natural killer cells. Each experiment was repeated with four different PBMC donors and data points represent donor means **(B,C)**. Statistical analysis was performed using Kruskal–Wallis test with Dunn's *post hoc* test ± SEM **(A)** or Mann–Whitney test ± SEM **(B,C)** (*$P \leq 0.05$, **$P < 0.01$, and ****$P < 0.0001$).

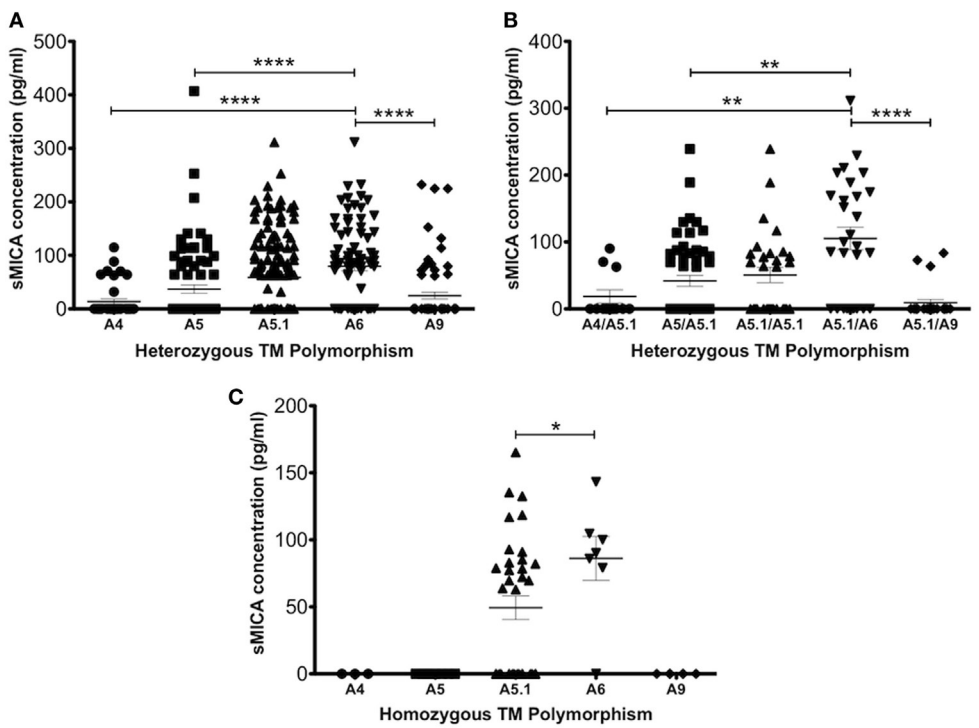


**FIGURE 6** | Influence of MHC class I-related chain A (MICA) transmembrane (TM) polymorphism on sMICA levels detected in umbilical cord blood (UCB) plasma samples. UCB samples were stratified according to the number of alanine residues encoded by GCT triplet (Ala) repeats in exon 5 of the MICA gene. **(A)** Heterozygous allele combinations with any other allele. **(B)** Heterozygous allele combinations with A5.1, which associates with MICA*008 with high frequency. **(C)** Homozygous TM polymorphism. Data represent MICA typing and ELISA measurement of sMICA obtained from 181 cord blood samples. Statistical analysis was performed using Kruskal–Wallis test with Dunn's *post hoc* test ± SEM (*$P \leq 0.05$, **$P < 0.01$, and ****$P < 0.0001$).

except MICA-P14. MICA-P14 was found uniquely during this study in two individual UCB samples from our cohort of 181 and is described in more detail elsewhere (29). Transcriptional activity of this promoter is high, around 75% relative to the SV40 control and higher than all other MICA promoters studied. Comparing the transcription level to MICA-P7, MICA-P14 was 20% higher ($P < 0.0001$).

MICB*005:02 promoter polymorphisms also result in variable levels of transcription. For example, MICB-P2 had the highest RRR of 58.5% compared with the next highest MICB*005:02-associated promoter MICB-P10, achieving 45% RRR ($P = 0.002$). MICB-P8 had the lowest activity of 23% and was lower than MICB-P9 with 31% ($P = 0.004$), which is known to have low expression (16). Thus, both highly frequent MICA and MICB alleles may be expressed differently depending on the polymorphisms present in the promoter region.

Next, we tested for MICA/B allelic promoter transcription levels of the *luciferase* reporter gene after heat-shock treatment using a previously described method (17), whereby HCT116 cells were cultured for 7 days to over-confluence followed by heat-shock or no heat-shock treatment of these quiescent cells. Venkataraman and colleagues demonstrated that for both MICA and MICB, this core promoter region contains heat-shock elements resulting in upregulation of *luciferase* reporter gene expression and our results using allelic variants are comparable. **Figure 8** shows the RRR of *luciferase* activity driven by promoter variants associated with MICA and MICB genes. Alongside these results are nucleotide alignments of polymorphic positions related to each promoter type and their relative position upstream of the ATG start codon. The unusually low transcription associated with MICA-P6 promoter remained after heat-shock and in quiescent cells. The difference between this promoter and the prevalent MICA-P7 is a nucleotide substitution of G to A at position -55 but this is not known to interfere with transcription factor-binding sites (TFBS). However, a TATA-like sequence and an activating protein 1 (AP-1)-binding site is located alongside at positions -61 to -75 as defined previously (17) and unknown transcription factors may also need to bind in this region, but this remains to be determined. MICA-P5 also has lower transcription than all other promoter types except P6 and has a C to G mutation at position -68 that interrupts a TFBS for AP-1, which may explain the lower transcriptional potential.

MHC class I-related chain B promoter types generally increased transcription under heat-shock conditions more than MICA promoters, whereas in quiescent cells *luciferase* activity was lower. MICB-P2 had the highest RRR in proliferating cells and was also much higher in heat-shocked cells at around sixfold relative to the SV40 control, compared with around threefold to fourfold for most other types. There are two consecutive nucleotide changes associated with MICB-P2 at positions -126 and -125 causing a C deletion and T to A mutation, respectively. These changes are very close to a TFBS for c-myb and the c-terminal domain of c-myb is a known transcriptional repressor (30), which may lead to overexpression of alleles driven by this promoter if c-myb binding is abrogated. The low expression indicated in proliferating cells

**TABLE 2** | MHC class I-related chain A (MICA) alleles grouped as MICA-129val or MICA-129met and their associated transmembrane polymorphisms encoding alanine repeats.

| MICA-129val | MICA-129met |
|---|---|
| *004–A6 | *001–A4 |
| *006–A6 | *002–A9 |
| *008–A5.1 | *007–A4 |
| *009–A6 | *011–A6 |
| *010–A5 | *012–A4 |
| *016–A5 | *015–A9 |
| *019–A5 | *017–A9 |
| *027–A5 | *018–A4 |
| *028–A5 | *020–A10 |
| *033–A5 | *026–A5 |
| *048–A5 | *029–A4 |
| *049–A6 | *030–A6 |
| *053–A5 | |
| *054–A5 | |
| *056–A5 | |

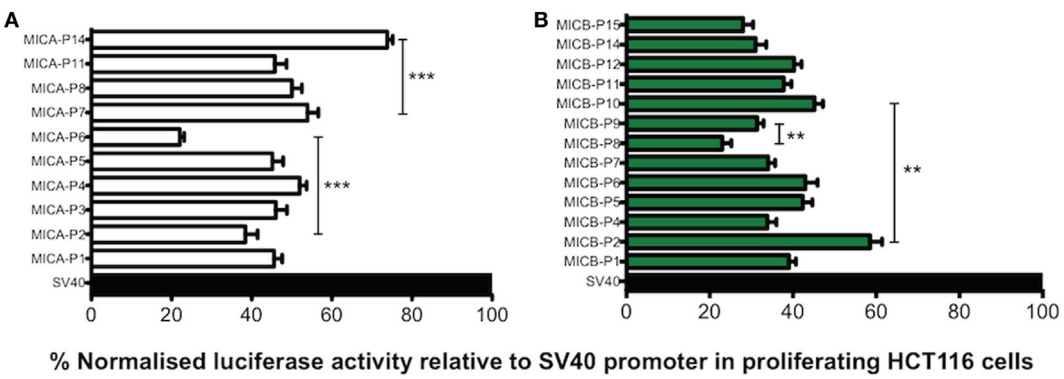


**FIGURE 7** | Transcriptional analysis of MHC class I-related chain A (MICA) and MHC class I-related chain B (MICB) promoter regions in proliferating HCT116 cells. Normalised percent *luciferase* activity relative to SV40 control plasmid transfection is shown. Assay conditions were performed in triplicate with three independent preparations of plasmid construct DNA and repeated for a total of four independent experiments. **(A)** MICA promoter driven *luciferase* transcription relative to SV40 promoter in proliferating HCT116 cells 24 h post-transfection. **(B)** MICB promoter driven *luciferase* transcription relative to SV40 promoter in proliferating HCT116 cells 24 h post-transfection. Statistical analysis was performed using Mann–Whitney test ± SEM (**$P < 0.01$ and ***$P < 0.001$).

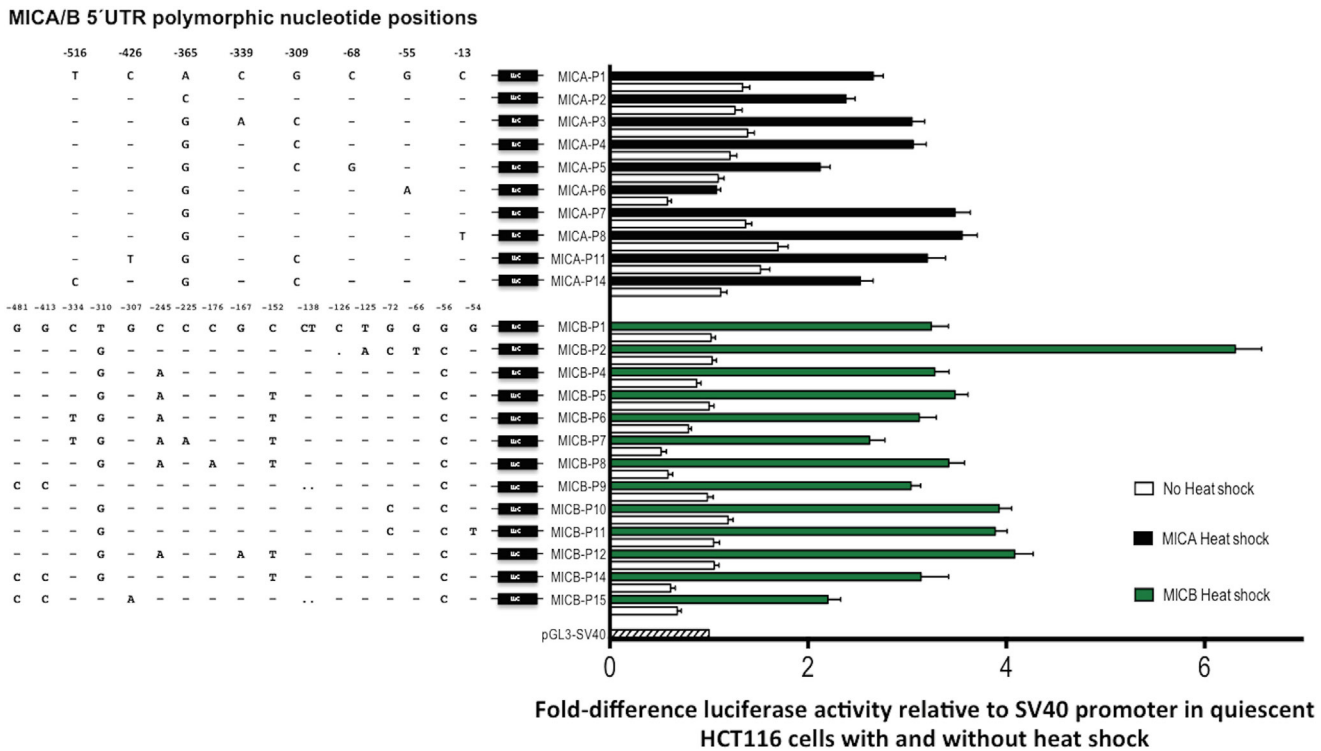


**FIGURE 8** | Transcriptional analysis of MHC class I-related chain A (MICA) and MHC class I-related chain B (MICB) promoter regions. 5′UTR polymorphic nucleotides associated with each type of MICA/B promoter are shown on the left and fold transcriptional activity relative to SV40 promoter is shown alongside. Quiescent HCT116 cells were grown to over-confluence for 7-day post-transfection before being heat-shock-treated (black/green bars) or isotype-treated without heat-shock (white bars). Fold-difference relative to SV40 control plasmid transfection is indicated. Assay conditions were performed in triplicate with three independent preparations of plasmid construct DNA and repeated for a total of four independent experiments. Nucleotide positions relative to ATG start codon are indicated for each polymorphic position. Abbreviations: UTR, untranslated region; LUC, *luciferase* gene.

under control of MICB-P8 was also seen in quiescent cells but heat-shocked levels of transcription were comparable to most other MICB promoter types.

Overall, these results indicate that differences in levels of transcription can occur not only between different NKG2DL genes but also between different promoter alleles of these genes, giving rise to variable expression level potential depending on an individual's genotype. Furthermore, transcriptional regulation may vary depending on the conditions the cells are subjected to as well as the tissue origin of the affected cell.

## Differences in Levels of Transcription for Different Promoter Types Are Not Influenced by 3′UTR Polymorphisms Potentially Affecting miRNA Binding

There are currently 9 and 7 known polymorphic positions or deletions within the 3′UTR of MICA and MICB genes, respectively. Some polymorphisms occur together and have been grouped in a similar way to MICA/B promoter types, giving rise to seven types for each gene. A study of 104 unrelated, healthy Chinese Han individuals found only two types for each gene comprised the majority of this diversity (18, 19). MICA-3′UTR1 had an AF of 69.7% and MICA-3′UTR2 was 23.6%. Similarly, MICB-3′UTR1 had an AF of 79.8 and 13% for MICB-3′UTR2.

The 3′UTR harbours recognition elements for miRNAs. miRNAs are short, single-stranded noncoding molecules around 19–22 nucleotides long and either endogenous or virally encoded in origin (31). In most cases, they function to suppress gene expression during processing and *in vivo* could potentially alter results obtained using *luciferase* gene reporter assays investigating differences in promoter sequences.

To identify whether or not 3′UTR polymorphisms may affect results obtained in this study, we confirmed the results of the Chinese Han studies (18, 19) using IHW cell line DNA previously characterised for promoter and MICA/B types (14). 3′UTR polymorphism analysis was performed by PCR amplification of MICA-3′UTR (692 bp) and MICB-3′UTR (1,209 bp) followed by Sanger sequencing. The results, shown in **Table 2**, were in agreement with the previous studies and showed that MICA-P6 was on the same haplotype as MICA-3′UTR1, as were other promoters with higher transcriptional potential. Of particular interest, the strong promoter MICB-P2 was also on the same haplotype as MICB-3′UTR1, which was also seen with MICB-P5, P6, P9, P10, and P12. Consequently, the low expression driven by MICA-P6 or high expression driven by

MICB-P2 is unlikely to be affected by polymorphisms in the 3′UTR influencing miRNA binding.

## Promoter Type-Specific Transcription Potential and Levels of sNKG2DLs Are Similar for sMICB Ligands but Not sMICA

**Figure 9** shows relative *luciferase* activity detected using individual promoter types for MICA (**Figure 9A**) and MICB genes (**Figure 9B**) alongside the corresponding levels of sMICA/B detected among 181 UCB samples. Owing to a lack of homozygous promoter types for MICA, we analysed different promoter types in combination with the common MICA-P7 promoter. We found that MICA-P6 and -P2 relative *luciferase* activity was significantly lower in both proliferating and heat-shocked HCT116 cells. By contrast, levels of sMICA in MICA-P6-positive samples were significantly higher. In addition, levels of sMICA among MICA-P11 samples were significantly lower than MICA-P7 alone but transcription levels in both experimental conditions for promoter P11 were similar to most other promoter types. Furthermore, the results shown in **Figure 5** show CBP with detectable sMICA associated with MICA-129val and the data presented in **Figure 6** and **Table 2** show a relationship between TM alanine repeats and sMICA levels. These results strongly suggest that rather than differences in transcription potential determining the amount of sMICA detected in CBP, their presence may be related to structural differences.

The analysis of sMICB ligand concentration levels from homozygous promoter typed samples and relative *luciferase* activity for individual promoter sequences showed similar profiles to both proliferating and heat-shocked HCT116 cells. In heat-shocked cells, MICB-P2 had highly significantly increased levels of *luciferase* activity compared to MICB-P1, P5, P6, and P9, with lowest levels related to MICA-P9. The profile seen relating to sMICB concentration is very similar to these transcription levels with MICB-P2 levels significantly higher and MICB-P9 significantly lower. Therefore, in contrast to MICA, it is likely that levels of sMICB are directly related to promoter strength.

## DISCUSSION

We have previously shown that CBP contains soluble ligands for NKG2D that can influence the function of NK cells and other cells by decreasing cytotoxicity *via* their interaction with the NKG2D receptor (10). Furthermore, we demonstrated that at the time of birth, significantly higher levels of sMICA and sMICB were present in CBP than in plasma from the paired maternal peripheral blood. By contrast, higher levels of sULBP1 were found in maternal plasma than CBP, indicating a possible fetal source of sMICA/B and maternal source of sULBP1. Differences were also detected in the physical form of the ligands as sMICA/B were

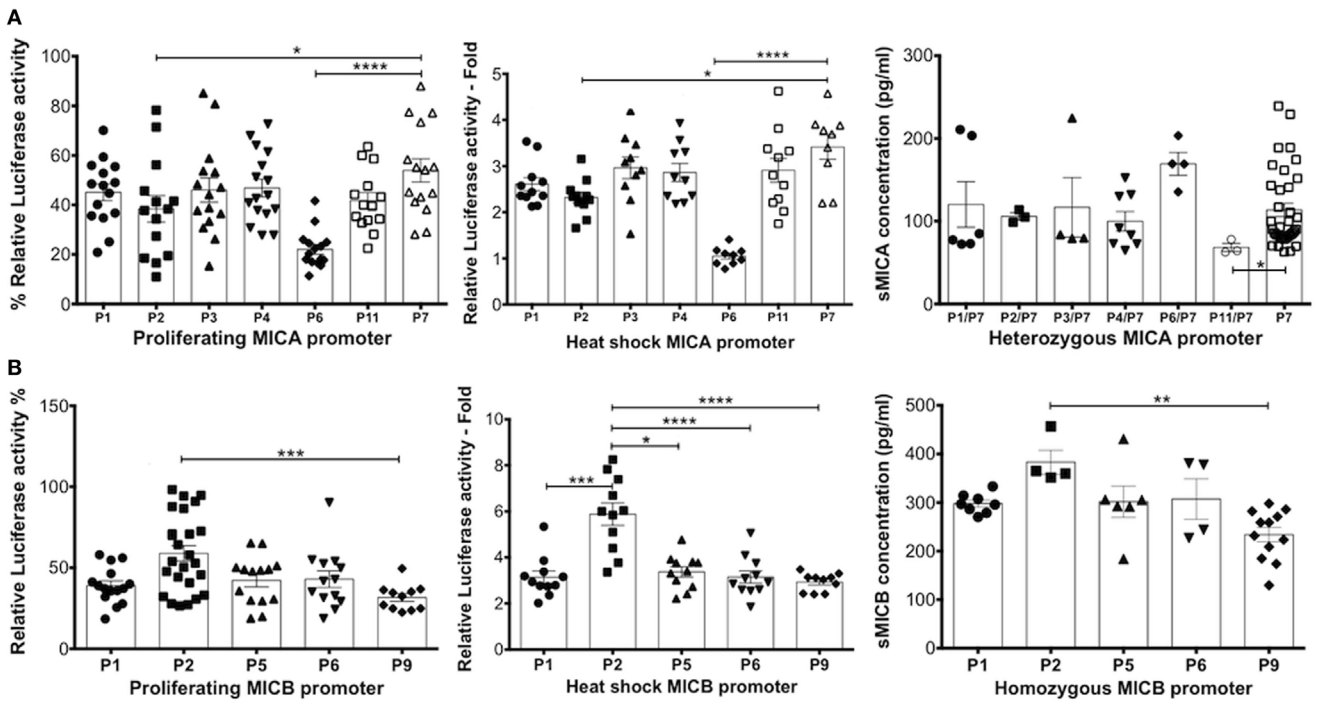


**FIGURE 9** | Comparison of relative *luciferase* transcription potential of MHC class I-related chain A (MICA)/MHC class I-related chain B (MICB) promoter types in proliferating and heat-shocked HCT116 cells and comparisons with levels of sMICA/B in cord blood. **(A)** Relative *luciferase* activity obtained using MICA promoter types. Owing to a lack of MICA homozygotes, heterozygous MICA promoter types were analysed in combination with the highly frequent MICA-P7 for comparison of sMICA levels with transcription levels. **(B)** Relative *luciferase* activity obtained using MICB promoter types associated with sMICB levels in homozygous MICB promoter typed cord blood. sMICA/B concentrations were obtained by ELISA for each cord blood plasma sample. Statistical analysis was performed using Kruskal–Wallis test with Dunn's *post hoc* test ± SEM (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$).

shown to be single soluble molecules whereas sULBP1 was found on isolated exosomes. In this study, we sought to determine which sNKG2DLs were responsible for the NK cell functional suppression. Did one type of ligand dominate in suppressive action or were other ligands needed and does polymorphism of MICA and MICB have any effect? Furthermore, can the information obtained from this analysis delineate the nature of this mechanism in terms of fetal–maternal tolerance or *in utero* immunity?

We utilised the ability of CBP containing sNKG2DLs to inhibit NK cell responses *via* NKG2D as previously demonstrated (10), choosing optimal conditions such as concentration of CBP and time of incubation to further define the biological role of the various sNKG2DLs. This time, a large cohort of 181 CBP and UCB DNA was collected and used to determine not only the type of sNKG2DLs present but also the MICA and MICB allelic and promoter types of the fetus. With this information we could then determine how differences in structure derived from allelic variants and levels of expression governed by polymorphic promoter sequences affected the functional potential of CBP to suppress the activation of NK cells, NKT cells, and T cells.

The data show that the main sNKG2DL correlating with functional suppression of NK cells and other cells expressing NKG2D is sULBP1, which is also the most abundant and, in contrast to sMICA and sMICB, monomorphic. However, stratification based on allelic types of MICB also shows a suppressive effect with some alleles having more of an effect than others. This is either a consequence of structural differences or a tendency for a particular allele to be expressed more abundantly due to promoter nucleotide polymorphisms. Indeed, the data show that differences relating to a particular allele can be explained by the variation in the promoter region. For example, sMICB*002 and *008 displayed lowest levels in CBP and both have MICB-P9 type promoter that also correlated with low relative transcriptional activity. Conversely, MICB*005:02 homozygotes had the highest concentration of sMICB in plasma and one of the promoter types associated with this allele, MICB-P2, also had high levels of expression and very high transcriptional potential. Contrary to what was expected, the two-nucleotide deletion of CT at positions -138-9 affecting MICB-P9 (and P15) did not result in substantial transcriptional reduction after heat-shock and was only slightly lower than other promoter types in proliferating cells. MICB-P15 was lower under all conditions but has an additional mutation of G to A at position -307, which may alter a TFBS. There are a number of reasons why our results may differ to those of Rodríguez-Rodero and colleagues (16). First, we used HCT116 cells, whereas Hela and CaCo-2 cells were used in the previous study, perhaps indicating that differences in cells or tissues can affect transcription. In addition, the expression plasmid used was not the same and heat-shock conditions were not tested previously. As we observed with other promoter types such as MICB-P8, consistently low expression in proliferating cells was not observed in heat-shocked cells.

Our data also showed that the higher plasma concentrations of sMICB induced the lowest levels of IFN-γ after incubation with PBMCs. However, this was not the case with sMICA allelic variants where higher levels of sMICA were associated with more activation and production of IFN-γ. Neither was there a clear relationship between the level of transcription potential and the concentration of sMICA detected in CBP. However, we did identify MICA promoters that caused high or low expression in association with the same MICA allele. In our previous study (14), we found MICA-P6 sometimes associated with MICA*008 (the most common MICA allele in most populations), rather than the more frequent MICA-P7 promoter (**Table 1**). Although the population frequency of this promoter type is largely unknown, one study investigating MICA allele and promoter haplotypes suggested an AF for MICA-P6 of around 3% in Chinese Han, on the same haplotype as MICA*008:01 (15). MICA-P13 and MICA-P14 were identified as a novel promoter types in our cohort of cord blood samples (29) and have the accession numbers KM358317 and KM358136, respectively. Although we cannot confirm the MICA allele haplotype association with MICA-P14, both individuals with this novel promoter were typed as MICA*008 so it is possible that this allele can be over-expressed in the presence of strong promoters such as P14. Overall, most individuals express MICA*008 moderately *via* MICA-P7 but those with the MICA-P6/*008 haplotype have very low expression and those with the MICA-P14/*008 have very high expression. Hence, MICA*008, currently defined as one allele type in terms of structure, may have diverse functional potential due to large differences in expression and have implications regarding MICA genotype and disease association studies, the immune response to tumours, infections, and transplantation in the unrelated setting.

Testing of CBP revealed that only samples typed as MICA-129val or MICA-129val/met had detectable levels of sMICA and this seemed to relate to the length of the TM region that may be influencing its ability to be shed from the as yet unconfirmed cellular source. These results may offer insights into mechanisms of *in utero* immunity that are currently very poorly understood. On one hand, immune tolerance and suppression is essential to maintain a *status quo* and avoid problems arising from allogenicity between the fetus and the mother. On the other hand, mechanisms must exist to allow immune challenge by the fetus against opportunistic pathogens in order to survive. We have derived a model based on the main findings of this study that allows sNKG2DLs to maintain tolerance under normal circumstances but also enable a break in tolerance and activation of NK cells to pursue an innate immune challenge.

The preliminary model, illustrated in **Figure 10**, allows NK cell suppression due to an abundance of sMICB and sULBP1 ligands within the fetal periphery. Furthermore, sULBP1 is exosomal, multivalent and able to crosslink NKG2D to provide strong signals *via* NKG2D. This is supported by the finding that sULBP1 is strongly correlated with decreased NKG2D expression, NK cell activation, and production of IFN-γ. High levels of sMICB also associated with some of these factors, but its high level of polymorphism may have prevented demonstration of a strong correlation. Macrophages are known to express surface MICA (32–36) and may offer a source of sMICA, detected in around a third of CBP samples, and an innate cellular mechanism of immunity *in utero*. In stark contrast to sMICB and sULBP1, the presence of sMICA resulted in increased activation of NK cells and production of IFN-γ, which was not expected. To understand these results, it is necessary to examine the properties of different NKG2DL and their interaction with NKG2D. MICB and ULBP1 have high affinity for NKG2D and, in

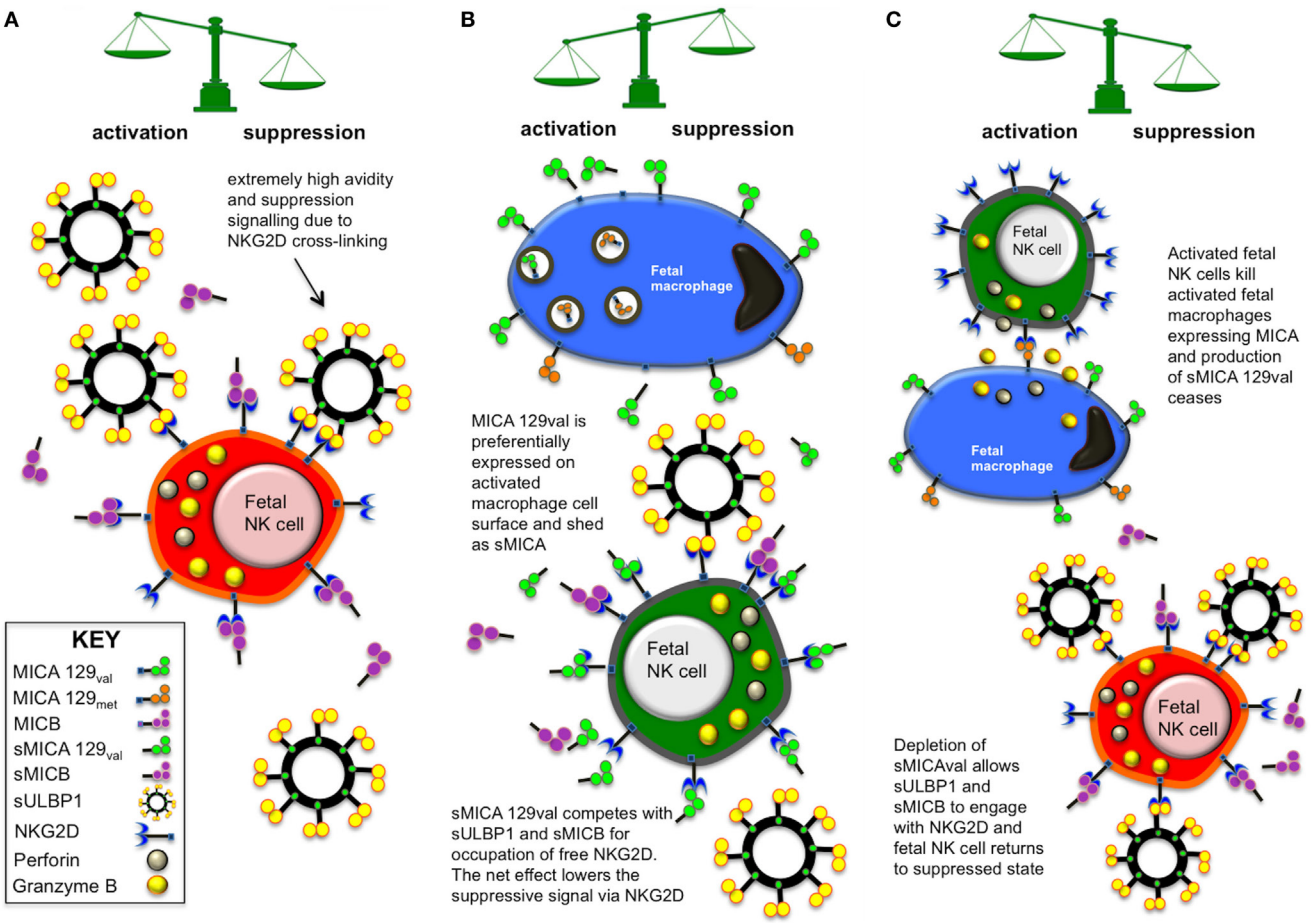


**FIGURE 10** | Model of *in utero* fetal immune suppression and tolerance mediated *via* soluble NKG2D ligand (NKG2DLs) interaction with fetal natural killer (NK) cells. **(A)** Under normal circumstances, an abundance of sMICB and sULBP1 binds with available NK group 2, member D (NKG2D) receptors on fetal NK cells and renders them hyporesponsive to activating stimuli at the maternal:fetal interface, promoting tolerance. In the particular, the very high concentration of high-avidity multivalent exosomal sULBP1 is a powerful suppressor of NK cells and may crosslink NKG2DLs. **(B)** In situations where opportunistic infection arises, innate immune cells such as macrophages may upregulate surface expression of MHC class I-related chain A (MICA) when activated but mechanisms exist preventing expression of MICA-129met variants, which are retained in cytoplasmic vesicles and degraded. The macrophage expression of MICA-129val may be a source of sMICA due to metalloprotease cleavage from the cell surface. Low-avidity sMICA-129val is then able to compete for binding with NKG2D, reducing the overall net suppressive signal to the NK cell. This may result in the NK cell hyporesponsiveness or tolerance being broken, enabling the NK cell to become activated and help in clearing the opportunistic infection. **(C)** Once the immunological threat has been dealt with, the remaining activated macrophages can be eliminated *via* interaction of NKG2D with cell surface MICA, which stops the production of sMICA-129val. NK cells are then able to return to a suppressed state as sMICB and sULBP1 engage available NKG2D receptors.

the case of sNKG2DLs, deliver a strong suppressive signal to the NK cells. However, MICA can bind NKG2D strongly or weakly depending on the presence of met or val, respectively, at residue 129 (26). In addition, an NK cell-mediated immune response has been described (27, 28, 37), whereby certain mechanisms prevent surface expression of MICA-129met variants, which are retained in cytoplasmic vesicles and degraded. Furthermore, with the exception of MICA-P6, variation in the promoter region of MICA genes does not substantially alter the expression potential as most promoter types resulted in similar transcription levels. The upregulation of MICA-129val by activated fetal macrophages may be a source of sMICA due to metalloprotease cleavage from the cell surface and may also explain the fact that only MICA-129val variants were detectable in CBP and that they were not always present. However, no study has yet demonstrated that macrophages can release sMICA, although little is known of fetal immune system mechanisms. The sudden release of soluble MICA-129val ligands may allow competitive binding with sMICB and sULBP1 for free NKG2D on fetal NK cells, especially as new NK cell progeny emerge. Other unknown mechanisms may also decrease or stop the proteolytic release of fetal sMICB. Although the weaker binding affinity with NKG2D may not be able to outcompete the strong affinity sMICB and sULBP1, it may still be able to bind and prevent their occupation of NKG2D. With NKG2D engagement by sMICA, the net suppressive signal to the cell may be reduced, releasing the NK cell from suppression and enabling it to become activated. The activated fetal NK cells can then proliferate and deal with the infection and once cleared, the release of sMICA-129val

ceases. The activated fetal macrophages can then be killed *via* their surface expression of MICA. Soluble MICB and sULBP1 can then dominate occupation of NKG2D on fetal NK cells, which return to their suppressed state due to strong signalling *via* NKG2D.

One problem with this model is that not all individuals express MICA-129val variants as they may be homozygous for MICA-129met or indeed express no MICA at all due to homozygosity of the MICA null allele (38). In this situation, the redundancy of the NKG2DL system may be important to maintain the balance of signals. RAET1G, also known as ULBP5, is an NKG2DL shown to have a similar expression pattern to MICA and can be transcribed as two isomeric forms, RAET1G1 and RAET1G2 (39). RAET1G2 is released as a soluble molecule from the cell and may be used as an alternative to sMICA in this situation. Moreover, RAET1G2 also has a very low affinity for NKG2D, similar to MICA-129val. Although we cannot confirm the presence of soluble RAET1G2 in CBP due to lack of availability of suitable ELISA systems, it may be expressed as we also found sULBP2 and sULBP3 in some CBP samples (10). Therefore, although sMICA may be preferred in immune regulation for unknown reasons, a deficiency of MICA expression with low NKG2D affinity could be compensated by soluble RAET1G2, as it is capable of fulfilling the same function. It is also possible that other sNKG2DLs that were not tested for in this study play a role in modulating NKG2D signalling to release fetal NK cells from suppression. One candidate is sULBP4 as this is the second most polymorphic of the UL-16 binding protein (ULBP) type ligands (3, 5, 40, 41). Binding affinity of ULBP4 for NKG2D is unknown so could be weak and this ligand has recently been shown to have capacity to be expressed as soluble isoforms, either by alternative splicing or proteolytic cleavage (42). We have already typed our cohort of 181 CB samples for ULBP4 allelic polymorphism and are awaiting a suitable and reliable ELISA assay to confirm the presence and level of expression of sULBP4 in CBP.

The preliminary model of fetal NK cell immunity discussed above remains to be confirmed by specific studies to identify the cellular source of sNKG2DLs in cord blood. In addition, the combined effect of different sNKG2DL and their level of expression on the suppression of NK cells need to be investigated in detail as this may represent an important mechanism in fine-tuning the regulation of NK cell immunity in both health and disease.

## CONCLUSION

Overall, we have found that the main sNKG2DL present in CBP contributing to NK cell suppression is sULBP1. sMICB also reduces NK cell cytotoxicity but it is variable depending on the allelic polymorphism of the promoter or coding region. However, the presence of sMICA in some CBP samples results in increased NK cell function. These findings may relate to mechanisms of fetal–maternal tolerance and *in utero* immunity that are currently poorly understood. Future work should focus on determining the cellular origins of the soluble NKG2DL and also characterise the profile relating to the remaining ULBP4, 5, and 6 ligands and their roles in fetal and maternal immunity.

## ETHICS STATEMENT

This study was carried out with the full approval of the local Research Ethics Committee (reference HC71/IU). Peripheral blood was obtained from healthy donors with prior written informed consent. UCB units were obtained from the Anthony Nolan Cord Blood Bank with prior written consent from pregnant mothers and ethical committee approval (Research Ethics Committee reference 10/H0405/27).

## AUTHOR CONTRIBUTIONS

SC, AS, and JM conceived and designed the study. SC designed and performed experiments, acquired data, performed statistical analysis, interpreted the data, and wrote the manuscript. DH and RD contributed to the administrative, technical, or material support of the study and critically revised the manuscript for important intellectual content. RL-B and HP carried out extensive assay procedures and results analysis. All authors approved the final version of the manuscript.

## FUNDING

Anthony Nolan funded the work undertaken for this study (Charity no. 803716/SC038827).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01282/full#supplementary-material.

**FIGURE S1** | Gating strategy for determination of natural killer (NK) ($CD56^+CD3^-$) bright and dim cells, NKT cell ($CD56^+CD3^+$), and T cell ($CD56^-CD3^+$) subpopulations and frequency of CD107a-expressing cells. Lymphocyte gating using forward (FSC) and side scatter (SSC) is shown in panel **(A)**. Lymphocyte subpopulations were distinguished using CD56-APC and CD3-PE-Cy7 fluorochromes **(B)**. **(C)** CD107a-expressing cells were gated using SSC against CD107a (FITC) and $CD56^+CD3^-$ NK cells, $CD56^+CD3^+$ NKT cells, and $CD56^-CD3^+$ T cells were gated from this population **(D)**. Double CD56 and CD3-negative cells were designated as "others" for use in **Figure 3**

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**Conflict of Interest Statement:** Author AS is currently employed by company GlaxoSmithKline. All other authors declare no competing interests.

frontiers in Immunology

CORRECTION
published: 04 February 2020
doi: 10.3389/fimmu.2020.00087

# Corrigendum: Functional Characterisation and Analysis of the Soluble NKG2D Ligand Repertoire Detected in Umbilical Cord Blood Plasma

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**Approved by:**
Frontiers Editorial Office, Frontiers Media SA, Switzerland

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

**Received:** 07 January 2020
**Accepted:** 13 January 2020
**Published:** 04 February 2020

**Citation:**
Cox ST, Danby R, Hernandez D, Laza-Briviesca R, Pearson H, Madrigal JA and Saudemont A (2020) Corrigendum: Functional Characterisation and Analysis of the Soluble NKG2D Ligand Repertoire Detected in Umbilical Cord Blood Plasma. Front. Immunol. 11:87. doi: 10.3389/fimmu.2020.00087

**Keywords: NK cell, pregnancy, tolerance, NKG2D, ligand, luciferase, MICA, ULBP1**

**A Corrigendum on**

**Functional Characterisation and Analysis of the Soluble NKG2D Ligand Repertoire Detected in Umbilical Cord Blood Plasma**
*by Cox, S. T., Danby, R., Hernandez, D., Laza-Briviesca, R., Pearson, H., Madrigal, J. A., et al. (2018). Front. Immunol. 9:1282. doi: 10.3389/fimmu.2018.01282*

**Raquel Laza-Briviesca** and **Hayley Pearson** were not included as authors in the published article. The corrected Author Contributions Statement appears below.

## AUTHOR CONTRIBUTIONS

SC, AS, and JM conceived and designed the study. SC designed and performed experiments, acquired data, performed statistical analysis, interpreted the data, and wrote the manuscript. DH and RD contributed to the administrative, technical, or material support of the study and critically revised the manuscript for important intellectual content. RL-B and HP carried out extensive assay procedures and results analysis. All authors approved the final version of the manuscript.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

frontiers in Immunology

MINI REVIEW
published: 18 June 2018
doi: 10.3389/fimmu.2018.01324

# New Insights Into the Regulation of Natural-Killer Group 2 Member D (NKG2D) and NKG2D-Ligands: Endoplasmic Reticulum Stress and CEA-Related Cell Adhesion Molecule 1

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

**Received:** 17 February 2018
**Accepted:** 28 May 2018
**Published:** 18 June 2018

**Citation:**
Hosomi S, Grootjans J, Huang Y-H, Kaser A and Blumberg RS (2018) New Insights Into the Regulation of Natural-Killer Group 2 Member D (NKG2D) and NKG2D-Ligands: Endoplasmic Reticulum Stress and CEA-Related Cell Adhesion Molecule 1. Front. Immunol. 9:1324. doi: 10.3389/fimmu.2018.01324

Natural-killer group 2 member D (NKG2D) is a well-characterized activating receptor expressed by natural killer (NK) cells, NKT cells, activated $CD8^+$ T cells, subsets of $\gamma\delta^+$ T cells, and innate-like T cells. NKG2D recognizes multiple ligands (NKG2D-ligands) to mount an innate immune response against stressed, transformed, or infected cells. NKG2D-ligand surface expression is tightly restricted on healthy cells through transcriptional and post-transcriptional mechanisms, while transformed or infected cells express the ligands as a danger signal. Recent studies have revealed that unfolded protein response pathways during endoplasmic reticulum (ER) stress result in upregulation of ULBP-related protein *via* the protein kinase RNA-like ER kinase-activating factor 4-C/EBP homologous protein (PERK-ATF4-CHOP) pathway, which can be linked to the pathogenesis of autoimmune diseases. Transformed cells, however, possess mechanisms to escape NKG2D-mediated immune surveillance, such as upregulation of carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1), a negative regulator of NKG2D-ligands. In this review, we discuss mechanisms of NKG2D-ligand regulation, with a focus on newly discovered mechanisms that promote NKG2D-ligand expression on epithelial cells, including ER stress, and mechanisms that suppress NKG2D-ligand-mediated killing of cancer cells, namely by co-expression of CEACAM1.

**Keywords: natural-killer group 2 member D, natural-killer group 2 member D-ligand, murine UL16-binding protein like transcript 1, UL16 binding protein 1, endoplasmic reticulum stress, CEA-related cell adhesion molecule 1**

## INTRODUCTION

Natural killer (NK) cells were originally identified as lymphocytes with cytotoxic reactivity against several types of cancer cells (1, 2), and are considered to be part of the innate immune cell compartment/family/pool because of lack of rearranged antigen-specific receptors by somatic recombination. Both activating and inhibitory receptors can regulate NK cells activity and recognize target cells (3). Inhibitory receptors, including killer cell immunoglobulin (Ig)-like receptors (KIRs) and

leukocyte Ig-like receptors in humans, Ly49s in mouse, and CD94-Natural-killer group 2 member A receptors in human and mouse, recognize major histocompatibility complex (MHC) class I. If NK cells encounter cells that express MHC class I, an immune response against these cells is prevented by the inhibitory signals through receptor–ligand interactions. In contrast, cells in which MHC class I is downregulated, for example, in virus-infected cells or cancer cells, NK cells are activated by the lack of inhibitory signals, which makes the "diseased" cells prone to NK cell-mediated killing (the missing-self hypothesis) (4). The inhibitory function is mediated through immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain of these inhibitory receptors (5).

Besides inhibitory receptors, numerous activating NK receptors, such as CD94-Natural-killer group 2 member C, natural-killer group 2 member D (NKG2D), NKp44, NKp46, KIRs in humans, Ly49 receptors, in mice have been identified (5). The combination of the activation receptors can synergistically mediate natural cytotoxicity (6). NKG2D, a type II transmembrane-anchored C-type lectin-like activating receptor, is a well-characterized activation receptor expressed by NK cells, NKT cells, activated $CD8^+$ T cells, subsets of $\gamma\delta^+$ T cells, and innate-like T cells, which are $TCR^+$ $NK1.1^+$ $CD49a^{high}$ $CD103^+$ tissue-resident T lymphocytes with innate cytolytic activities, transcriptionally related to ILC1 (7–11). NKG2D can recognize multiple ligands (NKG2D-ligands), which are homologous to MHC class I molecules; MHC class I chain-related proteins A (MICA), MICB, UL16 binding protein 1 (ULBP1)–ULBP6 in human; retinoic acid early inducible 1 (RAE-1) (isoforms α–ε), H60 (isoforms a–c), and murine UL16-binding protein like transcript 1 (MULT1) in mouse (12). Interaction of these ligands with NKG2D results in NK cell cytotoxicity *via* signal transducing adapter molecule DAP10 in human and both DAP10 and DAP12 in mouse (10). Surface expression of NKG2D-ligands on healthy cells is tightly restricted by regulation at transcriptional and posttranscriptional levels, to ensure that healthy cells are not recognized by the innate immune system. The mechanisms involved in NKG2D-ligand expression regulation have been studied extensively [reviewed in Ref. (12, 13)].

Emerging evidence shows that intracellular stress can also induce the NKG2D-ligand expression. In this review, we summarize the mechanisms of NKG2D-ligand regulation. We focus specifically on recent advances in our understanding of how endoplasmic reticulum (ER) stress leads to NKG2D-ligand surface expression, and eventually group 1 innate lymphoid cells (ILCs)-mediated inflammation, particularly inflammatory bowel diseases, which are associated with several ER stress-related genes. In addition, we discuss the mechanisms by which NKG2D-L are suppressed on the other hand and specifically through carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1).

# REGULATION OF NKG2D-LIGANDS

## Regulation of NKG2D-Ligands by Cellular Stress and ER Stress

As NKG2D-ligand expression signals the immune system to recognize infected or transformed cells, a variety of stress pathways have been demonstrated to regulate NKG2D-ligand expression *via* different mechanisms (**Table 1**). Oxidative stress leads to accumulation of $H_2O_2$, which induces NKG2D-ligand including MICA/B and ULBP1–4 *via* activation of the mitogen-activated protein kinases pathway (14, 15). In contrast, heat shock can transcriptionally regulate MICA/B, as the promoter regions of the MIC genes have heat shock elements that can be recognized by heat shock factor 1 (HSF1) (15–17). Knockdown of HSF1 has been shown to suppress MICB, but not MICA, membrane expression leading to a reduction in NK cell-mediated cytotoxicity (18). In mice, heat shock induces MULT1 protein expression in fibroblasts and transformed cells by altering protein stability (19). One of the mechanisms associated with regulation of MULT1 surface expression by heat shock could be the membrane-associated RING-CH (MARCH) family of E3 ubiquitin ligase. While MULT1 is post-transcriptionally regulated by ubiquitin-dependent degradation by the MARCH family in unstressed cells, MULT1 ubiquitination and degradation are reduced in response to heat shock stress (19, 20).

Until recently, little was known about how cellular stress that leads to disturbances in proteostasis, and eventually ER stress, interact with regulation of NKG2D-ligand expression. ER stress is caused by the accumulation of unfolded and misfolded proteins in the ER arising from either primary (genetic) or secondary (environmental) factors (21). Highly secretory cells are highly susceptible to ER stress. These include Ig-producing plasma cells, insulin-secreting β-cells in the pancreas and intestinal epithelial cells, in particular Paneth cells and goblet cells (22). ER stress leads to the accumulation of unfolded or misfolded proteins within the ER lumen, which triggers three ER stress sensors to induce the so-called unfolded protein response (UPR). These include inositol-requiring transmembrane kinase-endonuclease 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activated transcription factor 6 (22). The main goal of UPR activation

**TABLE 1** | Natural-killer group 2 member D (NKG2D)-ligands regulation by cellular stress.

| Type of cellular stress | Regulation mechanisms | Mouse NKG2D-ligands | Human-NKG2D ligands | Reference |
|---|---|---|---|---|
| Oxidative stress | MAPK pathway | | MICA/B, ULBP1-4 | (14, 15) |
| Heat shock | Transcriptional (HSF1) | | MICA/B | (15–20) |
| | Ubiquitin-dependent degradation (E3 ubiquitin ligase) | MULT1 | | |
| Endoplasmic reticulum stress | Transcriptional (ATF4, CHOP) | MULT1 | ULBPs | (25, 26) |

*ATF4, activating factor 4; CHOP, CCAAT/enhancer-binding protein (C/EBP) homologous protein; HSF1, heat shock factor 1; MAPK, mitogen-activated protein kinases; MICA/B, MHC class I chain-related proteins A/B; MULT1, murine UL16-binding protein like transcript 1; ULBP1, UL16 binding protein 1.*

is to restore proteostasis and enhance the secretory capacity of the ER. In the IRE1 arm of the UPR, phosphorylated IRE1 possesses endoribonuclease activity that excises a 26-nucleotide sequence of *X-box binding protein 1* (*Xbp1*) mRNA resulting in a frame shift and generation of a transcriptionally active isoform that functions as a transactivator of UPR target genes.

In the PERK arm, translation is suppressed by phosphorylation of elongation initiation factor 2α (eIF2α) to allow the cell to temporarily cope with excessive ER stress. Paradoxically, translation of some proteins, such as activating factor 4 (ATF4) is favored. Initially, ATF4 can induce several protective cellular pathways, among others autophagy (see below). However, if ER stress is excessive or prolonged, ATF4 induces apoptosis-related transcription factors, such as CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) (23).

Besides cell death, ER stress elicits inflammatory responses, and hence it was hypothesized that UPR-related proteins can induce surface expression of NKG2D-ligands. Surprisingly, Xbp1 knockdown in the mouse immortalized small intestinal epithelial cell line MODE-K (24) (*shXbp1* MODE-K by a short hairpin Xbp1 lentiviral vector), which causes ER stress (25), was shown to induce very strong induction of NKG2D-ligand MULT1 (both on mRNA level and surface protein expression), whereas inflammatory signals induced after stimulation with a variety of TLR ligands did not (25). Even more interesting was the fact that it appeared to be specific for MULT1, as both RAE-1 and H60 were not strongly induced. In contrast, expression of MHC class I, which is recognized by NK cell inhibitory receptors, was not affected by *Xbp1* knockdown *in vitro* and knockout *in vivo*. The effect was not specific for *Xbp1* deletion, as ER stress induction by administration of thapsigargin similarly induced strong upregulation of MULT1 surface expression. In addition, similar induction of ULBPs (the human ortholog of MULT1) was observed in a variety of human cell lines, including intestinal, gastric, esophageal, and hepatic cancer cell lines.

Intriguingly, ER stress protein ATF4 was found to be important in NKG2D-ligand upregulation using a completely different approach in a human cancer cell line HAP1 (26). This cancer cell line constitutively expresses ULBP1 and after treatment with a retroviral promoter trap vector, which randomly knocks out genes, the cell lines that had significant downregulation of ULBP1 surface expression were screened for gene enrichment. This screen revealed that ATF4 is important for the induction of ULBP1, which was confirmed by demonstrating that knockdown of ATF4 strongly decreased ULBP1 transcription. In addition, ATF4 was shown to have direct ULBP1 promotor binding sites and directly transactivates the ULBP1 promoter (26). In contrast to this study in human cancer cell lines, we have identified CHOP as a transcription factor that binds the promoter of the mouse ortholog of ULBP1, MULT1, using chromatin immunoprecipitation and luciferase assays. CHOP is downstream of PERK-ATF4, but can also be induced by other ER stress-associated pathway elements (27). Interestingly, MODE-K cells with silenced CHOP using *shRNA*, or primary intestinal epithelial cells from *Chop*$^{-/-}$ mice examined *ex vivo* show downregulation of ER stress-dependent induction of MULT1 on the surface of intestinal epithelial cells (**Figure 1**).

Together, these two recent studies indicate that the PERK–ATF4–CHOP pathway of the UPR is a highly conserved ER-stress-specific mechanism of regulation of NKG2D-ligands. Interestingly, ER stress is a common pathophysiological phenomenon in the two disease processes that have been mostly linked to NKG2D-ligand expression, namely cancer, discussed below, and (viral) infection. Viral infection can strongly induce NKG2D-ligands. For example, human immunodeficiency virus type 1 (HIV-1) induces DNA stress/damage checkpoint arrest initiated by the DNA damage-sensing protein kinase (ataxia telangiectasia-mutated and Rad3-related: ATR) (28), resulting in upregulation of NKG2D-ligands (29, 30). Recognition of viral products, such as double-stranded (dsRNA), by retinoic acid-inducible gene I and melanoma differentiation-associated gene 5, both cytoplasmic pattern recognition receptors, also upregulate MICA and ULBP2 expression (31). Since viral replication requires the host ER for the production of their structural and non-structural proteins, viral infection has been strongly associated with activation of the UPR as well, and further research is required to investigate how the UPR in viral infection affects NKG2D-ligand expression.

## Consequences of ER Stress-Induced Regulation of NKG2D-Ligands in the Gastrointestinal Tract

In genome-wide association studies, MICs gene have been associated with susceptibility to a variety of autoimmune diseases, including systemic type 1 diabetes, rheumatoid arthritis, and lupus erythematosus (32–34). Consequently, NKG2D/NKG2D ligand interactions have been hypothesized to be involved in their pathogenesis (35–38).

In the intestine, NKG2D/NKG2D-ligand interactions have mainly been studied in celiac disease. Celiac disease is a gluten-driven innate and acquired immune cell-mediated enteropathy characterized by findings of intraepithelial lymphocytosis, crypt hyperplasia, and villous atrophy, and a positive response to a gluten free-diet (39). The fractions of NKG2D$^{+}$ NK cells and NKT cells among the intraepithelial mononuclear cells in active celiac disease are significantly increased as compared to inactive celiac disease or control subjects (40). Increased MICA expression on the intestinal epithelium in active celiac disease *via* a pathway involving IL-15 induction by gliadin triggers activation of intraepithelial T cells, allowing for the killing of intestinal epithelial cells (41). Details of the MICA/B expression pattern showed that the expression was observed not only on epithelial cells but also in the cytoplasm of intraepithelial T lymphocytes in patients with active celiac disease, suggesting extensive stress conditions are present in active celiac disease (42). Interestingly, some studies have suggested that ER stress could be involved in NKG2D-ligand expression regulation in celiac disease (42).

In Crohn's disease, several studies have also suggested involvement of the NKG2D/NKG2D-ligand pathways in the pathogenesis of this disorder (43–45). Specifically, MICA and MICB expression has been demonstrated to be increased on intestinal epithelial cells in patients with inflammatory bowel disease (46, 47). Moreover, a subset of CD4$^{+}$ T cells expressing NKG2D was increased in patients with Crohn's disease and functionally active

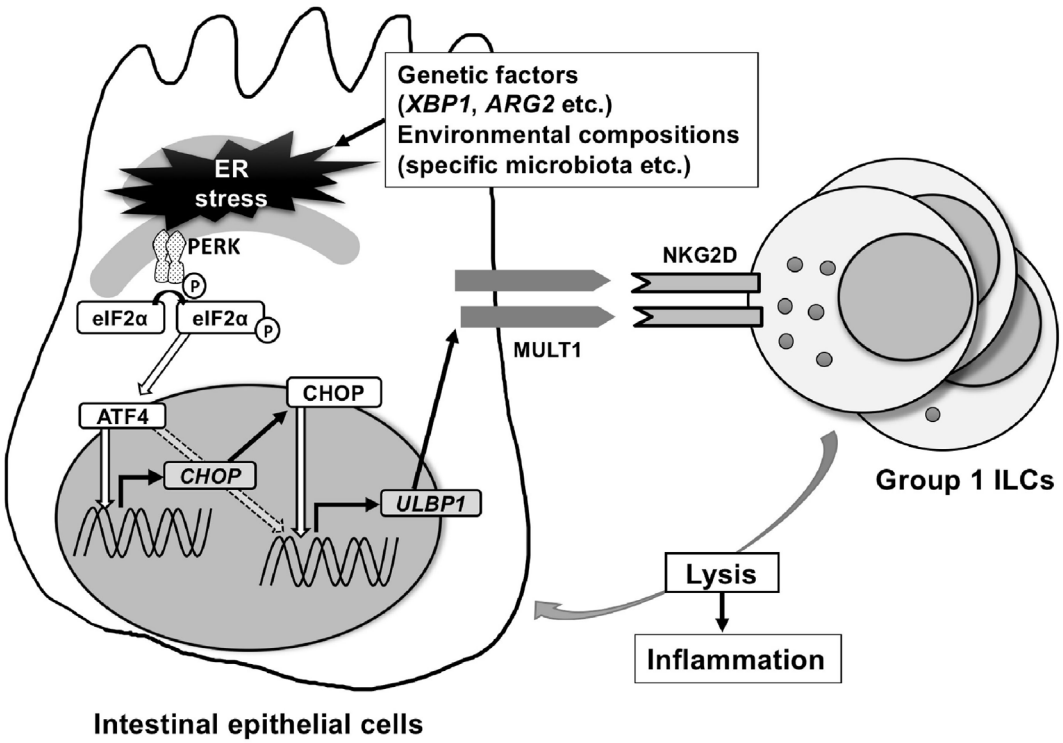


**FIGURE 1** | Endoplasmic reticulum (ER) stress-inducing murine UL16-binding protein like transcript 1 (MULT1) in mouse. MULT1 [encoded by UL16 binding protein 1 (*Ulbp1*)], but not other natural-killer group 2 member D (NKG2D)-ligands, mRNA, and surface expression are upregulated on ER-stressed mouse small intestinal epithelial cells. This occurs through activating factor 4 (ATF4) and/or C/EBP homologous protein (CHOP) presumably downstream of the protein kinase RNA-like ER kinase pathway. CHOP as a transcription factor binds directly to the promoter area of *Ulbp1*, which upon expression subsequently activates NKG2D-expressing intraepithelial group 1 innate lymphoid cells (ILCs) [natural killer (NK) cells and ILC1] that accumulate within the intestinal epithelium and promote small intestinal inflammation.

through MICA–NKG2D interactions, leading to interferon-γ production. In pediatric Crohn's disease and ulcerative colitis, ULBP1 and ULBP2 is upregulated on infiltrating immune cells in active inflammatory lesions (48). Interestingly, a recent clinical trial revealed efficacy of a blocking anti-NKG2D IgG4 antibody in patients with active Crohn's disease (49), suggesting that NKG2D/NKG2D-ligand interactions are of importance in the pathogenesis of Crohn's disease.

As ER stress has also been linked to the pathogenesis of Crohn's disease (50), and as we demonstrated that ER stress regulates NKG2D-ligands on intestinal epithelial cells, we subsequently investigated the functional consequences of epithelial ER stress-induced NKG2D-ligand expression and its role in the development of spontaneous enteritis in a mouse model of this condition. We demonstrated that increased MULT1 expression on intestinal epithelial cells of $Xbp1^{\Delta IEC}$ mice was accompanied by increased quantities of NKG2D-expressing intraepithelial group 1 ILCs (NK cells and ILC1) (25). The group 1 ILCs within the epithelium also exhibited evidence of increased activation as demonstrated by increased surface expression of NKG2D and CD25. Indeed, IELs from $Xbp1^{\Delta IEC}$ mice exhibited increased cytotoxicity in comparison to the activity observed with IELs from wild-type mice, suggesting an involvement in inflammation, which was supported by amelioration of inflammation upon NKG2D blockade. Furthermore, ER stress-induced intestinal inflammation due to *Xbp1* deletion in the intestinal epithelium was uniquely comprised of a significant component of innate immune activation as concomitant loss of the adaptive immune system in $Rag1^{-/-}Xbp1^{\Delta IEC}$ double mutant mice did not affect the severity or kinetics of the inflammation. However, depletion of NK cells significantly diminished inflammation that emerged from tamoxifen-driven, and thus temporally controlled induction of ER stress during adult life in $Xbp1^{T\text{-}\Delta IEC}$ mice. Similarly, spontaneous enteritis was reversed by NKG2D blockade in $Xbp1^{T\text{-}\Delta IEC}$ mice, in line with what has been shown for mouse colitis models (51, 52). Thus, our study demonstrates that ER stress in the small intestine leads to spontaneous enteritis that depends significantly on the presence of an innate immune system. Further, the development of ER-stress-mediated inflammation in this context involves the CHOP-dependent induction of NKG2D-ligand MULT1 and its recognition by NKG2D on group 1 ILC, which are increased in the intestinal epithelium. Future studies are required to investigate how ER stress is linked to NKG2D-ligand expression in the human gut, particularly in the setting of complex diseases including inflammatory bowel disease. Methods to resolve pathologic levels of ER stress could, therefore, be a potential target for therapies in this still uncurable disease.

## Regulation of NKG2D-Ligand Expression in Cancer

NKG2D-ligand expression has been extensively studied in the setting of cancer. The expression of NKG2D-ligands in cancer can be hypothesized to serve in activating the immune system for elimination of the excessively proliferating cancer cells. E2F transcription factors, for example, regulate cell proliferation but at the same time can induce specific NKG2D-ligands such as RAE-1, but not MULT1 and H60 in mouse fibroblasts by directly binding the promoter region of *Raet1* genes (53).

DNA damage responses to genotoxic stress coordinates activation of transcription, cell cycle control, apoptosis, and DNA repair processes mediated by a number of protein kinases, including ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) protein kinases (54). In both mouse and human non-tumor cells, genotoxic stress and inhibition of DNA replication *via* the DNA damage pathway through ATM or ATR protein kinases, leads to increased surface expression of NKG2D-ligands (55). In tumor cell lines, constitutive ligand expression on the cell surface is suppressed by pharmacological or genetic inhibition of ATR, ATM, or Chk1 (55).

p53, induced in response to DNA damage, can also regulate human ULBP1 and ULBP2 by binding to p53-responsive elements in the promoter area of *ULBP1* and *ULBP2* gene (56–58). However, several microRNAs that are induced by p53 (miR-34a and miR-34c) have been shown to suppress ULBP2 expression by directly binding to the 3′-UTR of *ULBP1* mRNA (59).

Other mechanisms of NKG2D-ligand induction in response to DNA damage response include the stimulator of interferon genes (STING)-dependent DNA sensor pathway (60). The accumulation of cytosolic DNA by DNA damage response activates STING-dependent DNA sensors, leading to the activation of TANK binding kinase 1 and interferon regulatory factor 3, which in turn are associated with RAE1 expression (60).

## Inhibition of NKG2D-Ligand Expression on Tumor Cells by CEACAM1

As NKG2D-ligand expression on tumor cells is critical for the recognition and clearance of tumors, tumor cells have evolved by developing several mechanisms to escape from immune surveillance. Several studies have demonstrated that soluble forms of NKG2D-ligands that were derived from cancer cells by either proteolytic shedding (61–63), alternative splicing (64), or exosome secretion (65) can impair NKG2D-mediated cytotoxicity by negatively regulating NKG2D expression or recognition (66). In addition, metastasis-associated microRNA miR-10b (also known as metastamir), which promotes tumor invasion and metastasis by targeting multiple genes, downregulates MICB expression by binding directly to the 3′-UTR of *MICB* (67).

CEACAM1, a member of the CEA family of Ig like transmembrane glycoproteins (68), is involved in the negative regulation of NKG2D-ligands in cancer (69–71). CEACAM1 is expressed in mouse and humans and is characterized by numerous transmembrane isoforms that derived from alternative splicing mechanisms [reviewed in Ref. (68, 72)]. This mechanism generates CEACAM1 variants that share a membrane distal IgV-like domain (N-domain), which functions in homophilic or heterophilic interactions, that is coupled to variable numbers of IgC2 domains and linked to either a long (L) or short cytoplasmic domain. NK cells and T cells predominantly express CEACAM1-L isoforms that contain two immunoreceptor tyrosine-based inhibitory motifs in their cytoplasmic tail which serves to recruit Src homology phosphatase 1 (SHP1) and SHP2 after phosphorylation by Src-related kinases (68, 73). Ligation of CEACAM1-L isoforms on NK cells by CEACAM1 on tumor cells suppresses NK cytolytic function as CEACAM1 on the NK cells negatively regulates NKG2D signaling (74). Specifically, recruitment of SHP1 by CEACAM1 leads to dephosphorylation of the guanine nucleotide exchange factor Vav1, one of the most proximal elements associated with NKG2D-mediated cytolytic signaling (75). At the

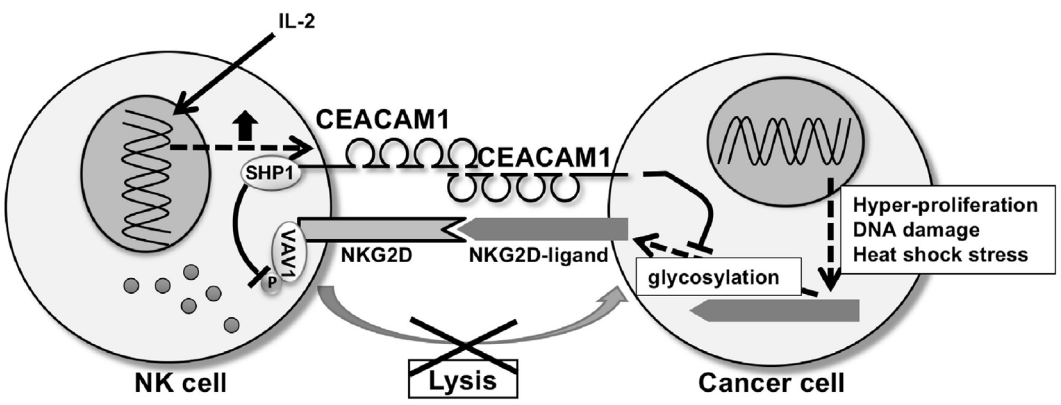


**FIGURE 2** | CEA-related cell adhesion molecule 1 (CEACAM1) regulating natural-killer group 2 member D (NKG2D)-ligands expression and NKG2D function. CEACAM1 regulates glycosylation of NKG2D-ligands, resulting in downregulation of the NKG2D-ligand expression on the cell surface of the tumor cell. CEACAM1 induced by interleukin-2 inhibits NKG2D-mediated cytotoxic function through recruitment of Src homology phosphatase 1 (SHP1) to phosphorylated CEACAM1 which leads to dephosphorylation of Vav1 on the natural killer (NK) cell. CEACAM1 on the NK cell and tumor cell interact homophilically through the N-domain of CEACAM1.

same time, CEACAM1 expression on the tumor cells has been shown to regulate NKG2D-ligand expression. Specifically, silencing of CEACAM1 in mouse and human tumor cells upregulates expression of NKG2D-ligands on the cell surface of the tumor cell and makes them more highly susceptible to NK cell-mediated cytotoxicity (**Figure 2**) (76). Silencing CEACAM1 did not alter the transcriptional levels of NKG2D-ligands. Indeed, cell surface expression of RAE-1 was increased in CEACAM-1 silenced cells, whereas intracellular RAE-1 protein was decreased in comparison to non-silenced cells. Furthermore, RAE-1 on the cell surface of CEACAM1 silenced cells possessed an increased quantity of carbohydrate side-chain modifications in comparison to CEACAM1 non-silenced cells, in which RAE-1 accumulates intracellularly as an incompletely glycosylated protein (76). Thus, CEACAM1 can regulate glycosylation of NKG2D-ligands resulting in the NKG2D-ligands retention in an intracellular compartment.

## CONCLUSION

NKG2D/NKG2D-ligands interactions play a critical role in the immune surveillance of sick cells, such as those that are infected or have undergone neoplastic transformation. In such contexts, the expression and function of NKG2D and NKG2D-ligands are critical for removal of the altered cells and resolution of the condition. However, in certain circumstances cancer and infected cells have developed the ability to avoid immune surveillance that is mediated by NKG2D/NKG2D-ligands which allows progression of the infection or tumor. Our recent studies indicate that CEACAM1 plays an important role in NKG2D/NKG2D-ligand function and expression, respectively, suggesting that manipulation of this pathway may be beneficial in such disorders. On the other hand, NKG2D/NKG2D-ligand expression and function may inappropriately be associated with distressed organs or cells in autoimmune disease, suggesting that their blockade would be an important means of resolving inflammation consistent with their current evaluation in clinical trials in inflammatory bowel disease (49). Our recent studies thus suggest that enhancement of CEACAM1 function might be beneficial in such disorders by enhancing inhibition of NK and ILC1-mediated activation due to NKG2D/NKG2D ligand interactions. This is consistent with previous studies that application of CEACAM1 ligands to mouse models of colitis can suppress inflammation (77). This is particularly interesting as a key feature of inflammatory bowel disease which is the common induction of ER stress within the intestinal epithelium, which can serve as nidus for development of inflammation (22, 50). As such, the recent evidence that ER stress is linked to the upregulation of specific NKG2D-ligand and activation of intraepithelial NK cells and ILC1, together imply that blockade of NKG2D and NKG2D ligand interactions may have wide benefit in inflammatory bowel disease and potentially other autoimmune conditions.

## AUTHOR CONTRIBUTIONS

All authors wrote and edited the manuscript and gave final approval of the manuscript.

## ACKNOWLEDGMENTS

The authors thank Aman Riar and Tara Traylor for administrative assistance in this manuscript.

## FUNDING

This work was supported by JSPS KAKENHI Grant Number 2689323 and 16K19162, Japan Foundation for Applied Enzymology (SH); Rubicon grant 825.13.012, Netherlands Organization for Scientific Research (JG); Wellcome Trust Senior Investigator Award 106260/Z/14/Z, the European Research Council HORIZON2020/ERC grant no. 648889 (AK); NIH grants DK044319, DK051362, DK053056, DK088199, the Harvard Digestive Diseases Center (HDDC) DK034854 (RB).

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**Conflict of Interest Statement:** RB is a consultant to Syntalogic Pharmaceuticals which is developing therapies that target CEACAM1. The other authors have no conflict of interest.

frontiers in Immunology

HYPOTHESIS AND THEORY
published: 13 August 2018
doi: 10.3389/fimmu.2018.01808

# The Paradoxical Role of NKG2D in Cancer Immunity

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The activating receptor NKG2D and its ligands are recognized as a potent immune axis that controls tumor growth and microbial infections. With regards to cancer surveillance, various studies have demonstrated the antitumor function mediated by NKG2D on natural killer cells and on conventional and unconventional T cells. The use of NKG2D-deficient mice established the importance of NKG2D in delaying tumor development in transgenic mouse models of cancer. However, we recently demonstrated an unexpected, flip side to this coin, the ability for NKG2D to contribute to tumor growth in a model of inflammation-driven liver cancer. With a focus on the liver, here, we review current knowledge of NKG2D-mediated tumor surveillance and discuss evidence supporting a dual role for NKG2D in cancer immunity. We postulate that in certain advanced cancers, expression of ligands for NKG2D can drive cancer progression rather than rejection. We propose that the nature of the microenvironment within and surrounding tumors impacts the outcome of NKG2D activation. In a form of autoimmune attack, NKG2D promotes tissue damage, mostly in the inflamed tissue adjacent to the tumor, facilitating tumor progression while being ineffective at rejecting transformed cells in the tumor bed.

**Keywords: NKG2D, cancer, hepatocellular carcinoma, inflammation, CD8$^+$ T cells, natural killer cells**

OPEN ACCESS

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

***Received:*** *28 April 2018*
***Accepted:*** *23 July 2018*
***Published:*** *13 August 2018*

***Citation:***
*Sheppard S, Ferry A, Guedes J and Guerra N (2018) The Paradoxical Role of NKG2D in Cancer Immunity. Front. Immunol. 9:1808. doi: 10.3389/fimmu.2018.01808*

Natural killer (NK) cells were discovered more than four decades ago, initially described as spontaneous cytolytic effector cells, operating rapidly without the T and B lymphocyte requirement for antigen presentation (1). The discoveries of multiple stimulatory and inhibitory NK cell receptors have helped refine our understanding of NK cell target recognition. These included the NKG2 family of transcripts identified by Houchins and colleagues, which encode type II integral membrane proteins (2–4). Unlike the majority of NKG2 receptors that form heterodimers with CD94, NKG2D forms a homodimer transmembrane C-type lectin-like receptor. NKG2D is highly conserved and although first identified on NK cells is expressed on both innate and adaptive lymphocytes, including NK cells, αβ T cells, γδ T cells, iNKT cells, and innate lymphoid cells (5–7). Identification of NKG2D ligands and their expression in response to cellular stress by the group of Thomas Spies revealed the critical link between NKG2D and stress-induced tissue damage (8–10). This potent activating receptor has been and remains the subject of intense research in cancer, infection, and autoimmunity.

NKG2D ligands are cell surface proteins structurally related to major histocompatibility complex (MHC). In humans, these ligands are the MHC class I-related chain A and B (MICA, MICB) proteins and the six unique long 16 (UL-16)-binding proteins (ULBP1-6) (11).

**Abbreviations:** HCC, hepatocellular carcinoma; MICA/B, major histocompatibility class I-related chain; RAE-1, retinoic acid early inducible gene-1; MULT1, murine UL16-binding protein-like transcript 1; MMP, matrix metalloproteinase; TRAMP, transgenic adenocarcinoma of mouse prostate; TME, tumor microenvironment; ULBP, UL16-binding protein; DEN, diethylnitrosamine; TIL, tumor-infiltrating lymphocyte; TRAIL, TNF-related apoptosis-inducing ligand; MCA, methylcholanthrene.

Their counterparts in mice are the retinoic acid early inducible gene-1 (RAE-1α-ε), minor histocompatibility H60a-c, and murine UL16-binding protein-like transcript 1 (MULT1) proteins. Our understanding of ligand regulation is far from complete with continuing discovery of regulatory mechanisms and pathways. Induction and upregulation of NKG2D ligands result from various stress signals including infection, DNA damage, heat shock, and hyperproliferation (12, 13). Regulation of ligand expression appears to occur predominantly at the transcriptional level (14–17); however, posttranscriptional (18–20) and epigenetic regulation have also been reported (21, 22). NKG2D ligands can be released from the cell surface by proteases of the matrix and desintegrin metalloproteinases families (MMP and ADAM) (23–25), alternatively, ligands can be secreted or released as exosome-bound ligands. Soluble or exosome bound ligands are often detected in the serum of patients with advanced cancer (26) and autoimmune diseases (27–29).

## NKG2D IN TUMOR IMMUNITY

A large body of experimental evidence established that NKG2D plays an important role in the surveillance of tumors by the immune system. NKG2D-dependent elimination of tumor cells that express at least one cognate ligand has been well documented *in vitro* (5, 8, 30, 31) and using *in vivo* models of transplanted tumors (16, 32–34). Direct evidence supporting a role for NKG2D in tumor surveillance came from studying tumor development in gene-targeted mice that lack NKG2D and carry transgenes that trigger tumorigenesis (35), mice with transgenic expression of human NKG2D ligand (36), and in a model of antibody-mediated NKG2D neutralization (37). Indirect evidence comes from model studies of failed tumor surveillance associated with the downregulation of NKG2D on NK cells. Constitutive expression of RAE-1ε led to systemic NKG2D downregulation that correlated with increased tumor burden in skin cancer (38) and an increased incidence of B cell lymphomas (39).

Expression of NKG2D ligands has been observed in human cancers arising from a variety of tissues. Variable expression of MICA, MICB, and ULBP1-3 ligands was observed in hematopoietic malignancies, including acute and chronic leukemias of lymphoid and myeloid origins (40), in addition to solid tumors such as neuroblastoma (41), colorectal (42), ovarian (43), cervical (44), breast (45), pancreatic (46), melanoma (47–49), and gastric cancers (50). One common feature is the heterogeneity in ligand expression between cancer types and individuals (42, 45, 47, 51), which hinders the prognostic value of NKG2D ligands in clinical assessment. Indeed, several reports have highlighted the paradoxical relationship between ligand expression and patient outcome. Studies of colorectal (42), cervical (44), and nasopharyngeal carcinoma (52) correlated high levels of surface ligand expression with improved disease-free survival, supporting the role of NKG2D in antitumor immunity. Conversely, high levels of cell surface ligand associated with poor prognosis in breast cancer (53), lung (54), and ovarian cancers (43, 55) suggest a failure in NKG2D-mediated tumor surveillance and/or that high levels of surface ligand drives disease progression. Specifically, Li and colleagues showed that high expression of ULBP2 detected by immunohistochemistry in 82 ovarian cancer patients correlated with less intraepithelial infiltration of T cells and poor prognosis (55). The authors found no correlation between the presence of soluble ligands and increased tumor stage undermining a role for soluble ligands in disease progression (55). McGilvray and colleagues corroborated the poor prognosis in ovarian cancer using a larger cohort of patients where expression of high levels of ULBP-1-5 correlated with decreased survival, whereas MICA expression did not correlate with disease progression (43). Madjd and colleagues studied a large cohort of 530 invasive breast cancer patients and showed that high intensity of MICA expression correlated with poor prognosis. In 50 cases studied for CD56 expression, the authors found absent or low NK cell infiltrate, yet, that did not correlate with MICA expression or prognosis (53). In non-small cell lung carcinoma, Chen and colleagues observed that 62% of 222 patients expressed high levels of MICA, which correlated with a decrease in median survival (54). Discrepancies might be accounted for by the variation in the nature of the ligand(s), i.e., their binding affinity to NKG2D (56, 57). de Kruijf et al. showed that ULBP-2 and major histocompatibility class I-related chain (MICA/B) expression, but not ULBP-1,3,4 or 5, correlated with longer relapse-free survival in breast cancer patients (45). The functional outcome of ligand variety on NK cell activation was recently evidenced using super-resolution microscopy (58). MICA and ULBP2 differentially affect NKG2D nanoscale reorganization at the NK cell membrane and subsequent NK cell activation. Binding to ULBP2, but not MICA, caused NKG2D nanoclusters to coalesce with the IL-2/IL-15 receptor beta subunit, leading to a greater production of IFN-γ (58). The function of NKG2D itself can also differ with different NKG2D (*KLRK1*) gene polymorphisms and associate with susceptibility to cancer. The low cytotoxic activity related to the NKG2D haplotype *LNK1/LNK1,* found in one-third of the general population, was associated with increased cancer development (59).

Many factors that constitute the tumor microenvironment (TME) impact the efficacy of the NKG2D-mediated antitumor response and with-it clinical outcome. These will be discussed throughout this paper, they include: (i) the presence of proteases that shed cell surface ligands, (ii) the quantity and quality of immune cells infiltrating tumors and (iii) the presence of cytokines that regulate the expression of NKG2D receptor and ligands. Clonal evolution of tumors is an additional variable likely to affect each of these parameters over the course of disease progression.

## NKG2D/NKG2D-LIGAND REGULATION IN COMPLEX AND DIVERSE TUMOR ENVIRONMENTS

NKG2D ligand expression can be downregulated from the cell surface due to hypoxia and intracellular retention by the CEACAM1 tumor-associated antigen, reducing sensitivity to NK cell lysis (60–62). Also, metalloproteases present in the TME have a negative impact on tumor surveillance by releasing soluble ligands known to downregulate NKG2D on NK and $CD8^+$ T cells

(41, 63–67). As a consequence, the presence of soluble ligands in the sera of cancer patients is often associated with poor prognosis (47, 68, 69), including in patients treated with checkpoint blockade therapy as recently shown in metastatic melanoma (70). Using an antibody to block ligand shedding, preclinical studies demonstrated the antitumor potential of NKG2D in rejecting metastases in mouse and humanized mouse models of MICA-transduced transplanted B16-F10, CT26, and A2058 mouse and human tumors (71). Nonetheless, the presence of soluble ligands is not always associated with NKG2D downregulation and impaired antiviral (72) and antitumor activities. Indeed, NKG2D expression on NK cells from stage IV melanoma patients did not significantly differ from age-matched healthy controls despite the presence of high sMICA (47). Soluble MICB present in the sera of patients with gastrointestinal tumors failed to alter NKG2D expression on NK cells *in vitro* (73). In ovarian cancer, high levels of sMICA and sULBP2 present in ascites samples did not correlate with a decreased expression of NKG2D on T cells or NK cells (74). Tumor-cell derived soluble ULBP2 did not induce NKG2D downregulation on NK cells *in vitro* as opposed to membrane-bound ULBP2 (75). Also, animal studies revealed that the secreted form of MULT1, the mouse equivalent of ULBP-1 with a unique high affinity, does not downregulate NKG2D but rather favors tumor rejection by stabilizing NKG2D expression and preventing NK cell desensitization induced by RAE-1 on myeloid cells (76).

An additional layer of complexity rests on the fact that non-tumor cells, including immune cells, can upregulate NKG2D ligands and negatively regulate NKG2D expression (77). mRNA transcripts for human and mouse NKG2D ligands are widely detected in healthy tissues (78), and protein expression is detected in low amounts in the intestinal tract (9), liver (79, 80), bronchial epithelial cells (81), endothelial cells (82), and on myeloid cells (83). In fact, contact-dependent and independent interactions between myeloid cells and NK cells have been shown to either enhance (84, 85) or impair (76, 86, 87) NK cell-mediated antitumor activity. Interestingly, NK cells express ADAM17, which can cleave NKG2D ligands from the cell membrane. Furthermore, in response to IL-15 + IL-12 + IL18, human NK cells can produce soluble NKG2D ligand in an NKG2D- and ADAM17-dependent manner (88). Thus, NK cells may play a role in shaping the NKG2D ligand environment.

Several cytokines present in the TME and adjacent tissue positively or negatively influence the expression of NKG2D receptor and ligands. IFN-γ was shown to decrease MICA and, in some cases, ULBP2 expression on human melanoma cell lines (89); Type 1 interferons can reduce H60 expression in the methylcholanthrene (MCA)-induced mouse model of fibrosarcoma (90) or increase MICA/B surface expression on human pancreatic cancer cell lines (91). TGF-β downregulates NKG2D on NK cells and $CD8^+$ T cells in cancer patients (79, 92, 93) and NKG2D ligands on cancer cells (94, 95). This was shown in glioma patients with heterogeneous expression of NKG2D ligands where TGF-β selectively downregulated MICA and ULBP2 transcription, but not MICB, ULBP1, and ULBP3 (95). IL-15 is well recognized for its ability to enhance NKG2D expression and activation (58, 96) whereas IL-21 that shares the common receptor γ chain operates in a context-dependent manner. Culturing human primary NK and $CD8^+$T cells with IL-21 was shown to downregulate surface expression of NKG2D and DAP10 transcription (97). However, a previous study showed an opposing role for IL-21 (98) in its capacity to enhance NKG2D-dependent tumor rejection in mice. This may be explained by additional signaling *via* mouse DAP12, which can pair with mouse NKG2D but not human NKG2D, highlighting the species-specific nature of NKG2D signaling (99). Thus, in model studies, it is possible that depending on the cytokine milieu, preferential activation of DAP10 versus DAP12 guides the outcome of NK cell activation toward cytokine release and/or cytotoxic activity (100). This difference in signaling between species is restricted to innate lymphocytes since NKG2D only signals *via* DAP10 in mouse $CD8^+$ T cells (34). Collectively, these studies highlight the heterogeneous expression of cell-bound and soluble forms of NKG2D ligands across cancer types and individuals, which ultimately challenges its benefit as a prognostic biomarker.

## HEPATOCELLULAR CARCINOMA (HCC)-NKG2D LIGAND EXPRESSION IN A TYPICAL INFLAMMATION-DRIVEN CANCER

The majority of HCCs develop from a background of chronic inflammation, which is now recognized as a hallmark of cancer (101). Persistent liver damage leading to hepatocyte death can trigger the production of IL-1α inducing the expression of TNFα, which in turn upregulates IL-6 (102, 103). In addition to its ability to modulate the immune response, IL-6 can also induce the production of hepatocyte growth factor, thereby stimulating hepatocyte proliferation to compensate for hepatocyte death (104, 105). Damaged hepatocytes will harbor genetic mutations that may either drive tumorigenesis (106, 107) or result in further cell death, creating a positive feedback loop (108, 109).

Chronic inflammation that precedes HCC is most commonly caused by chronic hepatitis B (HBV) or hepatitis C (HCV) infection, particularly in the developing world, although increasing numbers of cases develop from non-viral hepatitis induced by hepatic stress resulting from the excessive consumption of alcohol and or a high calorie diet (110, 111). The importance of the NKG2D axis in clearing liver infection has been well documented. In HCV or HBV infected patients, high levels of NKG2D expression on intrahepatic T cells (112), NK (79, 113–115), and iNKT cells (116) have been reported. During the course of HCV infection, MICA/B expression is elevated compared to healthy individuals (79) and HCV proteins were shown to enhance cell surface expression of ULBP-1 on human immortalized hepatocytes (117). Expression of MICA and MICB on transformed hepatocytes was also observed on hepatoma cell lines, HCCs (80, 118), and carcinoma cell lines (119). However, as seen in other tissues/diseases, expression of NKG2D ligands in hepatitis-associated conditions is heterogeneous, and there is still conflicting evidence as to the beneficial versus deleterious role of NKG2D in hepatitis. Various *MICA* alleles were shown to be over- or under-represented in infected individuals that develop HCC.

Specifically, MICA 251 Gln, MICA 175 Gly, MICA 129 Met, or a promoter region variant MICA rs259654A are significantly more prevalent in HBV-infected individuals that progress to HCC than in HBV-infected individuals who developed liver cirrhosis, but not HCC (120). Fang and colleagues demonstrated in a cohort of 96 HCC patients that low frequency of MICA/B surface expression correlated with high tumor grade and reduced overall survival (121). However, in a study including 47 HCC patients, Kamimura and colleagues demonstrated ULBP1 to be expressed in well-differentiated and moderately differentiated HCC, but absent from poorly differentiated HCC, which significantly correlated with early recurrence but not with overall survival (119). In this study, MICA was mainly expressed on endothelial cells and ULBP2-4 were not expressed (119). Soluble MICA/B have been reported to be elevated in patients with viral hepatitis compared to healthy individuals (79, 122) and correlated with markers of liver damage such as serum ALT and AST (120). Conversely, in HCV-infected patients, the SNP rs2596538 allele A has been linked to lower serum levels of sMICA and to the progression from hepatitis to HCC (123). Also, there is evidence that the NKG2D axis plays a role in non-viral autoimmune hepatitis. Patients diagnosed with non-alcoholic fatty liver (NAFL) or non-alcoholic steatohepatitis (NASH) showed significantly increased expression of MICA and MICB on hepatocytes. The increase in MICA/B mRNA levels correlated with decreased liver function, increased fibrosis, and hepatocyte apoptosis (124).

Together, these studies attest to the relevance of the NKG2D pathway in regulating immune responses in the liver and illustrate how sustained expression of NKG2D ligands can exacerbate liver tissue damage. In our view, this supports the idea that chronic NKG2D activation during viral or autoimmune inflammation could be a common feature of cancer driven by inflammation. Indeed, there is a growing body of evidence implicating the NKG2D/NKG2D ligand axis as a driver of inflammatory disorders *via* direct targeting of healthy tissues expressing NKG2D ligands and/or *via* secretion of cytokines that exacerbates the initial inflammation (125–127). We hypothesize that NKG2D could act as a dual player in cancer immunity in a context and time-dependent manner. We postulate that previously beneficial inflammatory responses against early neoplastic lesions or infectious agents contribute to tissue injury and tumor progression over time (128).

To test this hypothesis, we chose to focus on a model of liver cancer, diethylnitrosamine (DEN)-induced HCCs, due to the established causative link between chronic inflammation and tumorigenesis. A key feature of the DEN-induced HCC mouse model is the slow, physiologic development of autochthonous tumors over a 9- to 15-month period. This mimics important characteristics of humans advanced HCC, even though it does not recapitulate the liver fibrosis often associated with liver cancer. First, the DEN-induced HCC model mimics the heterogeneity of human HCC (129), demonstrating a high variation in the incidence rate of mutations in the B-raf and H-ras genes between mouse strains (130). Also, the upregulation of glypican-3 on transformed hepatocytes and other markers (glutamine synthetase and heat shock protein 70) in this model supports its relevance to recapitulate human HCC (131). Second, HCC comparative genomic studies have shown that DEN-induced HCC displays gene expression profiles with characteristics similar to those of human tumor biopsies taken from patients with a poor prognosis (132), including inflammatory signatures such as TNFα and IL-6 and activation of nuclear factor-κB (105, 133). Third, the DEN model also recreates the HCC gender bias observed in humans, where male:female incidence averages between 2:1 and 4:1 in most populations (105, 110). Finally, the DEN-induced HCC model recapitulates similar features described in human hepatitis and HCC including: (i) elevated levels of NKG2D and NKG2D ligands, (ii) expression of NKG2D ligands on healthy tissue, (iii) evidence for a high $CD8^+$ T cell infiltrate that correlates with tissue damage and HCC, and (iv) potential dysfunction in NK cell subsets. Although human and mouse NKG2D ligands differ (99), they have both evolved to be capable of binding strongly to NKG2D (134). Molecular modeling showed that the ligand-binding site of NKG2D is highly conserved between both species, with mouse NKG2D being capable of binding all the human ligands (135). Nonetheless, MULT-1 displays a high affinity for NKG2D that is unparalleled by human ligands.

## THE PARADIGM SHIFT: NKG2D CONTRIBUTES TO TUMOR PROGRESSION IN HCC

We treated NKG2D-deficient (*Klrk1*$^{-/-}$) and sufficient (*Klrk1*$^{+/+}$) mice with DEN to induce liver damage and study the progression of HCC, the expression of NKG2D ligands, and the immune composition of the liver tissue. Compared to *Klrk1*$^{-/-}$ mice, wild-type mice displayed reduced survival and increased tumor burden assessed according to three criteria: liver/body weight ratio, maximal tumor size, and tumor load (136). Histopathology analyses showed similar incidence of adenomas, benign nodules, and HCC in both genotypes, with malignant HCC developing in more than 70% of DEN treated mice by the time they reached the end point. These findings indicate that NKG2D did not impact tumor incidence in this model but significantly accelerated HCC progression once established (136). RAE-1 was highly expressed on tumors developing in both wild-type and *Klrk1*$^{-/-}$ mice indicating that tumor progression was not the consequence of escape *via* ligand editing.

In agreement with our findings, various model studies support the idea that NKG2D-expressing cells have the potential to drive, rather than resolve hepatitis. In the apolipoprotein E-deficient mouse model of lipid metabolic disorder, the presence of NKG2D led to high production of inflammatory cytokines (such as IL-6, IL-12, and IFNγ) and the accumulation of NK cells, iNKT cells, and macrophages, resulting in a higher level of liver damage when compared to *Klrk1*$^{-/-}$ mice and to wild-type mice treated with NKG2D blocking antibodies (137). Vilarinho et al. developed a mouse model of acute hepatitis B generated through hepatic expression of small, middle, and large envelope proteins of hepatitis B (138). They demonstrated that the development of hepatitis resulted in an increase in mRNA and cell surface expression of RAE-1 on hepatocytes. Furthermore, hepatitis only occurred in the presence of functional

B, T, and NKT cells and treatment with NKG2D neutralizing antibody dramatically reduced IFN-γ and IL-4 expression and liver damage. More recently, Huang et al. established a model of fulminant hepatitis induced by the double-stranded RNA mimic TLR3 agonist polyinosinic:polycytidylic acid in conjunction with the hepatotoxin D-galactosamine. In this model, mice treated with a plasmid coding for shRNA that inhibits the expression of all known mouse NKG2D ligands showed decreased liver damage and lower IFN-γ expression by NK cells, demonstrating that liver damage is reduced in the absence of ligands (139). In agreement with this, Chen and colleagues showed that RAE-1 and MULT1 expression on hepatocytes contribute to autoimmune liver injury similar to that observed in HBV-chronic infection (140). Using a model of Con A-induced hepatitis in the hepatitis B-Tg mouse, the authors showed an accumulation and activation of NK cells that could be prevented by blocking NKG2D (140).

The obvious commonality between these studies is the remarkably high level of expression of NKG2D ligands on hepatocytes, which suggests that the severity of hepatitis and consequent progression to HCC is dictated by the strength of the NKG2D response. A strong NKG2D-mediated immune response may be desirable to clear virally infected and transformed cells; however, it could be deleterious against uninfected hepatocytes causing more damage that sustain inflammation. Alternatively, downregulation of NKG2D and/or expression of NKG2D ligands with low binding affinity or at a low level may lead to a weak response unable to clear aberrant cells and consequently favors persistent infection associated with a smoldering inflammation that drives disease progression (92).

## Which Cell Types Are Involved in Tumor Rejection versus Promotion?

One key question relates to the cell type(s) involved in NKG2D-mediated promotion of tumor growth. The liver contains a large population of lymphocytes, including $CD8^+$ T, $CD4^+$ T, NK, and iNKT cells, all potentially activated *via* NKG2D in the DEN model. In accordance with previous work, we observed a remarkable enrichment in memory $CD8^+$ T cells in the liver of wild type DEN-treated mice compared to age-matched non-treated control mice (136, 141). $CD8^+$ T cells, of which at least a quarter expressed NKG2D, represent the main source of IFNγ (136). Evidence of NKG2D downregulation suggests that $CD8^+$ T cells have been activated *via* NKG2D engagement in the TME and NTME. The idea that $CD8^+$ T cells contribute to tumor growth in this model is counterintuitive because: (i) DEN-treated RAG-deficient mice develop liver tumor nodules earlier than WT mice, at 6 months of age, supporting the importance of adaptive immunity in preventing early tumor development (141) and (ii) high $CD8^+$ T cell infiltration is a hallmark of so-called "hot" tumors, correlating with a greater antitumor response in various cancer types and with better responses to immunotherapy (142). Yet, in a large cohort of 302 HCC patients, the presence of $CD8^+$ T cell alone did not correlate with overall survival or disease-free survival (143). In fact, a number of recent model studies point to a role for $CD8^+$ T cells in accelerating liver damage and HCC. In a study of 63 HCV-infected HCC patients, Ramzan and colleagues reported that the number of tumor-infiltrating $CD8^+$ T cells significantly correlated with higher tumor recurrence and decreased overall survival of cancer patients (144). In a model of hepatitis-induced HCC, Haybaeck and colleagues examined the role of lymphotoxin α and β and their receptors in tumor growth. Breeding a lymphotoxin-expressing transgenic mouse onto a RAG-deficient background reduced HCC formation, demonstrating a detrimental role of adaptive immunity in this model (145). Wolf et al. demonstrated the negative impact of $CD8^+$ T cell infiltration and activation in the development of NASH-induced HCC. Using RAG-deficient, β2m-deficient mice and $CD8^+$ T cell depletion in wild-type mice, they showed that the infiltration and activation of $CD8^+$ T cells and NKT cells is directly linked to liver damage and subsequent HCC in this model (146). The critical role of lymphotoxin β and hepatic $CD8^+$ T cell was further demonstrated by Endig et al. in a mouse model of chronic liver failure leading to HCC development (147).

The analogy between $CD8^+$ T cell activation in HCC and in inflammatory disorders builds on increasing evidence for the presence of NKG2D ligands on healthy tissues (7, 80, 148) and their contribution to wound-associated inflammation (149) and autoimmune diseases (27, 127, 137, 150–152). The contribution of NKG2D in autoimmune attack has been shown against several tissues including the lung (153, 154), skin (149), pancreatic islets (150), brain (152), joints (27, 155), liver (137), and gut (127, 156–160).

With regards to the role of NKG2D in liver cancer, there is no direct evidence that $NKG2D^+$ $CD8^+$ T cells promote HCC, but robust data support a detrimental effect of $NKG2D^+$ $CD8^+$ T cells during viral hepatitis. In patients with chronic hepatitis B and C, intrahepatic $CD8^+$ T cells display a significantly increased expression of NKG2D compared to healthy controls (112). In acute hepatitis A virus (AHA)-infected patients, virus-specific $CD8^+$ T cells expressed significantly higher levels of NKG2D compared to healthy donors and MICA/B was overexpressed in infected liver tissues (161). This promoted innate-like $CD8^+$ T cell cytotoxicity and consequently mediated host injury in AHA (161).

We noticed that a significant fraction of $CD4^+$ T cells expressed NKG2D in DEN-induced HCC and that $CD4^+$ T cells in both TME and NTME may be a significant source of IFNγ and to a lesser extent of IL-17 (unpublished). As demonstrated in multiple studies, $CD4^+$ T cells could be a main contributor to the pro-inflammatory environment *via* IFNγ and IL-17 secretion, as seen in Crohn's disease patients (151, 162) and other autoimmune conditions (27). Second, they could help recruit CTL to the liver. Using the RMA/S-Rae-1 transplantable tumor model, Westwood et al. showed that $CD4^+$ T cells helped establish effective $CD8^+$ T cell memory against re-challenge (163). Additionally, they could directly cause cytotoxicity against hepatocytes as previously shown in HCC (164) and against melanoma (165, 166). Finally, $NKG2D^+$ $CD4^+$ T cells could act as suppressive cells in NKG2D ligand-expressing late stage tumors *via* the release of soluble Fas ligand (167). In patients with chronic HBV infection, MICA/B was induced on a small fraction of intrahepatic activated $CD4^+$ T cells, which correlated with an increased proportion of activated NK cells (115). It will be interesting to see whether this

is the case in HCC patients. While we did not observe NKG2D ligand expression on any lymphocytes in the DEN model of HCC, we cannot rule out that this is occurring for a short period of time during tumor progression, despite it being no longer present at end point.

The role played by NKG2D on NK cells in the DEN-induced tumor model is unclear. NK cells represent a small fraction of lymphocytes residing in the liver of 15-month-old mice (less than 5%) and were not enriched in DEN-treated mice compared to untreated age-matched control mice, which is in line with the IL-1R8 dependent impairment of NK accumulation in this model (168). Nonetheless, the fraction of CD107a-positive NK cells capable of IFNγ-production in HCC tumors is neither lower nor higher than in young control untreated mice, suggesting that NK cells are functional to some extent and may contribute to inflammation by increasing pro-inflammatory cytokines (136, 168). NKT cells are a predominant cell type residing in healthy liver tissues in mice and known to act as effective antitumor effectors in HCC in mouse models (169). NKT cells are likely to play a central regulatory function in the DEN-induced tumor model, contributing to NK cell and $CD8^+$ T cell recruitment and activation. NKT cells can also contribute to hepatitis and NKG2D-dependent liver damage in mouse models possibly *via* cytokine production and direct targeting of hepatocytes (138, 170).

## What Are the Mechanisms Involved?

The second key question relates to the function of $NKG2D^+$ lymphocytes. In wild-type mice, does NKG2D expression sustain the inflammatory milieu that drives further liver damage? Or does chronic NKG2D engagement cause anergy in the lymphocytes upon which it is expressed, repressing their antitumor function?

### Is There a Cell Subset With a Pro-Tumor Function?

A subset of $TCR\alpha\beta^+CD8^+CD44^+CD62^-$ T cells (T-pro) were shown to promote cutaneous carcinogenesis in the 7,12-dimethylbenz[a] anthracene (DMBA) and phorbol 12-myristate 13-acetate (PMA)-induced model of cutaneous carcinoma in a dose-dependent manner (171, 172). At high doses of DMBA in combination with PMA, the T-pro cells produced large amounts of IFNγ and TNFα and expressed elevated levels of NKG2D transcripts, but low amounts of perforin (172). In contrast, at low doses, the pro-inflammatory response was driven by a TH17-type response (173). In both cases, perforin was downregulated in the infiltrating $CD8^+$ T cells, suggesting that this T cell subset with limited lytic activity promotes tumor growth *via* cytokine secretion. These findings are consistent with the proposed role of NKG2D in promoting inflammation-associated cancer *via* $CD8^+$ T cells. Nonetheless, in the DEN-induced HCC model, $CD8^+$ T cells produced IFNγ and TNF-α, but not IL-17; $CD4^+$ T cells, however, were a potential source of IL-17 and could be acting as a subset with pro-tumor function *(unpublished).*

### Are NKG2D-Expressing Cells Activated or Desensitized *via* NKG2D Ligand Binding?

Sustained engagement of NKG2D by its ligands on tumor cells (174) or on myeloid cells can cause its downregulation and reduce NK cell responsiveness (76). Thompson et al. showed that RAE-1 expressed on lymph node endothelial cells and on tumor-associated endothelium can cause NKG2D internalization and desensitize NK cells. This led to higher tumor burden in models of subcutaneously transplanted tumors and in the transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer (82). NK cells in NKG2D-deficient mice, due to the lack of NKG2D-mediated desensitization, appear more responsive as they are capable of a better tumor rejection compared to wild-type mice in the TRAMP model. This suggests that NK cell desensitization *via* NKG2D binding constitutes a mechanism of tolerance in wild-type mice (82). Whether RAE-1 is expressed on endothelial cells in the DEN-induced HCC model and whether RAE-1 on healthy hepatocytes can desensitize hepatic NK cells remains to be determined, but this is a plausible explanation for the maintenance of a tolerogenic liver NK cell phenotype at steady state. It is also an attractive explanation for the increase in tumor burden seen in DEN-treated wildtype compared to NKG2D-deficient mice (136). However, in the DEN model, NKG2D was expressed on all hepatic NK cells and its level of expression (based on fluorescence intensity of anti-NKG2D staining) was similar to levels detected in the naïve liver of control mice *(unpublished).* In line with this, intrahepatic NK cells in HBV- and HCV-infected patients also maintain high levels of NKG2D expression during infection (113, 114). HCC patients showed high levels of cell surface NKG2D on liver-resident NK cells, although at a lower intensity on those infiltrating HCC compared to those residing in healthy tissue (175). Also, in the HBV model of acute hepatitis in transgenic mice developed by Vilarinho and colleagues, NKG2D was slightly downregulated on NKT cells found to be the main drivers of hepatitis, but not on NK cells (138). A possible explanation relates to the organization and location of NK cells in the diseased or tumor-bearing liver. In contrast with other CTL, there is no evidence to date that NK cells located in the liver sinusoids efficiently enter the space of Disse to make physical contact with hepatocytes (176, 177). If NK cells make contact with NKG2D ligand-expressing hepatocytes, it may not last long enough to induce NKG2D downregulation. Deguine et al. showed that NK cell interaction with transplanted $RAE\text{-}1^+$ transfected EL4 tumor cells are more dynamic and transient that $NKG2D^+CD8^+$ T cells/tumor interactions (178). In support of this, we noticed that NK cells infiltrating DEN-induced HCC do not express PD-1 in contrast with $CD8^+$ T cells, which may suggest a lack of chronic activation—assuming the mechanism of PD-1 expression is comparable in T and NK cells. Another possible explanation for the lack of NKG2D downregulation on NK cells and modest downregulation of NKG2D observed in $CD8^+$ T cells in the DEN model relates to the presence of sMULT-1 in the serum of DEN-treated mice concomitant with high levels of MMP-9, MMP-14, and ADAM-10. As shown by Deng et al. (76), sMULT-1 could counteract RAE-1-mediated NKG2D downregulation in the DEN-induced HCC model by competitive binding to the receptor.

One limitation in our studies is that direct cytotoxicity of NK and $CD8^+$ T cells against liver tumor cells could not be tested due to the difficulty of isolating viable hepatocytes for *in vitro* killing assay. Nonetheless, *ex vivo* analysis of CD107a expression

by tumor-infiltrating CD8$^+$ T cells showed that about a quarter of CD8$^+$ T cells are surface CD107a$^+$, which is higher than the frequency detected in non-treated liver controls, demonstrating that CD8$^+$ T cells are degranulating in the tumor (136). Further supporting this, a larger fraction of CD8$^+$ T cells produced IFN-γ in DEN-treated livers compared to young naïve mice (average 40 versus 20%) illustrating that these cells are responsive and that NKG2D downregulation in this model does not result in complete functional anergy (136). It is conceivable that NKG2D downregulation on CD8$^+$ T cells is actually a sign of activation associated with endocytosis and signaling of the NKG2D/DAP10 complex (179). With regards to NK cells, the percentage and amount of IFN-γ$^+$ NK cells in DEN-treated mice is similar to that observed in control naïve mice (136), suggesting that they are neither more active nor desensitized *via* NKG2D at this point of disease progression.

### Does the Pro-Inflammatory Milieu Boost NKG2D-Mediated Responses?

In the DEN-induced HCC model, RAE-1 was not only expressed on transformed hepatocytes but also non-transformed hepatocytes in DEN-treated and age-match control mice compared to young controls, indicating that ligand expression increases with aging in the liver. This is probably due to the ongoing exposure to gut-derived microbial agents and/or to the occurrence of metabolic disorders consequent to weight gain over time.

The sustained expression of RAE-1 on tumors, i.e., lack of editing, and mild downregulation of NKG2D could be the key combination to sustaining a loop of liver damage as seen in autoimmune diseases. In support of this, one critical observation made in patients with various autoimmune diseases is the high level of NKG2D expression despite the presence of soluble ligands in the serum (27, 137, 156). In patients with rheumatoid arthritis, soluble MICA failed to downregulate NKG2D on CD4$^+$ T cells and CD8$^+$ T cells, possibly due to counteractions of IL-15 and TNF-α (27). The mild, rather than substantial, downregulation of NKG2D and lack of functional impairment of CD8$^+$ T cells in the DEN-induced HCC model supports the idea that the inflammatory milieu in HCC, like in autoimmune diseases, thwart anergy. IL-15 present in the milieu is the best candidate to counteract NKG2D-mediated downregulation and NK cell and CTL desensitization as shown in autoimmune diseases and *in vitro* studies (58, 180, 181). Notably, under inflammatory conditions, the NKG2D-IL15 pathway leads to CTL infiltration and upregulation of NKG2D ligands associated with inflammatory myopathies (182). In rheumatoid arthritis, the substantial amount of soluble MIC released by synoviocytes failed to downregulate NKG2D on CD4$^+$ T cells, possibly due to high levels of IL-15 and TNF-α (27). When exposed to IL-15 in culture, liver-resident NK cells isolated from HCC patients displayed increased NKG2D expression and functionality against cocultured targets (175).

In conclusion, we show that RAE1 and MULT1 are expressed in the DEN-induced HCC model, supporting the idea that NKG2D has been persistently engaged on CD8$^+$ T cells leading to partial NKG2D downregulation from the cell surface. It remains to be determined whether NKG2D ligands expressed on hepatocytes or stromal hepatic cells contribute to maintaining a tolerogenic liver at steady state, an equilibrium likely lost in contexts of infection, sterile inflammation caused by obesity, or tumorigenesis.

## TME versus NTME: Does the Location of NKG2D-Expressing Cells Impact Their Function?

The ability for effector cells to migrate and reside in the TME is a key parameter in determining the positive or negative function of NKG2D expressing lymphocytes. CD8$^+$ T cells have the ability to infiltrate the liver and establish contact with hepatocytes in healthy and infected tissues (176, 177) probing for cognate MHC/peptide on hepatocytes (183). In the DEN-induced HCC model, an enrichment of CD8$^+$ T cells was observed in the NTME of wild-type mice compared to NKG2D-deficient mice, but not in the TME. This could be explained by a better recruitment and retention of CD8$^+$ T cells in the NTME.

In support of this scenario, we found a greater amount of chemokines including CXCL9, CXCL10, CCL3, and CCL5 in the NTME of wildtype than NKG2D-deficient mice. These chemokines may be involved in CD8$^+$ T cell chemotaxis to the DEN-treated liver and/or be produced by CD8$^+$ T cells upon NKG2D-mediated activation in wild-type mice. Ligand expression may play a role in CD8$^+$ T cell enrichment in DEN-treated liver. Indeed, transgenic RAE-1 expression on pancreatic islet cells was previously shown to favor the recruitment of CD8$^+$ T cells *in vivo* during pancreatic inflammation where CCL5 was significantly elevated (184). Also, CD8$^+$ T cells were activated and recruited in an NKG2D-dependent manner *in vitro* across a monolayer of ligand-expressing human intestinal endothelial cells (160). Furthermore, in the DEN model, we observed a significantly higher proportion of neutrophils in the NTME of wildtype compared to NKG2D-deficient mice supporting their role in NKG2D-mediated recruitment of CD8$^+$ T cells to NTME (136). In a transgenic mouse model of HBV, neutrophils were critical for the recruitment of antigen non-specific T cells to inflamed liver (185). Neutrophils recruit CD8$^+$ T cell due to their capacity to promote and amplify an initial inflammatory response by secreting chemokines, including CXCL9, CXCL10, CCL3, and CCL5 (186, 187). Overall, NKG2D contributes significantly to CD8$^+$ T cell enrichment in the inflamed liver tissue adjacent to tumors, seemingly *via* increased chemotaxis of T effector memory cells ($T_{EM}$) and persistence as tissue-resident T cells.

In addition to the above possibilities, it is conceivable that CD8$^+$ T cells are found in higher amount in the NTME compared to the TME of wild type because they do not optimally infiltrate HCC tumors, regardless of NKG2D and NKG2D ligand expression. Due to inherent differences between the tumor tissue and the NTME, such as increased cell density and necrosis, it is possible that the ability of CD8$^+$ T cells to traffic to or within the surrounding tissue is superior to their ability to infiltrate tumors. The vasculature of HCC is characterized by "disorganized, tortuous vessels" that decrease in number as histological grade progresses (188) and restrict T cell infiltration (189). Cytotoxic T cell density was shown to be markedly reduced in tumor-cell rich areas and most CTL were retained at the border of such regions by long-lasting contacts (190). Thus, NKG2D-mediated

recruitment, effective in the NTME, may be inhibited by such contacts in the TME. We do not currently have further evidence to prove this hypothesis in the present model, protein expression of chemokines and chemokine receptors will have to be quantified and compared in wildtype and NKG2D-deficient mice.

## Impact of NTME in HCC Progression

In HCC patients, the adjacent NTME has been described as the site of a pro-inflammatory immune response that enhances tumorigenesis (133). Hoshida and colleagues showed that pro-inflammatory gene expression (TNFα, IL-6, and nuclear factor-κB) in the NTME, rather than the tumor itself, strongly associated with poor prognosis and high recurrence rate in patients undergoing hepatic resection (133). Interestingly, a significant proportion of $CD8^+$ T cells detected in the NTME of HCC patients are organized in microniches referred to as ectopic lymphoid-like structures (ELS). While the function of ELSs remains understudied, they are known to develop at sites of inflammation and signify good prognosis in certain malignancies. However, the formation of ELSs was shown to foster tumor growth in the DEN model of HCC (191). ELS development was abolished following T cell depletion and hepatocarcinogenesis was attenuated, demonstrating the importance of adaptive immune cells and the cytokine-rich microniche (191). ELS could have functional implications on the action of NKG2D in the DEN-induced HCC model, fostering NKG2D-mediated $CD8^+$ T cell activation by ligand-expressing hepatocytes, which subsequently exacerbate proinflammatory responses in the NTME.

Hepatic stellate cells (HSC) are non-parenchymal cells that constitute 20–30% of the liver tissue and, along with Kupffer cells (KC), they contribute to liver pathogenesis due to their capacity to secrete various cytokines, recruit and activate lymphocytes including NKT and T cells. Interestingly, activated T cells can enhance HSC activation, resulting in IL-6, IL1-α, and TGFβ production (192), thus adding to the inflammatory milieu of cytokines promoting tumor growth.

## Impact of TME in HCC Progression

The TME is an immunosuppressive environment with the ability to impair antitumor responses. Transcriptional and epigenetic changes may underpin the generation of dysfunctional T cells in the TME. Single-cell RNA-seq revealed a distinct clonal population of exhausted $CD8^+$ T cells that was elevated in late-stage HCC compared to early stage tumors associated with enrichment in regulatory T cells (Tregs) (193). Tregs were markedly increased in DEN-induced HCC compared to age-matched liver tissue (**Figure 1A**). This correlated with an increase in CCL17 and CCL22 transcripts (**Figures 1B,C**), two chemokines involved in Treg recruitment (194). In addition to the numerous mechanisms employed by Tregs to repress NK and $CD8^+$ T cell antitumor functions, Tregs may play a role in the mild down-regulation of NKG2D in $CD8^+$ T cells through TGFβ production, as seen in patients with persistent HBV infection (92). It has been shown that adoptive transfer of Tregs into Rag1-deficient mice inhibited the NK cell antitumor function against RAE-1 expressing B16 cells, implicating an inhibitory role for Tregs in NKG2D-mediated surveillance (195) or NKG2D signaling (196).

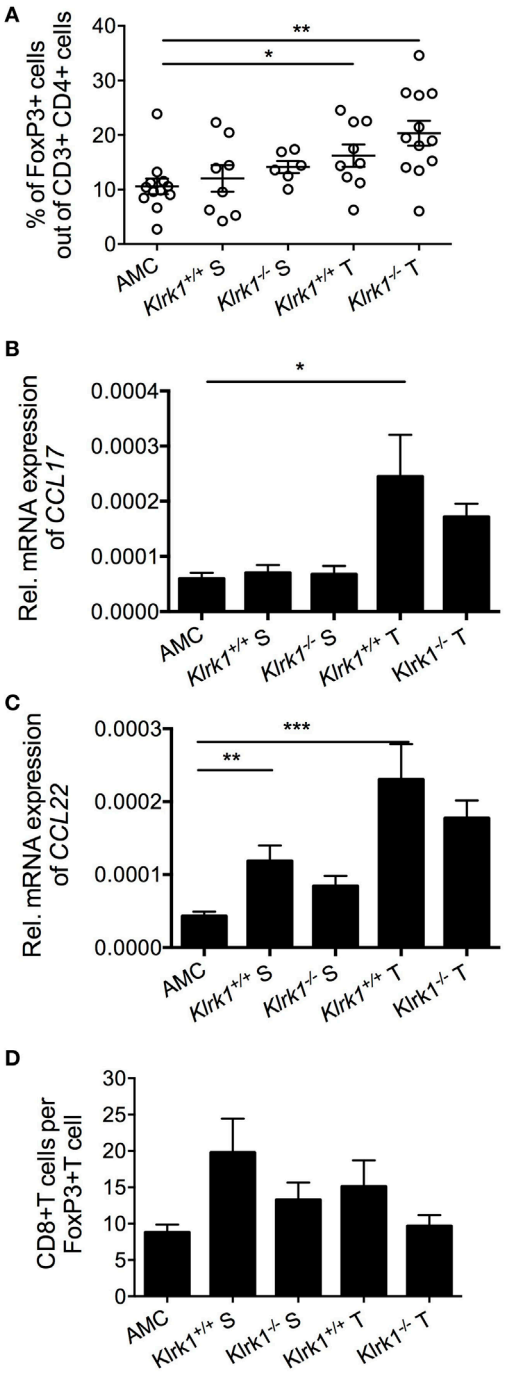


**FIGURE 1** | Accumulation of $Foxp3^+$ $CD4^+$ T cells in diethylnitrosamine (DEN)-treated liver. **(A)** Percentages of $FoxP3^+$ T cells (gated on $CD4^+$ cells) present within the tumor (T) and the surrounding tissue (S) in wildtype (*Klrk1*$^{+/+}$) and NKG2D-deficient (*Klrk1*$^{-/-}$) DEN-treated mice, and age-matched control mice (AMC). **(B,C)** Relative expression of **(B)** CCL17 and **(C)** CCL22 mRNA transcripts within tumors (T) and surrounding tissue (S) in wildtype and NKG2D-deficient DEN-treated mice ($n \geq 20$) and age-matched control mice (AMC) ($n \geq 8$). **(D)** Ratio of $CD8^+$ T cells to $FoxP3^+$ T cells in wildtype (*Klrk1*$^{+/+}$) and NKG2D-deficient (*Klrk1*$^{-/-}$) DEN-treated mice, and AMC. Graphs represent the mean ± SEM. Statistical analysis was performed by unpaired Student's *t* test, where statistically significant differences between groups are denoted as: $^*p \leq 0.05$ and $^{**}p \leq 0.01$.

However, Tregs were remarkably less represented than CD8+ T cells in DEN-treated liver (**Figure 1D**), suggesting that Treg-mediated suppression of CD8+ T cell antitumor activity may not be effective in this model. Also, NKG2D downregulation potentially mediated by sustained binding to NKG2D ligands, soluble ligands, and/or TGF-β could be counteracted by cytokines such as TNF-α, IL-15, IL-2, and IL-18 present in the milieu (79, 197), which would explain the mild, rather than drastic, downregulation we observed.

In line with clinical studies showing significantly higher levels of PD-1+CD8+ T cells in diseased human liver compared to healthy liver tissue and peripheral blood (198), the majority of CD8+ T cells infiltrating DEN-induced tumors expressed PD-1. Interestingly, a majority of PD-1+CD8+ T cells were functionally active as determined by GzB expression and IFNγ production. It is conceivable that the fraction of PD-1+CD8+ T cells that do not express IFNγ are dysfunctional and have impaired antitumor activity. Encouraging results were obtained from a clinical trial (checkmate40) using nivolumab to inhibit PD-1 in the treatment of advanced HCC patients (199, 200). It is tempting to postulate that the unleashed antitumor response against tumor-associated antigens (TAA) in treated patients is enhanced through NKG2D costimulation of the TCR. There are indications that this may be the case, as the presence of human soluble NKG2D ligand has been shown to correlate with poor response to checkpoint blockade therapy (70), with Ab neutralization of NKG2D ligands showing the potential to enhance efficacy and reduce autoimmune side effects (201). Furthermore, NKG2D-dependent, TCR-independent stimulation may also contribute *via* the elimination of NKG2D ligand-expressing tumor variants that have lost TAA expression.

Myeloid cells present in the TME could also impede antitumor responses in the DEN model as seen in other models of cancer (83). Recent data obtained by Vyas et al. in ovarian cancer patients, a type of cancer with a pro-inflammatory signature similar to HCC, demonstrated that high levels of soluble MICA and ULBP2 were associated with poor prognosis. Interestingly, this did not correlate with NKG2D downregulation, but rather with reduced infiltration of effector memory CD8+ T cells and increase infiltration of pro-tumorigenic macrophages (74).

With regards to NK cells, there is some evidence, from preclinical and clinical studies, that the degranulation response of hepatic NK cells is impaired in the DEN model (168) HCC patients (175) and chronic HCV-infected patients (114) is reduced. NK cells from HCV infected patients degranulated less, but produced equivalent levels of IFNγ and TNFα, in anti-NKG2D mAb mediated redirected cytotoxicity experiments in the presence of IL2 and IL12 (114). This suggests that the cytotoxic capacity of intrahepatic NK cells in patients with chronic hepatitis C may be reduced. Additional evidence was obtained by incubating peripheral blood NK cells from healthy donors with an HCV-infected HCC cell line for 18 h, this reduced degranulation and IFNγ production against K562 cells (202).

## Are CD8+ T Cells Involved in Antigen-Specific or TCR-Independent Responses?

In the DEN model, more than half of the memory CD8+ T cells infiltrating the tumors expressed PD-1, highlighting their prior encounter with antigens and chronicity of the TCR stimulation. This supports the idea that the majority of CTL in the TME are antigen-specific and may recognize a TAA still present on tumor cells. We detected a large amount of glypican 3 (GPC3)—one of the most frequently expressed known TAAs in human HCC (203)—suggesting that GPC3 could be responsible for CD8+ T cell infiltration and activation in the tumor bed (**Figure 2**) (136). Whether those cells are still functionally active in eliminating TAA+ tumors at the advanced stage of HCC in the DEN model (15-month end point) or whether they are ineffective against tumor variants that have lost TAA remains to be determined.

It is tempting to postulate the existence of a TCR-independent CTL response in the DEN-induced HCC model, especially in the NTME. NKG2D mainly acts as a co-stimulatory receptor on CD8+ T cells but can also act as a stimulatory receptor under certain circumstances (204–206). Markiewicz et al. showed that RAE1ε enhanced CTL IFN-γ secretion in response to IL-12 and IL-18 in the absence of antigenic stimulation (206). In human gut tissues, high expression of MICA can directly activate NKG2D in γδT cells and co-activate CD8+ T cells that constitute the

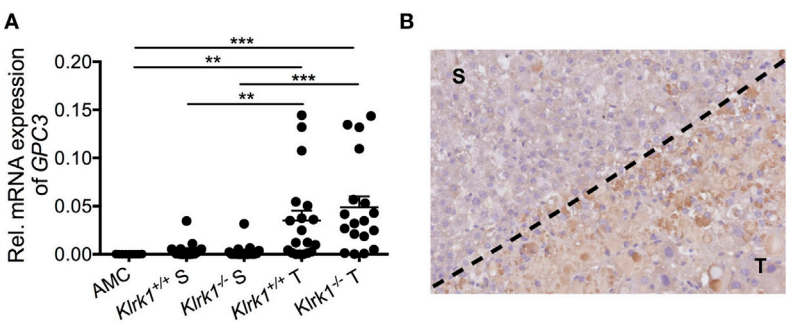


**FIGURE 2** | Tumor-specific expression of glypican3 in hepatocellular carcinoma. **(A)** Quantification of *Gpc3* mRNA transcripts within tumors (T) and surrounding liver tissue (S) in wildtype (*Klrk1*$^{+/+}$) and NKG2D-deficient (*Klrk1*$^{-/-}$) diethylnitrosamine (DEN)-treated mice ($n \geq 17$) and AMC ($n = 9$). **(B)** Representative IHC staining of GPC3 on a DEN-treated liver where T represents the tumor area and S represents the surrounding tissue. Graph represents the mean ± SEM. Statistical analysis was performed by unpaired Student's *t* test, where statistically significant differences between groups are denoted as: $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

intraepithelial lymphocytes (IEL). These effector cells were shown to target gut epithelial cells in a TCR-independent manner as well as *via* a gliadin antigen-dependent $CD8^+$ T cell response (156). Such lytic activity has led to villous atrophy in celiac disease caused by IEL-mediated damage likely *via* NKG2D activation, in a TCR-independent manner (127). Other studies have shown a direct role for NKG2D in a TCR-independent activation of gut IEL (156) in the presence of high does IL-2 (204). In the human intestinal epithelium, the presence of IL-15 might promote NKG2D expression or lack of downregulation (180) and could also be favorable to NKG2D signaling independently of the TCR (127). The established link between NKG2D and IL-15R signaling (207) led us to hypothesize that NKG2D-mediated activation of CTL in contact with NKG2D ligand-expressing normal hepatocytes, in the presence of IL-15, could lead to a self-damaging response in a non-cognate fashion. TCR-independent, NKG2D-dependent activation of memory $CD8^+$ T cells was recently demonstrated in hepatitis A virus-infected (HAV) patients where liver injury associates with high production of IL-15 and elevated expression of NKG2D ligands by infected hepatocytes (161). The severity of liver injury in these patients correlated with the activation of HAV-unrelated virus-specific $CD8^+$ T cells (specific for influenza A, Epstein–Barr, and cytomegalovirus) and the innate-like cytolytic activity of $CD8^+$ T cells, but not the activation of HAV-specific T cells (161). It was also shown that coculture of peripheral $CD4^+NKG2D^+$T cells from metastatic melanoma patients with sMICA + IL-15 induced IFNγ secretion in a TCR-independent manner (49).

The importance of antigenic recognition in CTL migration and location in the TME has been highlighted in tumor transplant models (208) showing that CTL were able to infiltrate tumors in depth only if tumors displayed cognate antigens; otherwise, they remain in peripheral regions (209). We, therefore, propose that when there is a significant influx of tumor-specific CTL into an inflamed liver, only tumor-antigen-specific T cells infiltrate the tumor, possibly becoming anergized through persistent TCR engagement.

## Adverse Effects of NKG2D in HCC: Working Model

We postulate that early stage precancerous liver lesions are rejected *via* activation of NK and NKT cells through NKG2D engaging NKG2D-ligand expressing neoplastic hepatocytes. Over time, sustained tissue damage initiated by the mutagen creates an environment enriched in myeloid cells and chemokines such as CXCL9, CXCL10, CCL3, CCL5, and Mip1-a, which drive the recruitment of memory $CD8^+$ T cells and inflammatory macrophages. Elevated perforin and granzyme B coupled with metalloproteinase activity amplifies the immune response and promotes liver injury. MP can also contribute to the shedding of ligands such as sMULT1, detected in HCC-bearing mice, that can counteract RAE-1-mediated desensitization. Continuous exposure of T cells to NKG2D ligands feeds the pro-inflammatory milieu *via* TNFα and IFNγ production in a TCR-independent, NKG2D-dependent fashion. Persistent hepatic injury, hepatocyte death, and regeneration of mutated hepatocytes ultimately results in tumor growth. This NKG2D-mediated inflammatory response is likely beneficial in rejecting early neoplastic tumors, yet, over time, feeds the loop of tissue injury-repair-proliferation that are the hallmark of HCC. In addition, the build-up of an immunosuppressive milieu within the TME and high levels of PD-1/PD-L1 expression is likely to impair tumor clearance by NK cells, NKT cells, and TAA-specific CTL (**Figure 3**).

## Tumor-Promoting Effect of NKG2D in Other Cancer Models

Previous studies have described how other immune components can act as antitumor versus pro-tumor effectors in the same model. For example, MyD88 was shown to promote tumor growth in the MCA-induced fibrosarcoma model, while simultaneously mediating a protective host response *via* TNFα and IFN-α/β signaling (210). IL-1α and IL-1β showed opposite functions in the development of fibrosarcomas induced by MCA. IL-1β acted as driver of oncogenic inflammation, IL-1β-deficient mice developed less tumors due to reduced pro-tumorigenic inflammation compared to wild-type mice. In contrast, IL-1α acted as a key mediator of immune surveillance; tumor cell lines derived from IL-1α-deficient mice showed reduced invasiveness when transplanted in wild-type hosts, demonstrating a lack of tumor editing in its absence. Loss of tumor editing in IL-1α-deficient mice was attributed to impaired NK cell maturation and a suboptimal killing capacity of effector immune cells resulting in reduced tumor editing (211). Also, αβ T cells have been shown to contribute to skin papilloma formation, reinforcing the idea that adaptive immunity plays a dual role in models of chemically induced inflammation and tumorogenesis (171, 212).

In a BALB/c mouse model of chronic pulmonary inflammation that progresses to lung adenocarcinoma due to the concomitant lack of IFN-γ and the β-common cytokines GM-CSF and IL-3, Dougan and colleagues showed that IL-6 is a key driver of oncogenic inflammation (213). These mice are also highly susceptible to tumor growth due to the lack of IFN-γ-driven tumor editing as demonstrated by their clearance upon transplant into immune-competent hosts. This model demonstrates the importance of IFNγ-driven tumor editing even in inflammation-driven cancer (213).

With regards to the NKG2D axis, tumor progression rather than rejection consequent to NKG2D ligands expression has been observed in other models of cancer. Our group has reached similar conclusions in a mouse model of intestinal polyposis and colon carcinoma. In this model, RAE-1 is expressed on the gut epithelium and associates with a significantly reduced survival of wildtype compared to NKG2D-deficient littermates *(unpublished data)*. In the TRAMP model, some TRAMP mice developed large, rapidly growing aggressive tumors (type I) that were rejected *via* NKG2D (35). However, some TRAMP mice developed a second type of tumor (type II) that progressed much slower, expressed high amounts of cell surface NKG2D ligands but were not rejected by NKG2D. Type II tumors actually grew faster in the presence of NKG2D, though the trend was not statistically significant. Although the TRAMP tumor

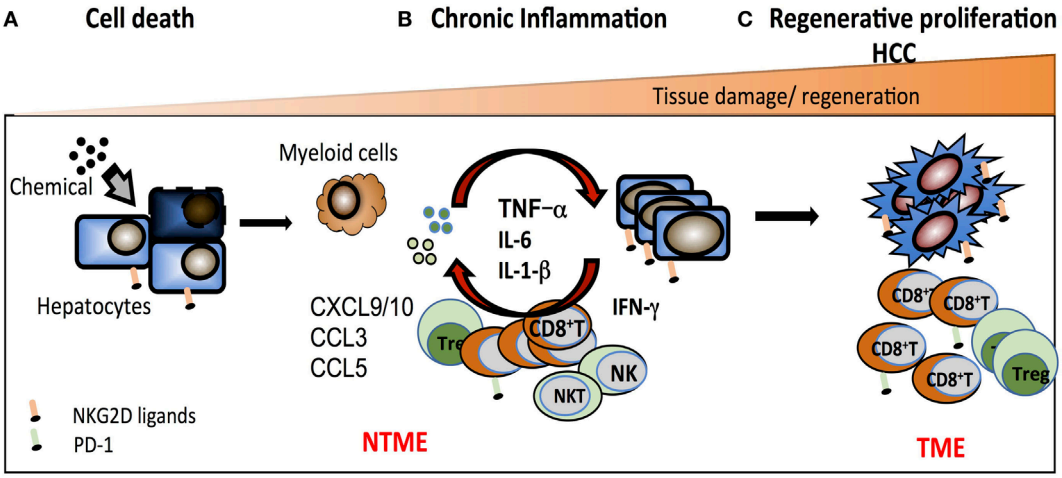


**FIGURE 3** | Working model of the pro-tumorigenic effect of NKG2D in hepatocellular carcinoma. **(A)** Chemical and/or viral injury causes cell stress that results in DNA damage, increased expression of NKG2D ligands, and hepatocyte cell death. NKG2D$^+$-resident effector cells [natural killer (NK) cell, NKT cell, CD8$^+$ T cells] participate in the elimination of stressed hepatocytes and contribute to shaping an innate inflammatory environment that further drives the recruitment and activation of antigen-specific CD8$^+$ T cells. **(B)** In response to a continuous exposure to NKG2D ligands in the non-tumor microenvironment (NTME), NKG2D$^+$ effector cells exacerbate the local inflammation *via* direct secretion of inflammatory components (such as TNFα, IFNγ, and MIP1-α) and chemoattractants leading to an enrichment in CD8$^+$ T cells. This indirectly favors the recruitment of myeloid cells that further the inflammatory milieu with pro-inflammatory cytokines such as IL-6, TNFα, and IL-1α. **(C)** In this context, cycles of persistent tissue injury characterized by the death of hepatocytes consequently drives the process of tissue regeneration, which encompass the proliferation of mutated hepatocytes or epithelial cells and ultimately promotes tumor growth. In the TME, the majority of CD8$^+$ T cells expresses PD-1 and may become partially impaired in their antitumor function due to antigen-specific chronic stimulation.

milieu has not been extensively studied, an obvious difference between these tumors is that type II tumors displayed a high immune infiltrate that was almost non-existent in type I tumors *(unpublished)*. The presence of soluble NKG2D ligands was not tested, hence their expression cannot be ruled out and may differentially impact the control of type I versus type II tumors (36). Another model worth discussing is the MCA-induced fibrosarcoma where MyD88-dependent inflammation was shown to promote tumorigenesis (210). We observed that slow-growing tumors induced by a low dose of MCA (5 μg) expressed high levels of cell surface NKG2D ligand and showed a trend to develop earlier in NKG2D-sufficient than NKG2D-deficient mice (35). This was not the case in fibrosarcoma induced with higher dose of MCA (25 μg), which progressed significantly faster regardless of NKG2D (35). The high level of ligand expression and lack of editing in both TRAMP type II tumors and fibrosarcoma supports the idea of a tumor-promoting effect through NKG2D activation. Further studies to characterize the TME and NTME and eventual release of soluble ligands would be valuable. Collectively, these studies indicate that the extrinsic pro-tumor effect associated with NKG2D–NKG2D ligand signaling is not restricted to HCC and could broadly apply to cancer driven by chronic inflammation. Dissecting the function of NKG2D on CD8$^+$ T versus NK versus NKT cells in this model will be the key to fully understand the function of NKG2D in inflammatory cancer. Experimental approaches that involve antibody depletion and/or adoptive transfer of specific cell types will be critical to this end in addition to the more definitive approaches of using conditionally genetically targeted mice.

## CONCLUDING REMARKS

In line with clinical studies that correlate high levels of NKG2D ligands with poor prognosis (43, 53–55), we observed in some mouse models that tumors were not efficiently rejected and even progressed in NKG2D-sufficient mice, despite high levels of surface NKG2D ligands expression on the tumors (35, 136, 214). Our studies of the DEN-induced model of HCC demonstrate a paradoxical role of the NKG2D axis in promoting tumor growth. NKG2D-mediated antitumor activities may exist in this model and we argue that antitumor and pro-tumor effects *via* NKG2D may occur in a time- and context-dependent manner (**Figure 4**). We propose that during HCC progression in DEN-treated mice, NK cells and NKT cells, *via* NKG2D, are likely to act as the main effectors against early neoplasia, although falling short of complete tumor elimination. Subsequently, CD8$^+$ T cells may represent the main drivers of the pro-tumor effect, as seen in chronic inflammatory diseases and recently in HCC promoting inflammation in the NTME in an NKG2D-dependent, TCR-independent manner. Concomitantly, persistent expression of PD-1 on memory CD8$^+$ T cells in the TME, along with other immunosuppressive components and tumor-intrinsic features, could reduce and/or prevent antigen-specific antitumor activity of CD8$^+$ T cell.

There is no doubt about the potential of NKG2D/NKG2D ligands as targets in immunotherapeutic strategies. To deliver an effective and tailored NKG2D-based therapy, several questions remain to be addressed regarding the influence of the TME and the impact of radiotherapy and chemotherapeutic agents on NKG2D ligand expression—as these could potentially impair or

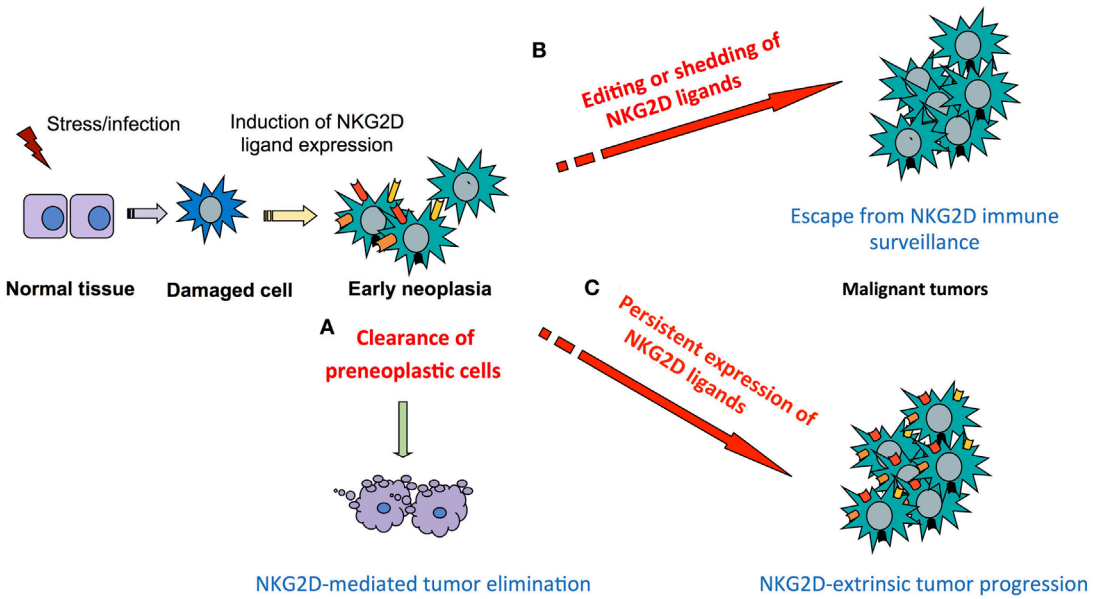


**FIGURE 4** | Principles of NKG2D dual function in tumor immunity. Induction of NKG2D ligand on stressed or damaged tissues initiates a local immune response *via* NKG2D-expressing cells that progresses according to four non-exclusive scenarios. **(A)** Clearance of neoplastic cells preventing or delaying tumor formation as seen in mouse models of Eμ myc driven B-cell lymphoma and leukemia and aggressive prostate tumors in the transgenic adenocarcinoma of mouse prostate (TRAMP) mouse model. **(B)** Failure to eliminate tumors due to NKG2D ligand editing as seen in a fraction of aggressive prostate tumors in the TRAMP mouse model or to ligand shedding and subsequent NKG2D downregulation and effector desensitization, as described in advanced human cancer with poor prognosis. **(C)** Persistent expression of NKG2D ligands at high levels exacerbates the pro-tumorigenic milieu *via* NKG2D-mediated immune responses in models of inflammation-driven cancer such as hepatocellular carcinoma and fibrosarcoma models.

eventually worsen the treatment. The kinase inhibitors Sorafenib and recently approved Regorafenib, used as second line treatment against advanced HCC, offer a limited survival benefit to advanced HCC patients. With the recent FDA approval of the anti-PD-1 mAb nivolumab for treatment of HCC, one might hope to specifically target tumor-infiltrating $CD8^+$ T cells and potentiate tumor antigen-specific response in the TME, including NKG2D-mediated surveillance, without enhancing the detrimental pro-inflammatory effect of NKG2D in the adjacent healthy tissue.

The finding that cancer may progress as an adverse effect of high expression of NKG2D ligands and NKG2D activation represents a challenge to the design of NKG2D-based immunotherapies due to the potential toxicity against healthy tissues expressing NKG2D ligands (215, 216). Approaches aiming at enhancing NKG2D ligands expression could contribute to the deleterious cycles of tissue damage and repair known to favor tumor growth over rejection in inflammation-driven cancer. Several clinical trials testing NKG2D-based chimeric antigen receptor (CAR) have been initiated; a first report of objective response to NKG2D-CAR (CYAD-01) was recently described in a relapsed/refractory acute myeloid leukemia patient (217). It will be interesting to see what type of solid tumors may benefit from this approach since it has the capacity to also target NKG2D ligand$^+$ immunosuppressive cells present in the TME. Advanced HCC and other types of cancer associated with chronic inflammation may require NKG2D-based approaches that target tumor cells with minimal damage to the NTME. Alternatively, blocking NKG2D as a means to reduce hepatic chronic inflammation is an approach worth considering during hepatitis and is currently being tested in Crohn's disease (159).

## MATERIALS AND METHODS

### Mice

*Klrk1*$^{-/-}$ mice on a C57BL/6J background were bred and maintained at the Imperial College London's animal facility, in a specific pathogen-free environment. The health status was regularly monitored throughout the study.

### HCC Induction

Cohorts of male age-matched wildtype (*Klrk1*$^{+/+}$) and NKG2D-deficient (*Klrk1*$^{-/-}$) mice received a single intraperitoneal injection of DEN (Sigma) (25 mg/kg body weight) or PBS at 14–21 days of age to induce HCC. Mice were euthanized at 15 months of age. Mice that became terminally ill prior to the endpoint were humanely sacrificed.

### Histology and Immunohistochemistry

Liver tissues were fixed in 10% neutral buffered formalin, paraffin embedded, and cut into 4 μm sections. Glypican-3 (GPC3) expression was analyzed using a rabbit polyclonal antiserum raised against GPC3 (ab66596) (Abcam, Cambridge, UK). Slide images were captured using a Nanozoomer slide scanner (Hamamatsu) and analyzed using Image J software.

## Tissue Dissociation and Flow Cytometry

Tumor and surrounding regions were delineated macroscopically. Tissues were dissociated through 100 μm cell strainers in PBS with 3% bovine serum albumin (BSA). Hepatocytes were removed by centrifugation on a 35% Percoll gradient at 700 × *g* at 21°C for 12 min. Leukocytes present in the pellet were resuspended in red blood cell lysis buffer (0.15 M $NH_4Cl$, 0.1 mM $KHCO_3$, 0.1 mM $Na_2$-EDTA in water; pH 7.2) for 1 min, washed, and then resuspended in PBS with 3% BSA. Cell suspensions were incubated with anti-mouse CD16/CD32 (Becton Dickinson, BD, USA) to block Fc receptors and Fixable Viability Dye eFluor 506 (eBioscience, San Diego, CA, USA). Cells were then stained with a cocktail of directly conjugated mAbs [CD3 (17A2) BD, CD4 (RM4-5) eBioscience, CD8 (53-6.7) BioLegend, CD45 (30-F11) BioLegend and Foxp3 (NRRF-30) eBioscience] for 30 min at 4°C. Intracellular staining was performed with a transcription factor staining buffer set from (eBioscience). The relevant fluorescence-minus-one labeling conditions including the appropriate isotype-matched mAb were used as controls. All samples were acquired on an LSR Fortessa flow cytometer (BD) and analyzed with FlowJo version 9.3.1 or above (Tree Star, Ashland, OR, USA).

## RNA Isolation and Quantitative RT-PCR

Tumor and surrounding liver tissue were collected in RNA-later (Sigma) and stored at −80°C as per the manufacturer's instructions. RNA was extracted using Qiagen's RNeasy kit (Hilden, Germany) and reverse transcribed into cDNA with High capacity cDNA RT kit (Life Technologies). For some genes, an amplification step was performed using the TaqMan PreAmp Master Mix Kit (Applied Biosystems). Quantitative real-time PCR was carried out using the TaqMan system (Applied Biosystems), all values were normalized to GAPDH expression.

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 5.03 (GraphPad Software Inc.). Two-tailed unpaired Student's *t*-test was performed with Welch's correction when appropriate. Differences at $p \leq 0.05$ were considered significant.

# ETHICS STATEMENT

All animal work was carried out in compliance with the British Home Office Animals Scientific Procedures Act 1986 (Project license number 70/7129).

# AUTHOR CONTRIBUTIONS

JG and SS performed the experiments and analyzed the data. SS and AF wrote the manuscript. NG designed and supervised the studies, analyzed the data, and wrote the manuscript.

# ACKNOWLEDGMENTS

We thank Prof. Lewis Lanier, Sophie Curio, and Dr. Jean-Pierre Couty for critical reading of the manuscript. This work was supported by the Wellcome Trust RCDF (RCDF088381/Z/09/Z) to NG, a Wellcome Trust ISSF Inflammation Science (IS) Studentship (JG) and a fellowship from the Biotechnology and Biological Sciences Research Council (BBSRC) (SS). NG receives funds from the AstraZeneca Innovation funds (RSRO_P71752).

# FUNDING

NG received funds from the Wellcome Trust awards RCDF088381/Z/09/Z and 097816/Z/11/ZR and from the Astrazeneca Innovation funds RSRO_P71752.

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**Conflict of Interest Statement:** NG receives funds from AstraZeneca. All other 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 August 2018
doi: 10.3389/fimmu.2018.01820

# The Biological Influence and Clinical Relevance of Polymorphism Within the NKG2D Ligands

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NKG2D is a major regulator of the activity of cytotoxic cells and interacts with eight different ligands (NKG2DL) from two families of MIC and ULBP proteins. The selective forces that drove evolution of NKG2DL are uncertain, but are likely to have been dominated by infectious disease and cancer. Of interest, NKG2DL are some of the most polymorphic genes outside the MHC locus and the study of these is uncovering a range of novel observations regarding the structure and function of NKG2DL. Polymorphism is present within all NKG2DL members and varies markedly within different populations. Allelic variation influences functional responses through three major mechanisms. First, it may drive differential levels of protein expression, modulate subcellular trafficking, or regulate release of soluble isoforms. In addition, it may alter the affinity of interaction with NKG2D or modulate cytotoxic activity from the target cell. In particular, ligands with high affinity for NKG2D are associated with down regulation of this protein on the effector cell, effectively limiting cytotoxic activity in a negative-feedback circuit. Given these observations, it is not surprising that NKG2DL alleles are associated with relative risk for development of several clinical disorders and the critical role of the NKG2D:NKG2DL interaction is demonstrated in many murine models. Increased understanding of the biophysical and functional consequences of this polymorphism is likely to provide insights into novel immunotherapeutic approaches.

**Keywords: polymorphism, single nucleotide, NKG2D ligands, binding affinity, cytotoxicity, immunologic, natural killer cells**

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

***Received:*** *28 April 2018*
***Accepted:*** *24 July 2018*
***Published:*** *16 August 2018*

***Citation:***
*Zuo J, Mohammed F and Moss P (2018) The Biological Influence and Clinical Relevance of Polymorphism Within the NKG2D Ligands. Front. Immunol. 9:1820. doi: 10.3389/fimmu.2018.01820*

## INTRODUCTION

NKG2D is a dominant activating receptor on cytotoxic lymphocytes, including natural killer (NK) cells, γδ T cells, NKT cells, and αβ T cell subsets (1). Engagement of NKG2D with NKG2D ligands (NKG2DLs) on target cells triggers cytotoxicity or cytokine production and plays an important role in both innate and adaptive immune responses, including control of viral infection, tumorigenesis and pathogenesis of autoimmune diseases (2–4).

A striking feature of the NKG2D:NKG2DL interaction is that only a single gene encodes NKG2D while there are eight *NKG2DL* genes within the human genome. These NKG2D ligands comprise six cytomegalovirus glycoprotein UL16 binding proteins and two major histocompatibility complex class I polypeptide-related sequences (MICA/B). In mice, NKG2D ligands comprise five RAET1 family members, three H60 proteins and MULT-1 (5–8). MICA and MICB contain α1, α2, and α3 domains together with a transmembrane domain and short cytoplasmic tail. In contrast, ULBP ectodomains comprise only the MHC-like α1 and α2 domain and ULBP1, ULBP2, ULBP3, and

ULBP6 are GPI-anchored receptors while ULBP4 and ULBP5 encompass a membrane anchor and cytoplasmic tail (9).

Several crystal structures of the NKG2D/NKG2DL interaction have been resolved, including NKG2D–MICA (10, 11), NKG2D–ULBP3 (12), and NKG2D–ULBP6 (13). These reveal that the symmetric NKG2D homodimer binds to a monomeric NKG2D ligand *via* a diagonal mode of engagement which is similar to TCR–pMHC interaction. In particular, the saddle-shaped NKG2D homodimer sits astride the NKG2D ligand helices with the NKG2D monomers A and B focused on the NKG2D ligand α2 and α1 helices, respectively (**Figure 1**) (13).

DAP10 is the binding partner for NKG2D and ligation and cross-linking of NKG2D leads to the recruitment of the p85 subunit of PI3K and Grb2-Vav1-Sos1 complex to the phosphorylated DAP10. This elicits the phosphorylation of the kinases Jak2, Stat5, Akt, MEK1/2, and Erk. This in turn induces calcium flux, actin reorganization, degranulation and, finally, cytotoxicity to target cells. PI3K inhibitors can thus completely block cytotoxic activity (14).

All *NKG2DL* genes demonstrate considerable polymorphism (15–18) and this variation is present within each of the domains and with a relatively random distribution, a feature in contrast to the focused variation that is observed with MHC class I genes (19, 20). Polymorphism is most significant within the *MICA* and *MICB* genes (17) where 62 single nucleotide polymorphisms (SNP) and 25 SNPs have been identified, respectively. In relation to the ULBP gene family, 28, 12, 10, 20, 10, and 14 SNPs have been reported within ULBP1-6 (15, 17, 21, 22). Interestingly, polymorphisms within MICA and MICB are distributed throughout the three extracellular α1–α3 domains and there are no identified coding polymorphisms within residues 40–89 within the section of the α1 domain that is predicted to mediate binding with NKG2D (9, 23). This profile is somewhat different to the profile of ULBP polymorphism where several variants are predicted to influence directly the site of NKG2D binding.

The frequency of individual *MICA* and *MICB* alleles varies in different populations around the world. In particular, *MICA*008* is the most frequent allele worldwide (9) (except in South American Indians) (24) followed by *MICA*002*, *MICA*010*, *MICA*009*, and *MICA*004*. The *MICB* gene is more homogeneous, with *MICB*005* being the most common allele and less representation from *MICB*002*, *MICB*004*, *MICB*008*, and *MICB*014* (9). Population-based variations in polymorphism are also a dominant feature and with wide variation in the frequency of SNPs within *ULBP* in Euro-Caucasoid, Afro-Caribbean, and Indo-Asian individuals (15) and most variation focused within *ULBP3*, *ULBP4*, and *ULBP6* (15, 16).

## POLYMORPHISM WITHIN NKG2D LIGANDS REFLECTS THE IMPACT OF DIFFERENTIAL SELECTIVE FORCES WITHIN EVOLUTION

An important question within NKG2DL biology relates to the evolutionary pressures that have driven gene duplication and

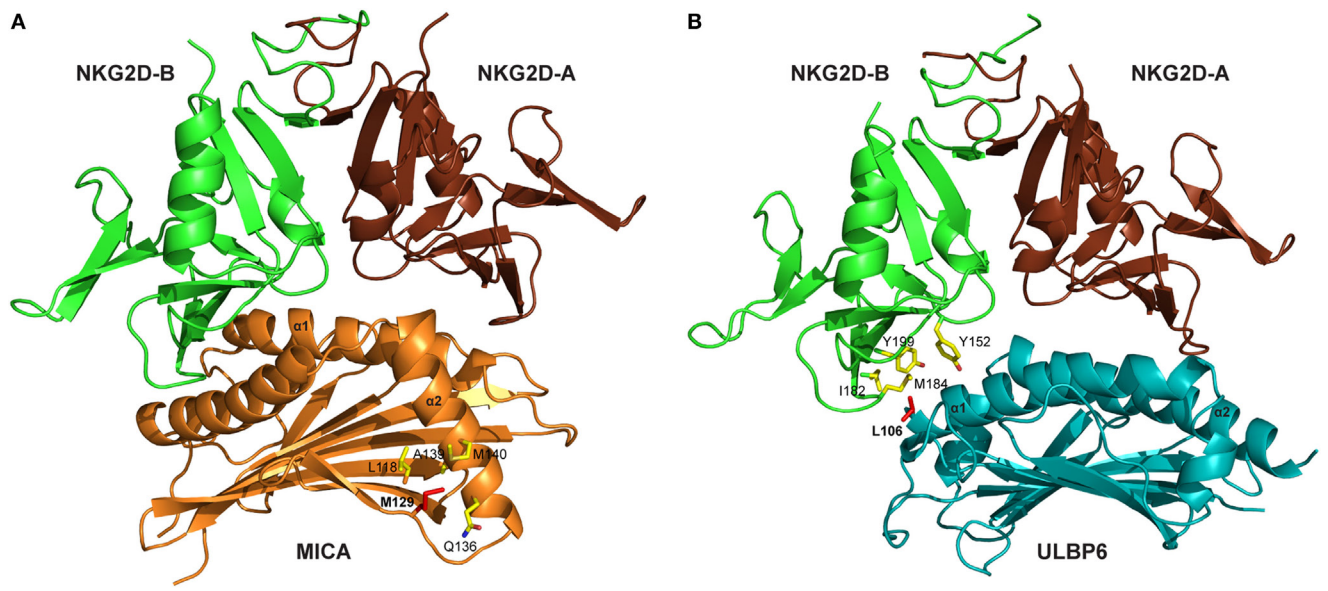


**FIGURE 1** | Crystal structures of NKG2D-ligand complexes. **(A)** Ribbon representation of the MICA–NKG2D complex [PDB code 1HYR, Li et al. (10)]. NKG2D homodimer [NKG2D-A (brown) and NKG2D-B (green)] interacts with the α1 and α2 domains of monomeric MICA (orange). The clinically relevant polymorphic residue Met129 (red) in MICA is located distal to the MICA–NKG2D interface. Partially buried Met129 mediates non-polar interactions with MICA residues (ball and stick format) that protrude from the α2 helix. The α3 domain of MICA (residues Thr181-Ser274) has been omitted. **(B)** Ribbon representation of the ULBP6–NKG2D complex [PDB code 4S0U; Zuo et al. (13)]. NKG2D homodimer [NKG2D-A (brown) and NKG2D-B (green)] interacts with the α1 and α2 domains of ULBP6 (teal). The disease-associated polymorphic residue Leu106 (red) in ULBP0602 is in close proximity to the ULBP6–NKG2D docking interface, inserting directly into the NKG2D hydrophobic pocket lined by several non-polar residues (ball and stick format). The figure was generated with PyMOL (Molecular Graphics System, Version 2.0 Schrödinger, LLC).

polymorphism within MIC and ULBP family members. The expression of each gene is differentially regulated within tissues and it is likely that temporal and tissue-specific regulation of NKG2DL has served to optimize control of infectious challenges while limiting the development of autoimmune complications (16). Evidence for this effect may be seen in relation to the ULBP0601 and ULBP0602 proteins, where ULBP0601 homozygosity is associated with increased cytotoxic activity in comparison to the ULBP0602 variant (13). Viral infections may have represented a particularly important selective force as viral immunoevasins generally bind only to specific ligands and no single viral immunoevasin has yet been described which can bind to all NKG2DL family members.

Assessment of NKG2DL polymorphism within different species might also provide some clues as to the pace and potential determinants of gene diversification. Meyer et al. (25) sequenced a range of *MIC* genes from non-human primates and demonstrated that these most likely derive from a single common MICB-like ancestor (26). Much of the polymorphism within ULBP genes appears to have arisen very early in the development of *Homo sapiens* and prior to migration out of Africa (15, 27). However, the pattern of diversity varies markedly across the globe and is likely to reflect differential selection to pathogens within different environments (9, 24). Some inferences may be drawn from the evolution of the KIR genes where, in addition to infection, a role in mediating successful pregnancy outcome also appears to be important (28).

## THE CLINICAL IMPORTANCE OF POLYMORPHISM WITHIN NKG2D LIGANDS

Polymorphisms within *NKG2DL* alleles have been identified as risk factors for a range of different clinical disorders. As discussed above, NKG2DL expression is increased by inflammatory stimuli (29) and as such the strong association of *ULBP6* SNP (rs9479482) with Alopecia Areata is compatible with its autoimmune pathogenesis (AA) (30, 31). Indeed, NKG2D$^+$ T cells have been identified as important mediators in the initiation of this disease (32). The first association of MICA polymorphism with autoimmune disease was observed by Mizuki et al. (33) in Behçet's disease and this has been followed by 12 further reports (**Table 1**).

Viral infection is a potent stimulus for NKG2DL expression and an early report identified that MICA polymorphism was associated with increased risk of CMV reactivation in HIV-seropositive patients (43). Strikingly, genome-wide association study analysis of 2,008 patients with pediatric Dengue shock syndrome and 2,018 controls from Vietnam demonstrated that the *MICB* polymorphism rs3134899 was one of only two associated risk alleles with a per-allele odds ratio of 1.34 (44). Moreover, a replication study in Thai patients confirmed these findings (46), which were also apparent in patients with less severe clinical phenotypes of dengue as well as an infant group (45, 46). As such, the association of *MICB* alleles with dengue is one of the most well characterized allelic associations of a non-HLA gene with any infectious disease.

Cancer may be been an important selective force in NKG2DL evolution and expression is increased markedly during cell transformation. Several reports have demonstrated an association between NKG2DL alleles and a range of different cancer subtype and it will be of interest to see if this is replicated in future studies (34–42). Tumor-specific immune responses are also central in disease control and are exemplified in stem cell transplantation (HSCT) (60) where the curative effect relies mainly on the graft versus leukemia effect. NKs contribute to alloreactive responses (61, 62) and NKG2DL polymorphism appears to play a particularly important role in this setting (63). Again, the MICA-129 Met/Val dimorphism is informative with higher relapse rates observed in association with the MICA-129Met/Met homozygous genotype (57) and increased incidence of cGVHD with MICA-129 Val/Val homozygotes (59). This has been interpreted as reflecting stronger NK and CD8$^+$ T cell activation in the presence of the MICA-129Met allele although the longer term effects may be mitigated by subsequent down regulation of NKG2D on effector cells. We also investigated the impact of ULBP in 371 SCT patient–donor pairs and related this to clinical outcome (58), observing a strong association between the ULBP0602 allele and overall survival. This effect might reflect either the direct consequence of cytotoxic activity of NK and T cells against tumor cells or a regulatory role of the NK subset on subsequent development of the alloreactive T cell response (64–67).

## THE FUNCTIONAL IMPACT OF POLYMORPHISM WITHIN NKG2D LIGAND PROTEINS

A major ambition is now to understand the biological importance of physiological variation within the NKG2DL proteins and how this information may be used both to understand established disease associations and to potentially develop novel immunotherapeutic approaches.

In this review, we address this challenge in relation to the influence of polymorphism (**Figure 2**) on (1) expression level of NKG2DL proteins, (2) differential affinity for NKG2D, and (3) modulation of cytotoxic activity.

### The Influence of Polymorphism on the Expression of Surface and Soluble Forms of NKG2DL

Perhaps the most immediate influence of NKG2DL polymorphism is that it may modulate the magnitude of protein expression at the cell surface. Indeed, use of a functional genomics system whereby a single copy of MICA cDNA can be stably integrated into CHO cells revealed differential transcriptional activity that varied by sixfold across four different alleles and correlated with protein expression (68). Two MICA polymorphisms associated with increased risk of HCV-related hepatocellular cancer are both located at the 5′ flanking region of MICA, rs2596542 (39) being 4.7 kb and rs2596538 (40) 2.8 kb upstream of the *MICA* gene. Of interest, the rs2596538 allele is located at a binding site for transcription factor specificity protein 1 and both SNPs modulate the level of soluble MICA protein due to relative transcriptional

**TABLE 1** | The clinical relevance of polymorphism within NKG2D ligands.

| | NKG2DL | Single nucleotide polymorphisms | Diseases | Reference |
|---|---|---|---|---|
| Malignancy association | MICA | 129-Met/Val | Nasopharyngeal carcinoma | Douik et al. (34) |
| | MICA | MICA-5.1 | Oral squamous cell carcinoma | Tamaki et al. (35, 36) |
| | MICA | MICA-5.1 | Breast cancer | Lavado-Valenzuela et al. (37) |
| | MICA | 213 thr and 251 gln | Cervical cancer | Jumnainsong et al. (38) |
| | MICA | rs2596542, rs2596538 | Hepatitis C virus (HCV)-induced hepatocellular carcinoma (HCC) | Kumar et al. (39); Lo et al. (40); Goto et al. (41) |
| | MCIA | rs2596542G/A 129Met/Val; 251Gln/Arg 175Gly/Ser; triplet repeat | Hepatitis B virus-induced hepatocellular carcinoma | Tong et al. (42) |
| Virus infection | MICA | MICA-5.1 | CMV reactivation in HIV-infected patients | Moenkemeyer et al. (43) |
| | MICB | rs3132468 | Dengue shock syndrome and non-severe dengue | Khor et al. (44); Whitehorn et al. (45); Dang et al. (46) |
| Autoimmune diseases | MICA | 129-Met/Val | Chronic Chagas heart disease | Ayo et al. (47) |
| | MICA | 129-Met/Val | Ankylosing spondylitis | Amroun et al. (48) |
| | MICA | rs1051794 | Rheumatoid arthritis | Kirsten et al. (49) |
| | MICA | 129-Met/Val | Inflammatory bowel disease | Lopez-Hernandez et al. (50); Zhao et al. (51) |
| | MICA | 129-Met/Val | Lupus erythematosus | Yoshida et al. (52) |
| | MICA | 129-Met/Val | Type I diabetes | Raache et al. (53) |
| | MICA | 129-Met/Val | Psoriatic disease | Pollock et al. (54) |
| | MICA | Triplet repeat microsatellite | Behçet disease | Mizuki et al. (33) |
| | MICA | MICA-A9 triplet repeat | Psoriatic arthritis | Gonzalez et al. (55) |
| | ULBP6 | Rs1543547 | Diabetic nephropathy | Mcknight et al. (56) |
| | ULBP6 | Rs9479482 | Autoimmune alopecia | Petukhova et al. (30) |
| HSCT | MICA | 129-Met/Val | Chronic GvHD | Boukouaci et al. (57) |
| | ULBP6 | ULBP0601/ULBP0602 | HSCT overall outcome | Antoun et al. (58) |
| | MICA | 129-Met/Val | HSCT clinical outcome | Isernhagen et al. (59) |

activity at the MICA locus (39, 40). The rs1051792 SNP of the MICA gene resulting in the MICA-129Met/Val dimorphism was the first MICA polymorphism for which a functional consequence was described and the MICA-129Met variant is associated with particularly strong NKG2D engagement. The effect of this exchange is more subtle, in that the MICA-129Met variant is associated with increased transcriptional activity but protein retention within intracellular compartments (69) and higher serum MICA levels are seen in patients with hepatitis B who are homozygous for the MICA-129Val allele (42). As such, polymorphism can influence not only the magnitude of protein expression but also the pattern of expression within the cell. Our own studies have focused on polymorphism within ULBP6 (13) where stably integrated CHO cell lines with a single copy of the ULBP0601 and ULBP0602 alleles, and primary patient samples, did not reveal any difference in surface expression or shedding according to genotype.

As such, polymorphisms within NKG2DLs that act to modulate protein expression, subcellular location, and surface shedding are important regulators of differential NKG2DL activity.

## The Importance of NKG2DL Polymorphism on Binding Affinity to NKG2D

Engagement of NKG2D ligands with NKG2D is a crucial step in the activity of many cytotoxic cells. Again, study of the MICA129 Met/Val dimorphism has been crucial in studies of how this may be influenced by allelic variation. Steinle et al. transfected a range of nine different MICA alleles into a reporter cell line and, after determining equivalent levels of cell surface expression, then interrogated their binding to soluble NKG2D. A hierarchy of fluorescent intensity was observed, with 5 alleles demonstrating stronger binding compared to the other 4 (70). Interestingly, the presence of methionine at position 129 (Met129) was the sole determinant of strong binding. In subsequent work, Isernhagen and colleagues expressed two MICA*0701 variants, with either methionine (the wild-type amino acid) or valine at position 129, and examined NKG2D affinity on a range of different cell types. The slope of NKG2D engagement in relation to increasing MICA expression intensity was steeper for the MICA-129Met variant, indicating higher avidity compared to the MICA-129Val variant (59). Based on the crystal structure of MICA*01 (10), this biallelic position does not participate directly in the MICA–NKG2D interface but may indirectly modulate NKG2D binding *via* a conformational change (**Figure 1A**). The methionine side chain protrudes from the β-sheet base and mediates an extensive network of non-polar interactions with Gln136, Ala139, and Met140 from the N-terminal helical stretch of α2, a region that is in close proximity to the MICA–NKG2D interface (11). It is conceivable that this conformation within the MICA-Met129 variant permits optimal contacts with bound NKG2D which is presumably lost in the MICA-Val129 form and this may account for the differing ligand binding affinities associated with the MICA-129Met/Val dimorphism.

We have also examined the binding of NKG2D to recombinant forms of the two major ULBP6 alleles (ULBP0601 and ULBP0602), using surface plasmon resonance (13). Strikingly, ULBP0602 demonstrated a very high affinity for its ligand with fast binding and slow dissociation. Indeed, the measured affinity of 15.5 nM is over 10-fold higher than the equivalent binding

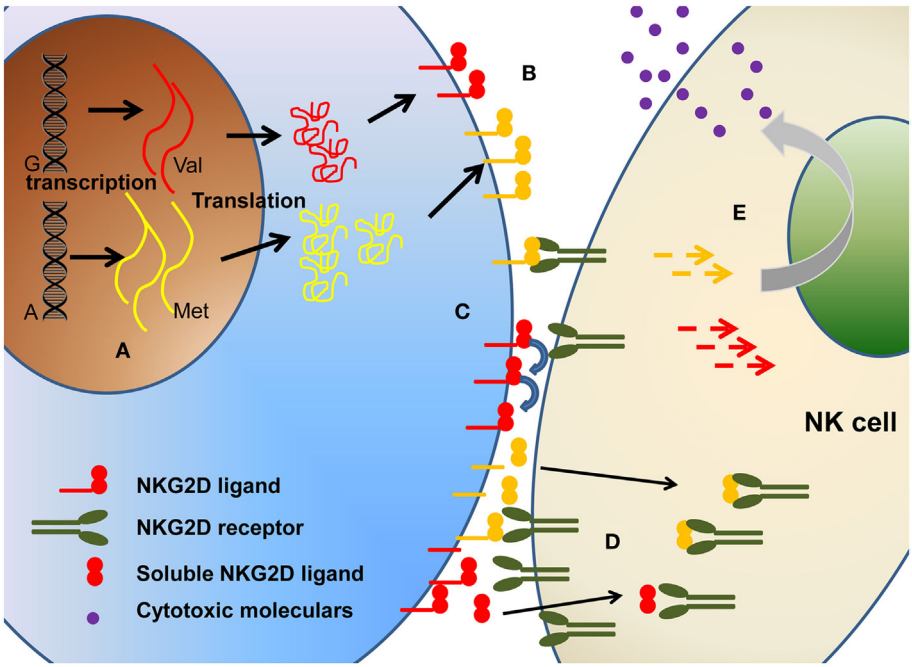


**FIGURE 2** | The polymorphism of NKG2D ligands will affect the biological function of NKG2D ligands in multiple levels, including **(A)** different transcription, **(B)** different expression level on cell surface, **(C)** different binding affinity, **(D)** different soluble ligands shedding. Collectively, these will ultimately affect the **(E)** downstream signaling and cytotoxicity function.

for ULBP0601 and substantially greater than any other ULBP family member. The structure of the NKG2D/ULBP0602 interaction revealed hydrophobic contacts at the ULBP0602/NKG2D interface sufficient to explain this difference as position Leu106 in ULBP0602, located at the NKG2D receptor ligand interface, inserts directly into the center of the hydrophobic patch B of NKG2D, forming numerous non-polar contacts with surrounding residues (Tyr152, Ile182, Met184, and Tyr199) (**Figure 1B**). In contrast, ULBP0601 has a charged and lengthy Arg at this position and its introduction within this predominantly hydrophobic environment is likely to be detrimental for NKG2D binding.

Collectively, these studies reveal that variation in the affinity of the interaction with NKG2D is likely to have acted as an important selective force in the polymorphism within NKG2D ligands.

## Polymorphism Within NKG2DL Have an Important Influence on Cytotoxic Activity of Effector Cells

The most important functional outcome of NKG2DL:NKG2D engagement is the degree of cytotoxic activity from the effector cell. A range of studies (13, 59, 68) have indicated subtle differences in NKG2DL structure can translate into significant variation in the cytotoxic capacity of effector cells. This has demonstrated clearly within the MIC-A family by increased levels of NK cell cytotoxicity and cytokine production following engagement with cells expressing the high-affinity MICA-129Met allele in comparison to those with surface expression of MICA-129Val (59). A comparable profile was observed in relation to co-stimulation of $CD8^+$ T cells where, again, the 129Met allele provided stronger signaling to $NKG2D^+$ $CD8^+$ cells in concert with TCR engagement.

In light of these findings it might be considered that the evolution of high-affinity NKG2DL alleles would result inexorably into positive selection within a population. However, as almost always in biology, there is clearly a balance in the relation to the optimal affinity for NKG2DL:NKG2D binding. In particular, as the level of NKG2DL protein expression increases on the surface of a target cell, as might occur in the setting of viral infection or transformation, this can lead to dramatic down regulation of NKG2D expression on the effector cell in situations where the NKG2DL ligand has a high affinity for NKG2D. As such, alleles such as MICA-129Met might offer a cytotoxic advantage against targets with low levels of NKG2D expression but become counterproductive as they drive the "NKG2D exhaustion" of effector cells following high levels of expression due to cellular stress.

Striking differences are also observed in the cytotoxic capacity of NK cells taken from different donors (68). A comparison of effector cell activity from 22 healthy donors revealed marked interindividual variation in the cytotoxic ability and, surprisingly, this was correlated both positively and negatively to MICA expression level in different donors. The authors argue that individual responses are "tuned" to different "dose bandwidths" of NKG2DL expression, again challenged the simple hypothesis that higher receptor levels promote greater responsiveness.

We also observed that effector cells expressing the high-affinity ULBP6 allele, ULBP0602, elicited weaker killing from a range of effector NK and T cells compared to ULBP0601, and was correlated with less downregulation of NKG2D (13). We suggest

that this may result either from the ultra stable binding nature of the NKG2D/ULBP0602 interaction, which equates to a $t_{1/2}$ of ~550 s at 25°C, acting to limit serial triggering of NKG2D receptors (71, 72). Alternatively, shedding of the high-affinity soluble ULBP0602 can inhibit NKG2D binding to the NKG2D ligands on the cell surface.

## Exploiting the Translational Potential of Polymorphism Within NKG2DL

The reports of polymorphism within NKG2D ligands, particularly MICA and ULBP6, and disease risk indicate that NKG2DL family members may represent an important therapeutic targets for treatment. The interaction of NKG2D and NKG2D ligands has been shown to be particularly important in the control of malignant disease. *In vivo* studies in murine models have shown that expression of NKG2D ligands on tumor cells is an important determinant of tumor control (73) and as such it is no surprise that tumor progression is associated with selection for tumor variants which are able to evade NKG2D-mediated immune recognition. In the setting of inflammatory disease, one potential strategy might be to downregulate NKG2D receptor expression on T cells and NK cells through soluble NKG2D ligands or immune suppressive cytokine (74, 75). In the setting of malignant disease, considerably more information is required on the profile of NKG2DL expression within individual tumor and how this is related both to the profile of somatic mutations within the tumor and the tumor microenvironment. Cytokines such as TGF-β have also been shown to regulate NKG2DL expression and represent a further influence on ligand regulation within the tumor microenvironment (76).

As such, interventions that can modulate the functional outcome of the NKG2D:NKG2D interaction may represent important and novel immunotherapeutic approaches (77). This indicates the requirement for continuing research to understand and exploit the lessons that can be derived from the extensive polymorphic variation within this remarkable ligand interaction.

## AUTHOR CONTRIBUTIONS

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

## FUNDING

This study was supported by Bloodwise Project grant funding to PM (grant code 17009).

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**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 September 2018
doi: 10.3389/fimmu.2018.02040

# NKG2D Ligands–Critical Targets for Cancer Immune Escape and Therapy

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DNA damage, oncogene activation and excessive proliferation, chromatin modulations or oxidative stress are all important hallmarks of cancer. Interestingly, all of these abnormalities also induce a cellular stress response. By upregulating "stress-induced ligands," damaged or transformed cells can be recognized by immune cells and cleared. The human genome encodes eight functional "stress-induced ligands": MICA, MICB, and ULBP1-6. All of them are recognized by a single receptor, NKG2D, which is expressed on natural killer (NK) cells, cytotoxic T cells and other T cell subsets. The NKG2D ligand/NKG2D-axis is well-recognized as an important mediator of anti-tumor activity; however, patient data about the role of NKG2D ligands in immune surveillance and escape appears conflicting. As these ligands are often actively transcribed, tumor cells are urged to manipulate the expression of these ligands on post-transcriptional or post-translational level. Although our knowledge on the regulation of NKG2D ligand expression remains fragmentary, research of the past years revealed multiple cellular mechanisms that are adopted by tumor cells to reduce the expression of "stress-induced ligands" and therefore escape immune recognition. Here, we review the post-transcriptional and post-translational mechanisms by which NKG2D ligands are modulated in cancer cells and their impact on patient prognosis.We discuss controversies and approaches to apply our understanding of the NKG2D ligand/NKG2D-axis for cancer therapy.

**Keywords: NKG2D, NKG2D ligands (NKG2DL), cellular stress response, cancer therapy, immunotherapy, post-transcriptional regulation, post-translational regulation, shedding**

OPEN ACCESS

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

***Received:*** *04 May 2018*
***Accepted:*** *20 August 2018*
***Published:*** *11 September 2018*

***Citation:***
*Schmiedel D and Mandelboim O (2018) NKG2D Ligands–Critical Targets for Cancer Immune Escape and Therapy. Front. Immunol. 9:2040. doi: 10.3389/fimmu.2018.02040*

## THE FAMILY OF STRESS-INDUCED LIGANDS COMPRISES HIGH DIVERSITY ON RNA AND PROTEIN LEVEL

The NKG2D receptor is, in several aspects, an outstanding immune receptor of major interest in research and immunotherapy: First, the NKG2D receptor is expressed on lymphocytes both of the innate immune system, Natural Killer (NK) cells, as well as cytotoxic, CD4 or γδ T cells, which are assigned to the adaptive branch of the immune system (1, 2). Being considered a genuine activating receptor on NK cells, NKG2D acts as a co-stimulatory receptor on T cells (1, 3). Thereby, NKG2D receptor triggering induces not only cytotoxicity (4), but also drives cytokine production (5–7), or impacts T cell differentiation and expansion (8, 9). Second, the NKG2D receptor recognizes eight different ligands in humans, MICA, MICB and ULBP1-6 (10). Collectively, these are termed "stress-induced ligands" since they are differentially expressed after different cellular stresses. This redundancy facilitates the immune surveillance: NKG2D alone can recognize cell suffering from infection, DNA damage, fluctuating oxygen levels, excessive

proliferation with active tumor-promoting signaling, or heat shock. NKG2D and its ligands are therefore key proteins to mount an immune response against unhealthy cells (10). However, it must be noted that expression occurs under certain conditions also on healthy cells, especially by immune cells for immunoregulatory purposes (11, 12). Third, the NKG2D ligand-NKG2D axis is widely recognized as anti-tumorigenic checkpoint. NKG2D-expressing immune cells are believed to reject transforming cells prior to immune-editing, which is a prerequisite for immune escape.

However, patient data of the past years revealed conflicting data and it appears that the importance of the NKG2D receptor in tumor immune surveillance and escape is far more complex as compared to other receptor-ligand interactions we are aware of (13).

Altogether, the wide range of NKG2D-expressing immune cells, the diverse ligand repertoire and its role in cancer therapy renders this receptor as an exceptional candidate for basic and applied cancer research.

## DIFFERENCES AND SIMILARITIES IN THE NKG2D LIGANDS IN RNA AND PROTEIN

The eight NKG2D ligands all belong to the family of MHC class I–like proteins. They share some degree of conservation, yet, they have distinct differences in their promoters, their RNA and protein sequences. Consequently, also their regulation is oftentimes unique and independent from each other.

As a variety of stresses induces differential expression of proteins of this family, it is not surprising that diverse cancer-associated transcription factors were previously shown to affect the expression of NKG2D ligands. Prominent examples of inducers of NKG2D ligand transcription are p53, that binds the promoter regions of ULBP1 and ULBP2 following DNA damage (14), Sp family transcription factors that influence the expression MICA, MICB and ULBP1 in proliferating cells (15, 16), or heat shock transcription factor 1 (HSF1) that binds its respective heat-shock elements in the promoters of MICA and MICB, respectively (17). An elaborate overview on the transcriptional regulation in humans and mice is given elsewhere (18). However, it is very clear that transcription is only the first controlled step in a multilayer of regulations that enables effective and fast induction of protein expression when required but suppresses undesired, excessive protein expression on healthy cells.

Several mechanisms how these ligands are regulated on post-transcriptional level were disclosed in the past few years that will be discussed more thoroughly below. Unsurprisingly, these important immune-modulatory molecules are complexly and mostly independently regulated and research is just on the verge of deciphering underlying regulatory networks. In the first part of this review, we will summarize known cellular, post-transcriptional mechanisms that impact NKG2D ligand expression that may be hijacked by cancer cells to evade from NKG2D-mediated surveillance. An overview on the different mechanisms is provided in **Figure 1**. As the biology of ULBP4 (RAET1E), ULBP5 (RAET1G), and ULBP6 (RAET1L) and their role in tumor immunity is poorly understood, we will focus on the well-studied five ligands MICA, MICB, ULBP1-3. In the second part, we will provide an overview on current approaches to use these ligands for cancer therapy which are summarized in **Figure 2**.

## REGULATORY CIRCUITS CONTROLLING NKG2D LIGAND EXPRESSION

Due to the fact that post-transcriptional regulation mostly occurs within the untranslated regions (UTR) spanning the coding sequence upstream (5'UTR) or downstream (3'UTR) of the coding sequence, we analyzed similarities of the common variants and assessed sequence homologies using MUSCLE (19); however, we want to stress that annotations differ between databases. Also, sequences are still undergoing updates. Below, we refer to sequences found in the NCBI nuccore database (as of July 9th, 2018; NM_000247.2, NM_005931.4, NM_025218.3, NM_025217.3, NM_024518.2).

All 5′UTRs of these mRNA transcripts of all NKG2D are fairly short (below 100 nucleotides). However, the 3' UTRs, which is considered the more important site on RNA regulation, shows striking differences: MICA and MICB share 90% homology, but about 1,000 nucleotides of the 3'UTR of MICB are missing in MICA. Similarly, ULBP1 and ULBP3 have extremely long UTRs of about 2,400 nucleotides, which are overall over 90% homologous. However, both UTRs contain unique regions stretching over 300 nucleotides that are not shared and therefore pose potential sites for differential regulation.

The ULBP2 3' UTR consists of only about 550 nucleotides. It contains many single nucleotide exchanges when compared to ULBP1 and ULBP3, with an overall homology of 70%. Interestingly, only the beginning and the end of the sequence show conservation, whereas the sequences in between are largely missing.

Although single nucleotide exchanges exist, the observed differences suggest that the diversification in the RNA regulation following gene duplication is driven by deleterious events in the UTRs.

### Post-transcriptional Regulators

MicroRNAs (miRNA or miR) were the first molecules described to impair the expression of the NKG2D ligands MICA, MICB and ULBP2 in cancer cells on mRNA level (20–24). In part, these miRNAs were shown to be overexpressed in the tumor itself ("oncomiRs"), like miR-93 that targets both MICA and MICB, but also in metastasis-associated miRNAs ("metastamiRs"), like miR-10b, that targets MICB expression (21). Interestingly, several binding sites for cellular miRNAs are overlapping with viral miRNAs (20), suggesting that the necessity to regulate stress-induced ligands using miRNA surpasses the need to evolve these sites to fight viral infections more efficiently. Next to the classical role of miRNAs to suppress protein translation, it was recently demonstrated that the 3' UTR of the stress-induced ligand MICA can switch from a short to a long version by

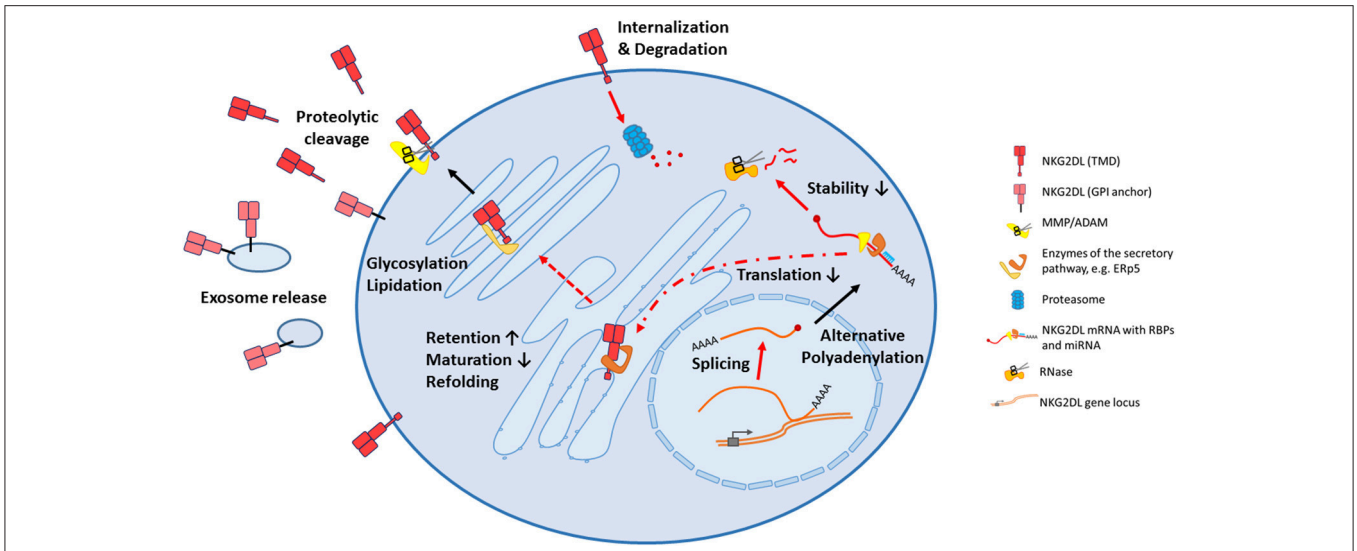


**FIGURE 1 |** All stages of biogenesis of NKG2D ligands can be affected in cancer cells. Following transcription, mRNA processing can be altered affecting splicing and alternative adenylation, therefore different isoforms of a ligand can be produced. After export to the cytoplasm, mRNA translation can be inhibited by miRNAs, and decay is frequently induced by RNA binding proteins. During their trafficking in endoplasmatic reticulum and golgi apparatus, the NKG2D ligands can be refolded, by instance with the help of the thioisomerase ERp5, or differentially modified by glycosylations or lipidations. Potentially, some of these modifications contribute to intracellular retention by an impaired protein maturation. Certainly, these alterations change the biological properties of these ligands once they reach the surface, with the consequence that their release from the cell surface by shedding or release in exosomes is facilitated. Alternatively, the can also be internalized and degraded by the proteasome. NKG2DL, NKG2D ligand; TMD, transmembrane domain; GPI, glycosylphosphatidylinositol; MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease; RBP, RNA binding protein; miRNA, microRNA.

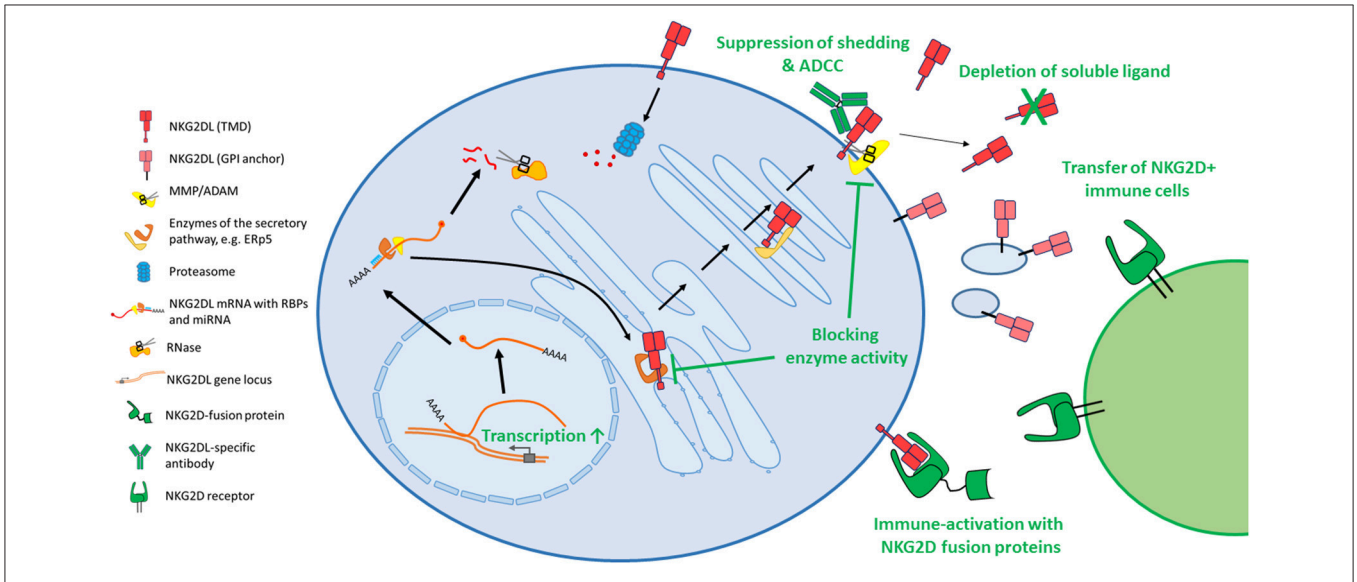


**FIGURE 2 |** Diverse approaches attempt to target the NKG2D axis for cancer therapy**.** As most cytotoxic drugs induce or constitute a cellular stress, several classes of drugs induce expression of the stress-induced NKG2D ligands, amongst DNA damaging agents, proteasome inhibitors or histone deacetylase inhibitors. In order to decrease shedding of these ligands, small molecule inhibitors targeting matrix metalloproteases were developed. Other enzymes, which are involved in protein maturation, also pose potential drug targets. Antibodies can bind the surface MICA and prevent shedding and induce ADCC. Others bind and block soluble ligands and prevent their harmful binding to immune cells. Similarly, also apheresis can reduce the load of shed ligands in serum of cancer patients. To activate the immune system toward NKG2D ligand expressing tumor cells, diverse fusion proteins were created that contain the extracellular domain of NKG2D and are linked to IL-15, anti-CD3 or an Fc portion to induce ADCC, or others. Also, the transfer of NKG2D expressing immune cells, like bone marrow grafts, donor NK cells or genetically modified T cells are approaches to fight NKG2D ligand expressing tumors. NKG2DL, NKG2D ligand; TMD, transmembrane domain; GPI, glycosylphosphatidylinositol; MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease.

means of alternative polyadenylation in dependence of miRNA binding (24).

What is the role of miRNAs in the regulation of NKG2D ligands in healthy conditions? One theory is, that miRNAs are capable to inhibit the translation of NKG2D ligand protein, if only little mRNA is present, for instance due to mild stresses. A minor trigger is therefore insufficient to render a healthy cell as a target for immune cell attack. According to this line of thought, miRNAs work as a "buffering capacity" and a fine-tuner of ligand expression that suppress ligand expression up to a certain threshold of stress (20).

Next to miRNAs, also RNA binding proteins (RBPs) were shown to interact with NKG2D ligand transcripts: MICB expression is affected by at least twelve confirmed RBPs that bind its 3′UTR and impact all post-transcriptional aspects like processing, turnover rate, localization or translation rate (25). Most of which suppress MICB expression and are therefore described as negative regulators. Recently, also a negative regulator binding the short 5′UTR of MICB was described (26). Interestingly, all described RBPs were shown not to affect the close relative MICA (25, 26). Also ULBP1 biogenesis in cancer cells is critically affected by RBPs (27), as is ULBP2 mRNA stability (28). For ULBP1 is was additionally reported that RBPs affect biogenesis of different isoforms alternative splicing (27).

**Figure 1** gives a summary on the post-transcriptional regulations mentioned above. Still, pathways and critical players that affect the fate of the RNA transcripts of NKG2D ligands are only fragmentarily understood. Additionally, in many occasions, we don't know if discovered mechanisms are tumor-specific, or at least enhanced in cancer cells, or if they are simply part of the "healthy" RNA processing pathway for these ligands.

## Post-translational Effects

Next to differences in the UTR regions of the ligands, major differences are also observable in the protein sequence. ULBP family members possess an α1/α2 domain structure, while MIC proteins possess an α1/α2/α3 structure (29). Although being classified as a MHC class I–like protein, both families lack peptide binding ability and association with β2-microglobulin (18, 29, 30). On top of this layer of diversity, over 100 MIC alleles and 16 ULBP allelic variants were discovered. Notably, MICA and MICB do not possess hypervariable regions like classical MHC molecules. Genetic shuffling and point mutations occur over all three domains (31, 32). Another notable distinctive feature is the membrane anchorage. ULBP1, ULBP3, ULBP6 and one prevalent allele of MICA, MICA*008, are attached to the membrane by a GPI (glycosylphosphatidylinositol) anchor (33), whereas ULBP4 and ULBP5, MICB alleles and all other allelic variants of MICA are embedded into the lipid bilayer with a transmembrane domain. Uniquely, ULBP2 can be expressed both as GPI- or transmembrane anchored protein (34). Whereas, the GPI-anchored ligands localize to lipid rafts, members possessing transmembrane domain appear not to do so (35). Altogether, these proteins belong to one of the most plastic families encoded in the human genome (33).

Yet, it is not understood to what extent differences in surface localization or affinities to NKG2D give rise to differential functional outcomes when triggering the receptor. Also, we don't understand yet why the MIC proteins, and MICA in in particular, are so superior regarding their potential to create new allelic variants compared to ULBP family members.

## Tackling the Ligands on the Protein Level

Like outlined above, the regulation of the eight different ligands on RNA level is multilayered and largely non-redundant. Therefore, many cancer cells can't efficiently inhibit the biogenesis of the transcripts or suppress their translation.

However, options to prevent a translated ligand from being surface-expressed exist as well. These findings are summarized in **Figure 1**.

First, there are several reports showing that NKG2D ligands are indeed expressed but retained intracellularly (36, 37). However, until now, we lack understanding which proteins are involved in this retention process that is exploited by cancer but most likely also a cellular mechanism in ligand homeostasis (37). A recent report showed that hypoxic conditions modulate MICA glycosylation and thereby prevent surface expression (38). Accordingly, glycosylation and therefore protein maturation may be one contributor in this process.

Second, surface expressed ULBP1 can be internalized and degraded by the proteasomal pathway (39). Thereby, both the levels of surface expression but also the duration of the stress-response can be controlled.

Third, these ligands can be released from the tumor cell surface, a process termed "shedding." Cells can shed ligands either by proteolytic cleavage or by releasing ligands in exosomes.

# SHEDDING-AN EFFICIENT WAY TO EVADE FROM NKG2D-MEDIATED SURVEILLANCE

Shedding constitutes a very beneficial mean for cancer cells to avoid surface expression of these ligands. Metalloproteases, most prominently ADAM10, ADAM17 (40, 41), and MMP14 (42) are frequently expressed in the tumor microenvironment but also on platelets (43), cleave and thereby remove MICA, MICB, or ULBP proteins from the tumor cell surface (44–48). The process of shedding is influenced by proteins that inhibit metalloprotease activity like TIMP3 (49), or that enable or facilitate the proteolytic cleavage, like the disulfide-isomerase ERp5 (50). Accordingly, high ERp5 and ADAM10 expression were shown to yield a high load of soluble NKG2D ligands in supernatants of primary cancer cell cultures (51). Ligands, which are linked to the membrane via a GPI-anchor, like ULBP1, ULBP3 or the MICA allele *008, are frequently released in exosomes (52, 53). For MICA, palmitoylation was shown to be crucial for co-localization with the exosome-forming protein caveolin-1 and therefore for the incorporation in exosomes (54). Although both soluble and exosomal-released NKG2D ligands bind the NKG2D receptor and mediate receptor internalization, a stronger internalization of the receptor is induced by exosomal-released ligands, probably due to their ability to crosslink the receptor on the surface (55, 56).

Shedding provides several major advantages for the cancer cell in terms of immune evasion. First of all, if one or several of these

ligands are released systemically into the bloodstream of patients, they are not cell surface-exposed and therefore unable to activate NKG2D receptor-bearing cells.

More importantly, the released ligands are still capable of binding the NKG2D receptor on NK or T cells. In consequence, the NKG2D receptor is internalized in both NK and CD8 T cells (57–59). NKG2D receptor internalization is a major downside of the promiscuity of the NKG2D receptor. Whereas, a diverse array of stresses can be recognized by a functional receptor, the shedding of a single ligand is sufficient to render immune cells blind to the entire ligand family. On top, chronic engagement of the NKG2D receptor was shown to downmodulate also the activity of other NK cell receptors (60, 61) which may be in part connected to the degradation of the CD3ζ signaling molecule that also impairs T cell activity (62). Therefore, shedding is a very powerful way to overcome NKG2D-mediated immune surveillance.

## NKG2D Ligands as Prognostic Marker in Cancer

Ultimately, these differences on RNA and protein level determine NKG2D ligand regulation and expression patterns and impact thereby their importance in tumor biology.

Histological analyses of tumor samples puzzled researchers and doctors alike for several years, as the expression of NKG2D ligands was sometimes favorable and sometimes unfavorable for disease prognosis–different studies appeared contradictory (63, 64). However, this diversity was due to the inability to discriminate soluble and membrane expressed ligands in histology. Today we know, that solely membrane-bound ligands on cancer cells are a positive predictor for patient survival (65). However, the levels of soluble stress-induced ligands in the serum cancer patients pose a valuable prognostic factor. First, they anti-correlate with NK and T cell activity; second, they correlate to staging of the disease and have an overall negative impact on patient survival (48, 65). In line with the decrease in immune cell activity due to soluble NKG2D ligands, checkpoint inhibition therapy using PD-1 antibodies in melanoma was found to be most effective in absence of shed ligands (66), supporting the view that NKG2D ligands need to be taken in consideration for therapies that are not "intentionally" involve the NKG2D-axis.

## NKG2D Ligands-A Promising Target for Immunotherapy

As surface-expressed NKG2D ligands promote tumor rejection and give a favorable survival prognosis, these ligands pose a promising therapeutic target for (immuno-) therapy. Diverse attempts to manipulate the expression of these ligands were undertaken in the past few years in order to harness the immune system against cancer. An overview of different strategies is given in **Figure 2**.

As most anti-cancer drugs act by inducing immediate cellular stress (with the ultimate goal to induce cell death), surface expression NKG2D ligands is frequently increased following treatment. Diverse compounds were identified that substantially increase stress-ligand expression and thereby render tumor cells more susceptible to immune cell attack. One prominent example is the histone deacetylase (HDAC) inhibitor valproic acid (67–69) which was shown to upregulate ligands *in vitro* and *in vivo* (68). But also other drugs like hydroxyurea (70), bortezomib (71), all-trans-retinoid acid (68) or sodium butyrate (72, 73) appear to enhance stress-ligand expression. Therefore, patients can benefit more of some regimens if the drugs not solely damage cancer cells but also lead to the loss of immune tolerance toward the tumor (73, 74). However, whereas valproic acid appears to increase only the level of membrane-bound but not of soluble ligand in cell cultures (69, 75), other HDAC inhibitors apparently induce metalloprotease expression and might therefore also increase shedding (76). Also, an impairment of NKG2D receptor expression in NK cells upon HDAC treatment was reported (77, 78). Future research in this field should address this issue and assess effects on NKG2D ligands more systematically and *in vivo*, as frequently used *in vitro* models don't reflect the complexity in the interplay of tumor cells, tumor microenvironment and NKG2D expressing immune cells which are also impacted by the treatment. It is important to choose proper models that may actually predict if NKG2D mediated immune surveillance can be restored, and if patients may actually benefit of these approaches.

Whereas induction of ligands might be beneficial to activate the immune system, many late-stage cancers release ligands in soluble form and are therefore inappropriate candidates for these kind of anti-tumor strategies. The use of different inhibitors of shedding proved the concept that soluble NKG2D ligands can be effectively reduced, and that their immune-disarming properties can be reversed. Examples are inhibition of the thioreductase ERp5 (50), or prevention of proteolytic cleavage by sheddases like MMP9 (79) or ADAM10 (80). Attempts of the past years yielded selective small molecule inhibitors for MMPs like ADAM10. These are considered for cancer-therapy also for other immune-modulatory purposes besides the manipulation of NKG2D ligands which are summarized elsewhere (81). In contrast, for other potential targets, like ERp5, solely unspecific inhibitors exist at present which therefore pose no therapeutic option in the near future.

But not every new target requires a new drug development: some clinically applied drugs appear to reduce shedding of ligands as a pleasant "side effect," as shown for hypomethylating agents (49) or tyrosine kinase inhibitors (82).

However, we should bear in mind that sheddase activity is also important for the mounting immune response, for instance, the release of TNFα and fractalkine is mediated by ADAM17 (83, 84).

Recently, a new antibody was developed to prevent the shedding of both MICA and MICB in order to restore NKG2D receptor activity and induce better killing of tumor cells by inducing antibody dependent cellular cytotoxicity (ADCC) (85).

However, while inhibiting shedding will be helpful in many cancer patients, exosome release of ligands is another issue that will need to be addressed. Another promising attempt uses adsorption apheresis or antibodies to reduce soluble MICA levels in the serum to restore functionality of NKG2D-bearing immune cells. In consequence, NK cell activity was successfully restored

following depletion of soluble NKG2D ligands in plasma of cancer patients (86, 87).

A third, commonly exerted approach to target NKG2D ligand expressing tumor cells is the development of fusion proteins that show promising anti-tumor effects in mouse models. By using the NKG2D extracellular domain (ECD) fused to an immune-activating component, diverse immune effector mechanisms can be targeted against NKG2D ligand expressing cells. By instance, a NKG2D receptor domain fused to the constant domain of an antibody mediates ADCC (88, 89) via engagement of Fc receptors on immune cells. The fusion of the ECD to a single chain targeting CD3 directs T cell immune responses against the tumor cells (90, 91). The fusion with cytokines like IL-21 or IL-15 can activate T and NK cell immunity in the tumor proximity and help tumor clearance (92–94).

Last but not least, hematopoietic stem cell transplantation (HSCT) and infusions of immune cells like NK cells or genetically modified T cells, were also studied with particular emphasis to the function of the NKG2D receptor. HSCT is frequently exerted to treat hematopoietic malignancies (95, 96). Due to conditioning regimens leading to cellular stress, transplanted NKG2D expressing cells are critically involved in graft vs. leukemia but also graft vs. host disease (97). Infusions of activated NK cells to treat cancer are generally considered safe (98), and can scavenge soluble MICA in the serum of cancer patients, thereby restoring NKG2D-mediated immune surveillance (99). Also, for several years, the anti-tumor efficacy of T cells with possessing a transduced NKG2D chimeric antigen receptor (CAR) with different signaling domains were assessed (100). However, whereas the efficacy of these engineered cells appears to be striking in mouse models (101–103), unwanted activation and fratricide (104) of CAR T cells combined with excess cytokine release (105, 106) pose severe problems that likely prohibit studies in patients. The sensitivity of NKG2D CAR T cells is apparently too high so also healthy cells pose targets and lead to excess CAR T cell activation.

## CONCLUSIONS

The past few years revealed a lot of new insights into the regulation of post-transcriptional and post-translational level of stress-induced ligands harboring an unforeseen complexity of regulation. We also gathered a wider understanding on how NKG2D ligands control immune responses by affecting immune cell activity, in health and disease, and opened paths how to use the NKG2D ligand axis for cancer therapy.

Yet, our understanding remains too fragmentary. Several regulatory mechanisms and factors can be attributed to determine the fate of a single ligand, but its importance for other members of the family is unknown. Certainly, our knowledge of factors affecting ligand biogenesis only scratches the surface of different layers of regulation. For instance, it appears that NKG2D ligands can be retained intracellularly, but we lack understanding how this takes place. Altogether, we miss a holistic picture, a systematic landscape of regulation, that determines which pathways (instead of single proteins or RNAs) regulating NKG2D ligands deteriorate in auto-inflammation or cancer. Such a landscape would also provide new targets, disclose new therapeutic options to harness the NKG2D axis in cancer therapy. On top, we still don't understand well, if and how different NKG2D ligands modulate immune responses differently. Can we utilize NKG2D ligands not only to kill tumor cells directly but also to orchestrate the immune response by impacting the crosstalk of immune cells? A very recent report disclosed that the NKG2D receptor triggered by ULBP2 exhibits a different nanoscale organization on the surface compared to an engagement with MICA, also leading to different functional outcomes (107), thereby giving insights to previously reported differential effects on NKG2D receptor endocytosis by binding to different ligands (108). Future studies will need to address these differences to understand the role of eight different ligands in immune homeostasis under healthy conditions as well as in cancer and autoimmunity. However, we should also bear in mind that the evolution of this diversity might be triggered by pathogens, and viruses in particular, that modulate stress-induced ligands as a mean of immune evasion (109).

Nonetheless, what we know now gives hope that the NKG2D axis might be a game changer—at least for some cancer patients. Arising methods to inhibit or deplete of soluble ligands may become more effective and easily applicable, neutralizing a strong immune inhibitor. Patients, that currently fail to mount an immune reaction when receiving checkpoint inhibitors, may regain responsiveness. Combinatorial approaches with NKG2D ligands are a very promising target to overcome the immune-suppressive tumor environment and re-activate the immune system for an anti-tumor reaction.

The next few years will show, how far we can reach out utilizing the NKG2D receptor in therapy, but we should not lose focus to advance also most basic knowledge on the NKG2D ligand and receptor axis, as every new evidence will help us to personalize (NKG2D mediated) therapies.

## AUTHOR CONTRIBUTIONS

DS outlined, wrote, referenced the manuscript, and prepared the figures. OM supervised and carefully edited the work.

## FUNDING

This work was supported by the German-Israeli Foundation for Scientific Research and Development (GIF) and the DKFZ-MOST program.

## ACKNOWLEDGMENTS

We apologize to all colleagues whose work was implemented into this review due to space limitations.

In line with publishing policies of Frontiers and with the policy of the Hebrew University of Jerusalem, content which first appeared in the dissertation of DS was included in this review [Disarming cellular alarm systems—How tumors and human herpesvirus 6 alike escape immune recognition by manipulating NKG2D ligands. [Dissertation] Hebrew University of Jerusalem, Israel].

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**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: 27 March 2019
doi: 10.3389/fimmu.2019.00569

# Histone Deacetylase Inhibitor Modulates NKG2D Receptor Expression and Memory Phenotype of Human Gamma/Delta T Cells Upon Interaction With Tumor Cells

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**Specialty section:**
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

**Received:** 02 December 2018
**Accepted:** 04 March 2019
**Published:** 27 March 2019

**Citation:**
Bhat J, Dubin S, Dananberg A, Quabius ES, Fritsch J, Dowds CM, Saxena A, Chitadze G, Lettau M and Kabelitz D (2019) Histone Deacetylase Inhibitor Modulates NKG2D Receptor Expression and Memory Phenotype of Human Gamma/Delta T Cells Upon Interaction With Tumor Cells. Front. Immunol. 10:569. doi: 10.3389/fimmu.2019.00569

The functional plasticity and anti-tumor potential of human γδ T cells have been widely studied. However, the epigenetic regulation of γδ T-cell/tumor cell interactions has been poorly investigated. In the present study, we show that treatment with the histone deacetylase inhibitor Valproic acid (VPA) significantly enhanced the expression and/or release of the NKG2D ligands MICA, MICB and ULBP-2, but not ULBP-1 in the pancreatic carcinoma cell line Panc89 and the prostate carcinoma cell line PC-3. Under *in vitro* tumor co-culture conditions, the expression of full length and the truncated form of the NKG2D receptor in γδ T cells was significantly downregulated. Furthermore, using a newly established flow cytometry-based method to analyze histone acetylation (H3K9ac) in γδ T cells, we showed constitutive H3K9ac$^{low}$ and inducible H3K9ac$^{high}$ expression in Vδ2 T cells. The detailed analysis of H3K9ac$^{low}$ Vδ2 T cells revealed a significant reversion of $T_{EMRA}$ to $T_{EM}$ phenotype during *in vitro* co-culture with pancreatic ductal adenocarcinoma cells. Our study uncovers novel mechanisms of how epigenetic modifiers modulate γδ T-cell differentiation during interaction with tumor cells. This information is important when considering combination therapy of VPA with the γδ T-cell-based immunotherapy for the treatment of certain types of cancer.

**Keywords: gamma/delta T cells, HDAC inhibitor(s), histone acetylation, NKG2D, memory T cells, tumor microenvironment, valproic acid**

## INTRODUCTION

Immune cells have the capacity to recognize self-antigens which are upregulated in response to viral infection, DNA damage, or cellular transformation. NKG2D (natural-killer group 2, member D) is one of the receptors recognizing such upregulated self-proteins. NKG2D is a C-type lectin-like type II transmembrane glycoprotein, expressed on almost all human NK cells and γδ T cells, substantial proportions of NKT cells and, CD8 T cells, and a small subset of CD4 T cells (1). Human NKG2D is an activating receptor that recognizes two families of ligands. The first family of NKG2D ligands includes the highly polymorphic MHC class I chain-related A (MICA) and B (MICB)

proteins, while another family comprises the UL16-binding proteins (ULBP1-6). The NKG2D receptor/ligand interaction plays an important role in regulating the γδ T-cell mediated cytotoxicity against a broad range of tumor cells (2, 3) and NK cell activity (4). In order to escape immune cell attack, tumor cells can release NKG2D ligands from the cell surface by proteolytic cleavage (shedding) (5). Shedding of NKG2D ligands varies among tumor entities and can involve different enzymes like a-disintegrin-and-metalloprotease (ADAM) proteases and matrix metalloproteases (MMP) (6, 7). However, it is still under extensive investigation whether shedding of NKG2D ligands is a pro-tumorigenic or anti-tumor immune response (8, 9).

Many biological processes are regulated by epigenetic mechanisms. Dysregulation of such fundamental processes may lead to the development of cancer. Targeting epigenetic mechanisms including DNA methylation and histone modifications by various inhibitors or small molecules holds the potential for novel therapeutic approaches in oncology (10). Proteins involved in epigenetic regulation are divided into three distinct groups based on broad functions: "writers," "erasers," and "readers." The most widely studied enzymes, DNA methyltransferases (DNMT) and histone acetyltransferases (HAT), are epigenetic writers, which set up epigenetic marks on DNA or associated histones. On the other hand, histone deacetylases (HDAC) are epigenetic erasers, which remove such marks (11). Pharmacological inhibitors specific for respective epigenetic enzymes have been identified as potential candidates for clinical application. The DNMT inhibitors Decitabine and Epigallocatechin-3-gallate (EGCG), the HAT inhibitor Curcumin and the HDAC inhibitors Valproic acid (VPA), Trichostatin A (TSA) and 4-Phenylbutric acid (4-PBA) are already in the clinic or in clinical trials for the treatment of various diseases (12–16).

In the present study, we show that an HDAC inhibitor significantly increases the expression and release of NKG2D ligands from pancreatic and prostate carcinoma cell lines. Specifically, the HDAC inhibitor VPA modulates NKG2D receptor gene and protein expression in γδ T cells upon co-culture with tumor cells. We established a flow cytometry-based method to analyze H3K9 acetylation in γδ T cells, which revealed substantial alterations in the subset distribution of memory γδ T cells as an effect of VPA treatment in co-cultures with tumor cells. Thus, we demonstrate that the functional plasticity of γδ T cells depending on H3K9 acetylation status is affected by *in vitro* tumor microenvironment and is additionally modulated by clinically approved epigenetic modifiers. These findings will help to optimize the clinical applicability of γδ T cells depending on the *in vitro* activity against distinct tumors.

## RESULTS

### HDAC Inhibitors Differentially Modulate NKG2D Ligand Surface Expression and Release From Pancreatic Carcinoma and Prostate Carcinoma Cells

Previous findings from our group have shown that the pancreatic carcinoma cell line Panc89 is heterozygous for MICA*009:01 (A6) and MICA*027 (A5), and the prostate carcinoma cell line PC-3 is heterozygous for MICA*008:01:01 (A5.1) and MICA*012:01:01 (A4). Based on these differences of MICA alleles, Panc89 cells shed MICA/B by proteolytic cleavage, whereas PC-3 cells release MICA via exosomes (6). To address the potential role of epigenetic regulation in the mechanism of NKG2D ligand shedding, we used six different epigenetic inhibitors (Decitabine, EGCG, Curcumin, VPA, TSA, and 4-PBA) specific for different important epigenetic processes. The experimental strategy to investigate the effect of epigenetic inhibitors on Panc89 and PC-3 cells is schematically illustrated in **Supplementary Figure 1**. All epigenetic modifiers were titrated for their cell type dependent effective dose concentrations (data not shown) (17, 18).

After 24 h of treatment, VPA concentrations of 5 and 2.5 mM significantly increased ULBP-2/5/6 cell surface expression on Panc89 cells (**Figures 1A–C**). PC-3 cells also showed a strong and highly significant increase in the expression of MICB and ULBP-2/5/6, however the increase in MICA expression was only moderate but still significant after 5 mM and 2.5 mM VPA treatment (**Figures 2A–C**). Representative histograms of NKG2DL cell surface expression on Panc89 and PC-3 are shown in **Supplementary Figure 2**. Analysis of cell culture supernatants by ELISA also showed a remarkable increase in the release of soluble NKG2D ligands from both cell lines after treatment with 5 and 2.5 mM VPA (**Figures 1D–F**, **2D–F**). In contrast, there was no increase in ULBP-1 cell surface expression and release from Panc89 and PC-3 cell lines upon treatment with epigenetic inhibitors (data not shown). Treatment with the HDAC inhibitor TSA also induced an increase in the release of MICA, MICB and ULBP-2 from Panc89 cell culture supernatants, but not in surface expression, and no effect was observed in PC-3 cells. Of note, the epigenetic modifiers did not induce notable cell death in the tumor cell lines at the concentration used (data not shown), in contrast to the effect observed on γδ T cells (17). Additionally, in a similar experimental set-up, a slight or no induction of surface NK2DL protein and/or release of NKG2DL from γδ T cells were observed (**Supplementary Figure 3**) reiterating the previously reported role of post-transcriptional regulation (19, 20).

Thus, out of six different epigenetic modifiers tested, only HDAC inhibitor VPA increased cell surface expression and/or release of MICA, MICB and ULBP-2 from the pancreatic carcinoma and prostate carcinoma cell lines irrespective of their allelic MICA variation.

### VPA Affects NKG2D Receptor Expression on the Cell Surface of Activated γδ T Cells Under *in vitro* Tumor Co-culture Conditions

The previous experiments showed that VPA induces a significant increase in MICA/B and ULBP-2 surface expression and release from tumor cells of different origin. Using a co-culture experiment setting (see **Supplementary Figure 1**), we tested the effect of VPA-stimulated NKG2D ligand release on effector cells, i.e., freshly isolated PBMC or short-term γδ T-cell lines established from zoledronic acid-stimulated PBMC. The nitrogen-containing bisphosphonate zoledronic

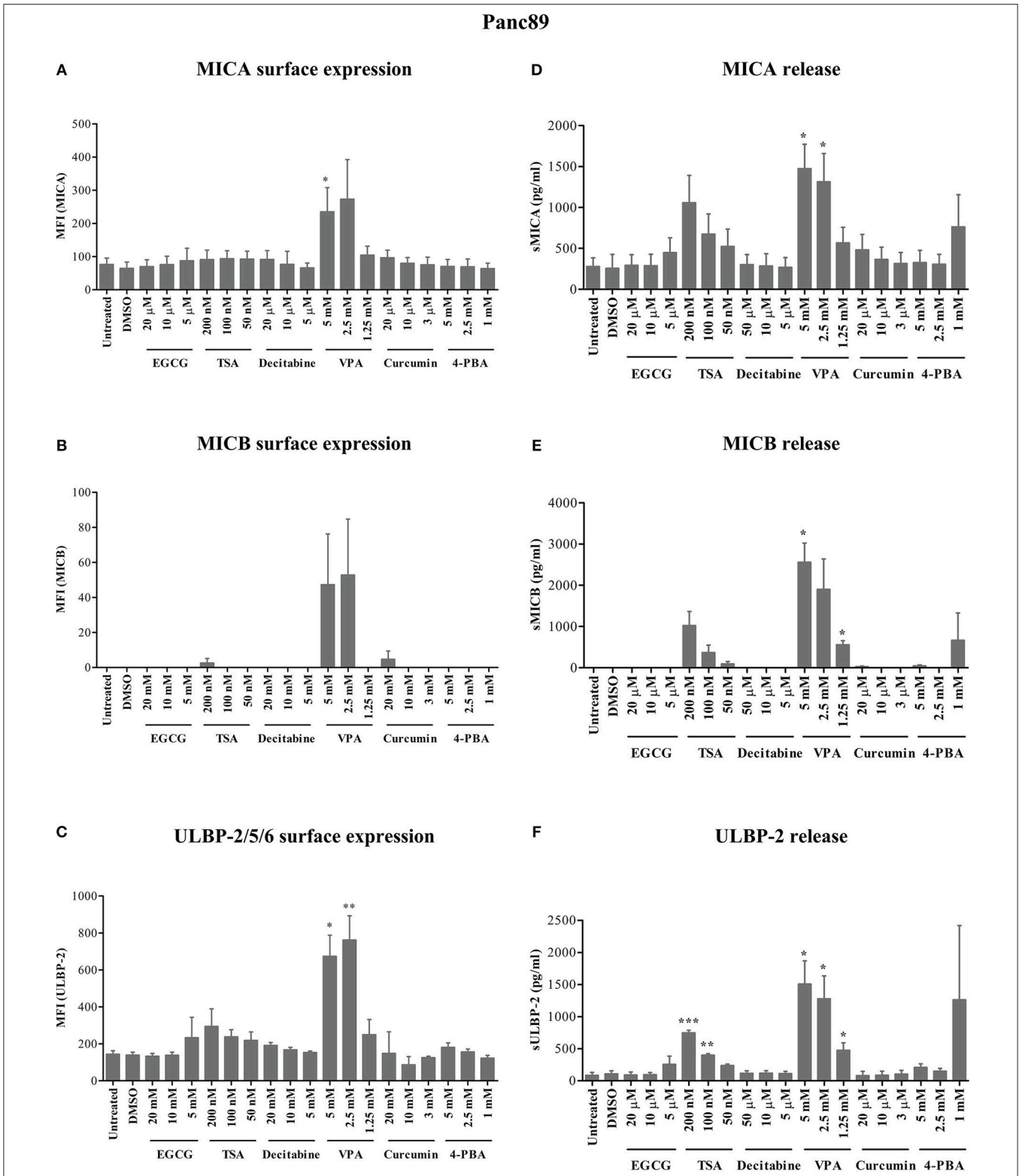


**FIGURE 1 |** Modulation of NKG2D ligand expression and release from a pancreatic cancer cell line by epigenetic modifiers. As schematically shown in **Supplementary Figure 1**, $0.8 \times 10^6$ Panc89 cells were treated with varying concentrations of inhibitors for HDACs, HATs and DNMTs. **(A–C)** After 24 h, cells were harvested for the analysis of MICA, MICB and ULBP-2/5/6 surface protein expression, respectively. **(D–F)** Culture supernatants from the same experiments were analyzed for the release of MICA, MICB, and ULBP-2 using respective ELISA. Data represents mean values ± S.E. of three independent experiments. Statistical significances with $p$-value ≤ 0.05, 0.01, and 0.005 in comparison to untreated or DMSO treated cells are indicated by *, **, or ***, respectively.

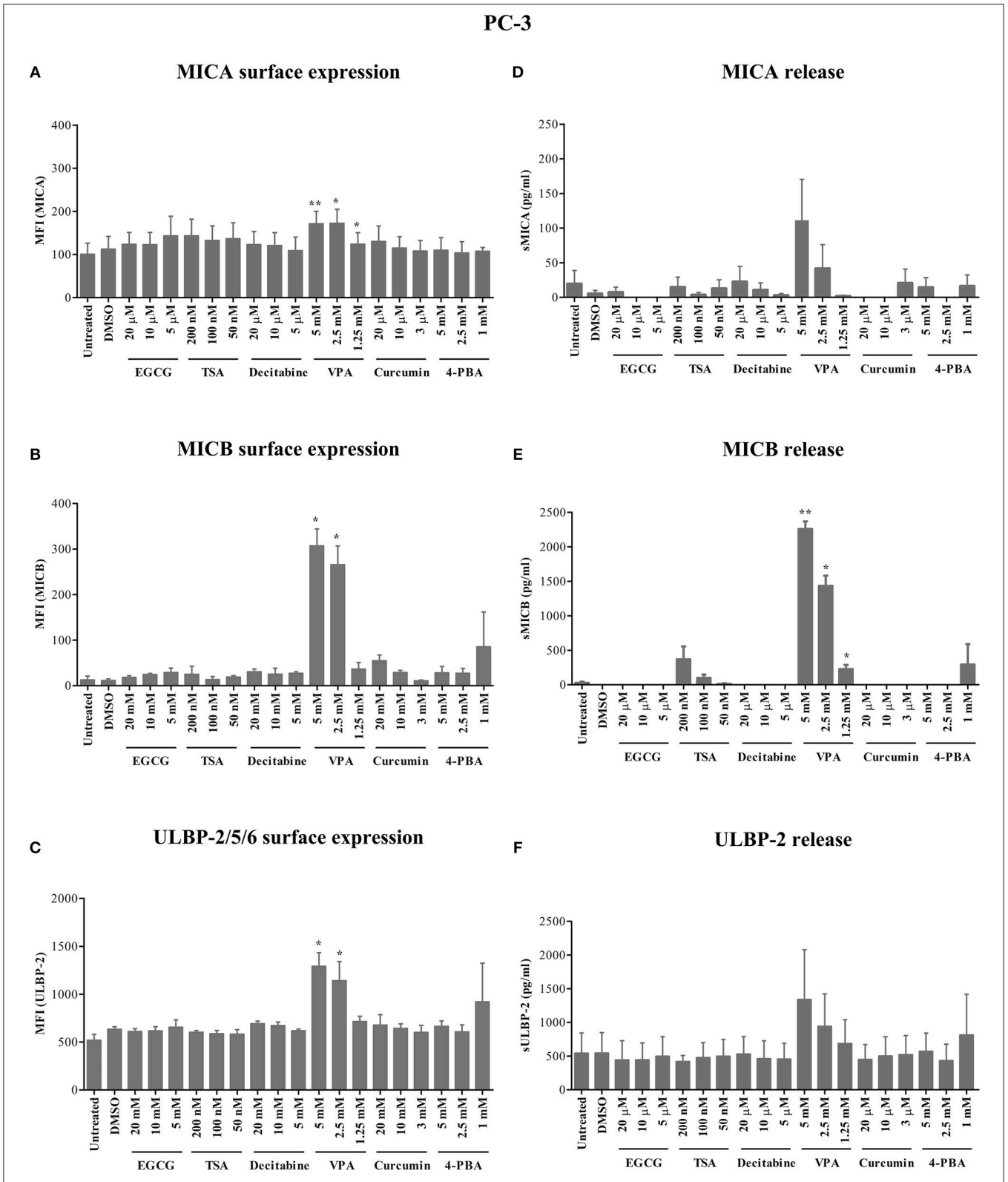


**FIGURE 2 |** Modulation of NKG2D ligand expression and release from a prostate cancer cell line by epigenetic inhibitors**. (A–C)** In a similar approach as shown for Panc89 cells in **Figure 1**, PC-3 cells were analyzed for MICA, MICB and ULBP-2/5/6 surface expression. **(D–F)** Culture supernatants were quantitated for respective soluble NKG2D ligand proteins using ELISA. Data are summarized as mean ± S.E. from three independent experiments and *p*-values for $<0.05$ and 0.01 in comparison to untreated or DMSO treated cells are shown as *, and **, respectively.

acid induces selective expansion of Vδ2 T cells due to the endogenous production of the γδ T-cell stimulating isopentenyl pyrophosphate (IPP) in the eukaryotic mevalonate pathway (21). As expected, γδ T cells down-modulated NKG2D receptor expression upon co-culture for 24 h with Panc89 and PC-3 cells (**Figure 3A**, upper panel). This effect was enhanced by VPA treatment of tumor cells for 24 h before co-culture (**Figure 3A**, lower panel). Corroborating our previous report (17), VPA treatment of γδ T cells significantly decreased NKG2D receptor expression. Interestingly, this effect was not observed in co-cultures with freshly isolated PBMC (**Figure 3C**, lower panel). A summary of three to four experiments is presented in **Figure 3B** (co-culture with γδ T cells) and **Figure 3D** (co-culture with PBMC). The selective downregulation of the NKG2D receptor on γδ T cells is of importance, specifically in the tumor co-culture, in addition to the dose-dependent effect of VPA treatment (17). Taken together, these results may support the previous notion that ligand binding induces down-modulation of NKG2D cell surface expression, which is further regulated by the HDAC inhibitor VPA during *in vitro* co-culture with tumor cells.

Cytotoxic effector activity of human γδ T cells against a broad range of tumor cells can be triggered via the T-cell receptor (TCR) or via the NKG2D receptor, and is known to be modulated by cell interaction molecules like lymphocyte function-associated antigen-1 (LFA-1) (2, 22). We analyzed degranulation (indicative of effector cell cytotoxicity) using the CD107a analysis within the same experimental set-up to study if decrease in NKG2D receptor expression on γδ T cells and/or increase in NKG2D ligand release by tumors affect the degranulation. The γδ T cells showed remarkable degranulation upon co-culture with Panc89 but not with PC-3 (**Figures 3E,F**). In line with our expectation, the CD107a expression was significantly enhanced after zoledronate treatment, but little additive/synergistic effect was observed upon VPA treatment. Given the obvious differences between co-cultures with Panc89 and PC-3 tumor cells (**Figures 3E,F**), it appears that the VPA-induced increase in NKG2D ligand expression and/or release from tumor cells and the decrease in NKG2D receptor expression on γδ T cells during co-culture does not directly reflect the level of cytotoxic activity of γδ T cells against tumor cells as revealed by the CD107a degranulation assay.

## VPA Regulates mRNA Expression of NKG2D Receptor and Its Ligands

Using overexpression systems and biochemical approaches, Karimi et al. recently showed that a truncated NKG2D isoform (referred hereafter as Tr_NKG2D) competitively interferes with the full-length form (referred hereafter as FL_NKG2D) resulting in decreased NKG2D cell surface expression (23). Since we observed a differential modulation of NKG2D receptor expression on the surface of γδ T cells and PBMC in the presence of VPA (**Figure 3**), we next determined the gene expression level of Tr_NKG2D in the co-culture setting. In contrast to protein expression, VPA at 2.5 mM had no impact on FL_NKG2D gene expression levels in γδ T cells (**Figure 4A**, upper panel). In PBMC both, FL_NKG2D and Tr_NKG2D transcript levels were increased after treatment with 2.5 mM VPA (**Supplementary Figure 4**). In γδ T cells or PBMC co-cultured with Panc89 and carefully removed from adherent tumor cells, FL_NKG2D and Tr_NKG2D transcripts were remarkably downregulated after treatment with 2.5 mM VPA. In PBMC co-cultured with PC-3, it was even further decreased (**Supplementary Figure 4**, upper panel) compared to VPA treatment, but unexpectedly this was not the case with γδ T cells (**Figure 4A**, upper panel). Thus, the gene expression level of NKG2D isoforms is not consistent in short-term expanded γδ T cell-lines and freshly isolated PBMC from such co-cultures with tumor cells.

Because our previous experiments showed a substantial release of the NKG2D ligands MICA, MICB, and ULBP-2, we also quantified the expression of these genes in the same co-culture set-up (**Figure 4A**, lower panel). In line with the increased protein levels, the gene expression of NKG2D ligands in both Panc89 and PC-3 was also increased upon treatment with 2.5 mM VPA. Of note, the level of expression was low in PBMC (**Supplementary Figure 4**) or γδ T cells co-cultured with untreated tumor cells, but was remarkably increased upon VPA treatment (**Figure 4A**). We applied a traditional *in silico* method, commonly used for graphical representation of microarray or sequencing experiments, to understand gene function and regulation. As a result of such clustering techniques, genes with similar expression patterns cluster together with similar cellular functions (24–29). Hence, we performed a statistical unsupervised hierarchical cluster analysis of NKG2D ligand and NKG2D receptor isoform genes under all experimental conditions (**Figure 4B**). Based on Euclidian distance method and average linkage rule, we found two distinct clusters, separating the full-length NKG2D receptor and ligands, representing functionally related genes. To our surprise, the truncated form of NKG2D receptor clustered together with ligands for NKG2D. As mentioned before, NKG2D ligand shedding and the truncated form of the NKG2D receptor are known to affect the expression and function of NKG2D receptor on effector cells. Thus, our results demonstrate a distinct association of NKG2D receptor transcript variants (truncated vs. full length), NKG2D ligand expression, and regulation upon HDAC inhibitor treatment.

## Flow Cytometric Analysis of H3K9 Acetylation in γδ T Cells

In addition to its effect on cell surface protein and intracellular cytokine expression (e.g., IL-4), VPA effectively targets class I HDAC proteins and induces H3K9 acetylation in human γδ T cells as previously shown by western blot analysis (17, 18). Considering the limitation in cell numbers and addressing the γδ T cell-tumor interaction by the use of co-culture setting in the present study, we addressed the combined effect of NKG2D ligand release during tumor cell co-culture and VPA-induced H3K9ac at the single cell level in γδ T cells using flow cytometry.

To this end, we adapted a previously reported method to investigate changes in protein expression associated with H3K9ac in γδ T cells based on the staining with a monoclonal anti-H3K9ac antibody (30, 31). The highest

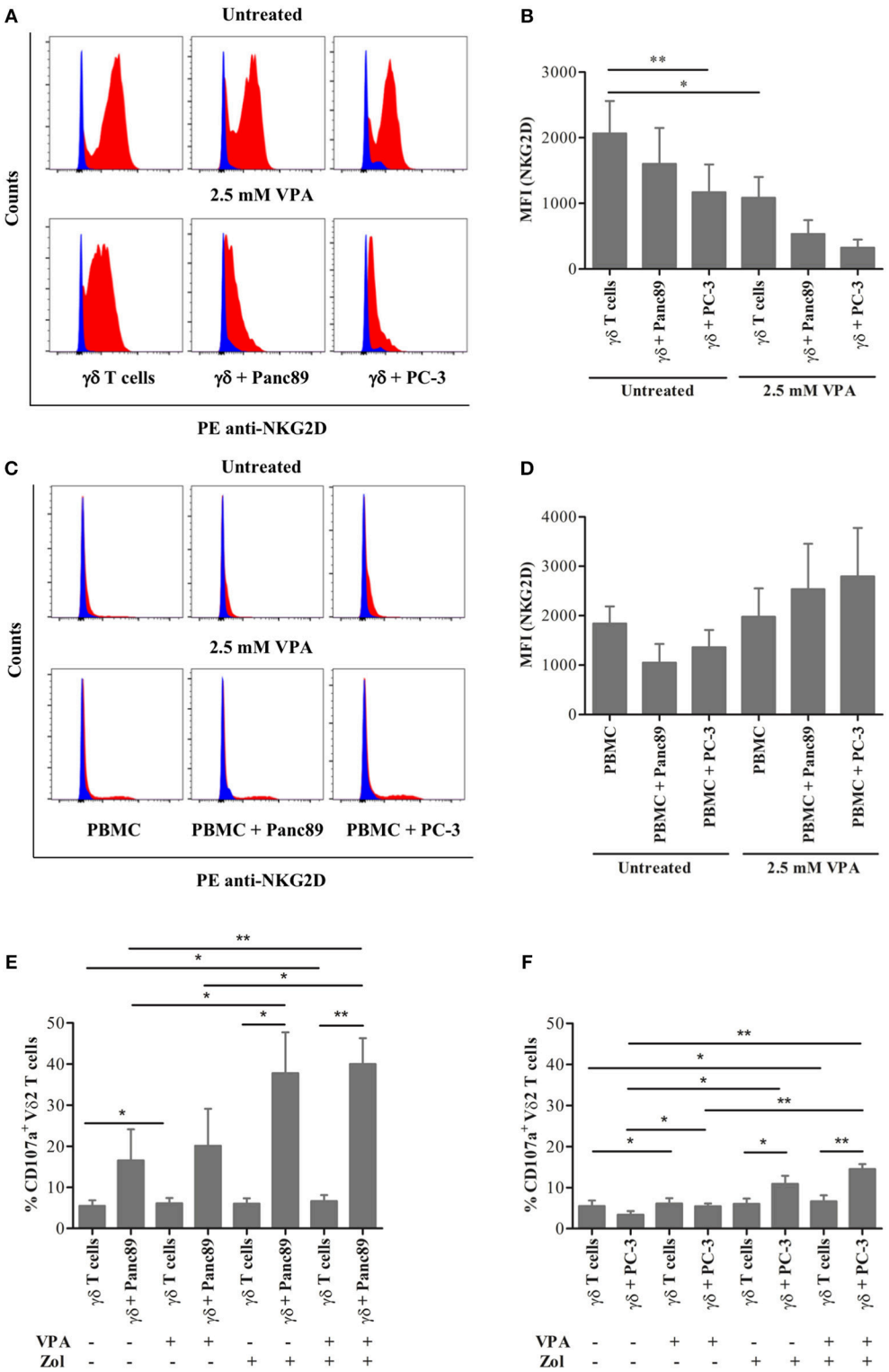


**FIGURE 3 |** Effect of the HDAC inhibitor VPA on NKG2D receptor expression on effector cells. γδ T cells from 12 days zoledronic acid-stimulated PBMC **(A–B)** or freshly-isolated PBMC **(C–D)** were co-cultured with Panc89 and PC-3 cells at a 1:1 ratio in the presence or absence of 2.5 mM VPA, as described in **Supplementary Figure 1**. After 24 h of co-culture, γδ T cells **(A–B)** or PBMC **(C–D)** were collected by gentle pipetting and analyzed for NKG2D receptor expression by flow cytometry. In the representative histograms **(A,C)**, the upper panel represents the untreated effector cells or co-culture with Panc89 or PC-3 cells, and the lower panel represents additional 2.5 mM VPA treatment. Blue histograms represent isotype controls, while red histograms represent anti-human PE-NKG2D staining.

*(Continued)*

**FIGURE 3 |** A summary of three to four independent experiments as bar plots is represented by Median Fluorescence Intensity (MFI) in **(B,D)**. MFI was calculated by subtracting MFI of isotype controls from MFI of test antibodies. Mean values ± S.E. of median fluorescence intensity (MFI) in the bar plot are shown in comparison to untreated solo γδ T cells with statistically significant *p*-value < 0.01 or 0.05 as ** or *, respectively. In a similar set-up, γδ T cells were analyzed for the expression of CD107a (degranulation marker) after 24 h co-culture with **(E)** Panc89 and **(F)** PC-3 in the presence or absence of VPA and Zol (Zoledronate). The data represented here is the mean values ± S.E. of $CD107a^+$ Vδ2 T cells from three independent experiments. The statistical significance is shown by ** or * for *p*-value < 0.01 or 0.05, respectively. Vδ2 T cells or lymphocyte-gated PBMC were acquired after respective staining. The results were further analyzed using FlowJo software.

concentration (0.1 μg/ml) of Pacific Blue-labeled H3K9ac antibody gave a higher background in untreated γδ T cells, which decreased substantially with decreasing antibody concentrations (**Figure 5A**, left; **Supplementary Figure 5A**). As shown by western blot detecting a single distinct band at 17 Kda, VPA-induced H3K9ac in a dose-dependent manner in γδ T cells (**Figure 5B**). For flow cytometry, H3K9ac antibody at 0.01 μg/ml concentration was chosen further based on the ratio of median fluorescence intensity (MFI) between untreated and 5 mM VPA-treated γδ T cells (**Figure 5A**, right). Our results with VPA-dose-dependent decrease in H3K9ac protein determined by flow cytometry (**Figure 5C**; **Supplementary Figure 5B**) further substantiated the use of 0.01 μg/ml H3K9ac antibody since such VPA-dose-dependent decrease was not observed with 0.005 μg/ml (data not shown). Overall, H3K9ac analysis by flow cytometry strongly correlated ($R^2 = 0.72$, $r = 0.85$, $p$-value = 0.0005) with the densitometric analysis of conventional western blot (**Figure 5D**), but was highly sensitive toward the co-staining with other antibody-conjugates in the flow cytometry.

To further validate the flow cytometry-based epigenetic analysis, we performed control experiments to verify the specificity of the H3K9ac antibody. ImageStream cytometry was used to analyze the co-localization of H3K9ac with DRAQ5™ (a DNA intercalating dye, which stains living cells only) in human γδ T cells treated or not with 5 mM VPA. Complete co-localization of H3K9ac within the nuclear stain confirms "nuclear specificity" of the H3K9ac antibody. Treatment with 5 mM VPA enhanced H3K9ac staining (**Supplementary Figure 5**). Moreover, the co-localization of H3K9ac and DNA was strikingly enhanced only after treatment with VPA (**Supplementary Figure 5C**). The weak expression of H3K9ac observed in untreated γδ T cells (**Supplementary Figure 5D**) corresponds to the low constitutive expression of H3K9ac in the cell and is referred to as "$H3K9ac^{low}$" (**Figure 5**; **Supplementary Figure 5C**). This can be clearly distinguished from 5 mM VPA-treated γδ T cells showing strong inducible expression of H3K9ac, referred to as "$H3K9ac^{high}$" (**Supplementary Figure 5E**). Taken together, the single cell epigenetic analysis revealed specificity for nuclear H3K9 acetylation distinguishing levels of H3K9ac expression in γδ T cells, thus further corroborating previous single cell epigenetic analysis of CD8 T cells (31).

## VPA Affects the Distribution of H3K9ac-Associated Memory γδ T Cells Under *in vitro* Tumor Co-culture Conditions

Having established the ultra-sensitive, flow cytometry-based method of H3K9ac analysis in γδ T cells, we further extended this approach to evaluate the distribution of γδ memory T-cell phenotypes. To this end, we used a similar experimental set-up as applied to address NKG2D receptor expression. γδ T cells were harvested from co-cultures with Panc89 and PC-3 tumor cells or from solo-cultures treated or not with 2.5 mM VPA. Consistent with the previous observations, we detected $H3K9ac^{low}$ and $H3K9ac^{high}$ expression in γδ T cells upon 2.5 mM VPA treatment, irrespective of tumor co-culture or solo-culture (flow cytometry gating strategy illustrated in the **Supplementary Figure 6**). The proportion of $H3K9ac^{high}$ cells among Vδ2 T cells was significantly increased in the co-culture with Panc89 and PC-3 cells after 2.5 mM VPA treatment (**Figure 6A**), while the percentage of $H3K9ac^{low}$ Vδ2 T cells was significantly reduced (**Figure 6B**). This clearly reflects the change in H3K9ac levels as an effect of VPA treatment upon co-culture with tumor cells. Interestingly, the constitutive expression of H3K9ac in γδ T cells was significantly increased in Panc89 and PC-3 co-cultures (**Figure 6B**). Thus, the *in vitro* co-culture with Panc89 and PC-3 tumors modulates epigenome-wide inducible histone acetylation levels in γδ T cells, which might have consequences for the functional cellular response. As a first step to address this, we analyzed memory subset phenotypes based on the surface markers CD27 and CD45RA (as described by Dieli et al. (32)) within $H3K9ac^{low}$ and $H3K9ac^{high}$ Vδ2 T-cell populations. Memory phenotype distribution of Vδ2 T cells associated with $H3K9ac^{high}$ remained unaffected and also the distribution of naïve ($T_N$) and central memory ($T_{CM}$) phenotypes of $H3K9ac^{low}$ Vδ2 T cells did not change remarkably (data not shown). However, a substantial change in the relative distribution of effector memory ($T_{EM}$) cells (defined as $CD27^-CD45RA^-$) and terminally differentiated ($T_{EMRA}$) cells (defined as $CD27^-CD45RA^+$) was observed (**Figures 6C,D**). Of note, the significant increase in the proportion of TEM within $H3K9ac^{low}$ Vδ2 T cells (**Figure 6C**) and the decrease in the proportion of TEMRA within $H3K9ac^{low}$ Vδ2 T cells (**Figure 6D**) were found only upon co-culture with Panc89 tumor cells treated with 2.5 mM VPA, and not in the case of PC-3 tumor cells. Thus, taking all results together, it appears that Vδ2 T cells constitutively expressing low levels of H3K9ac might revert to the $T_{EM}$ phenotype under the *in vitro* microenvironment of pancreatic carcinoma in response to inhibition of histone deacetylase enzymes.

## DISCUSSION

Previous reports from our group and others have shown that the differential mechanisms of NKG2D ligand release are related to the origin of tumor cells and the allelic polymorphisms of MICA (5, 6). In this study, we used epigenetic inhibitors

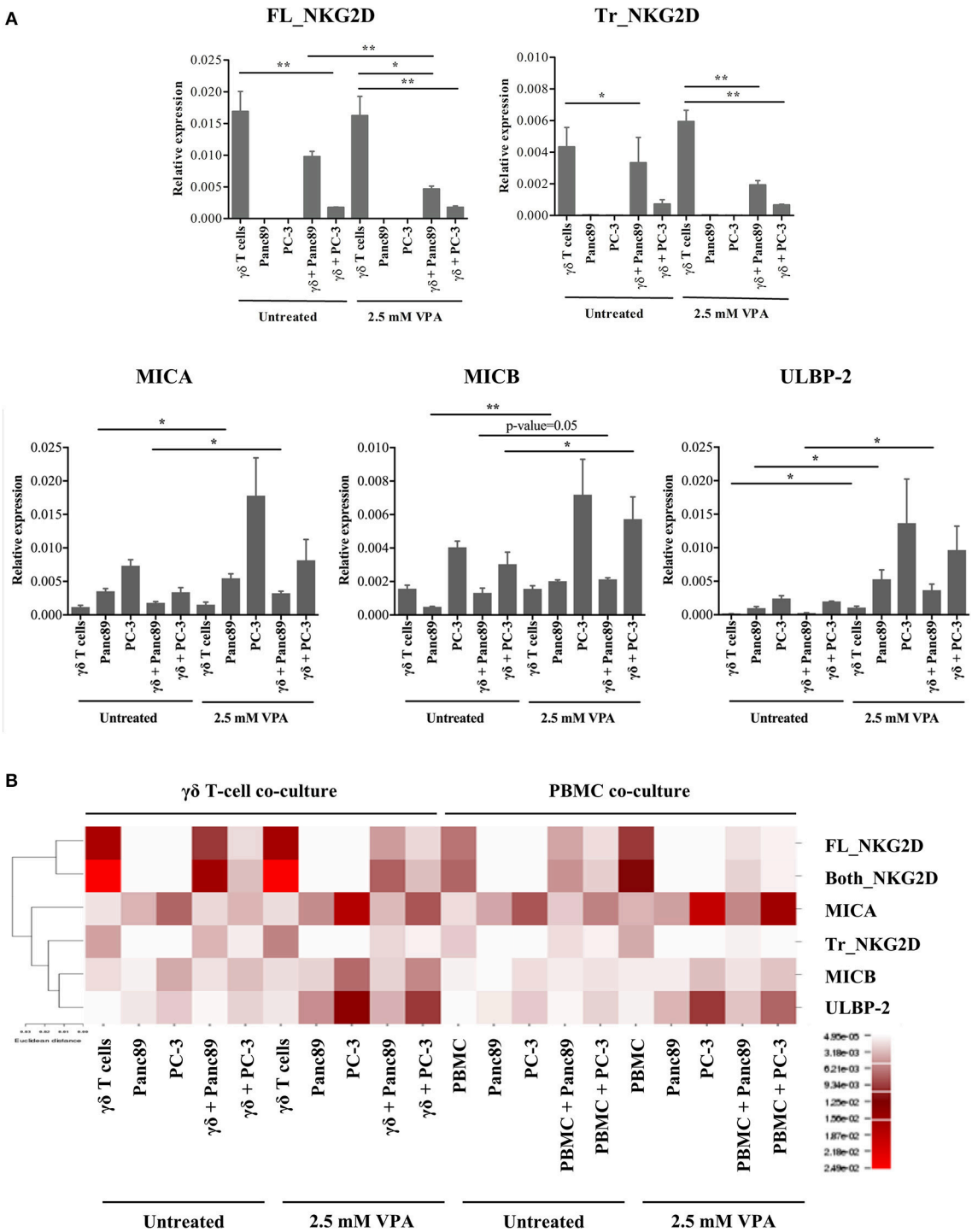


**FIGURE 4 |** VPA-mediated regulation of NKG2D receptor and ligand gene expression. **(A)** γδ T cells were treated with 2.5 mM VPA or left untreated and co-cultured with or without Panc89 and PC-3 cells. After 24 h, γδ T cells from co-cultures and solo-culture, or adherent tumor cells from solo-cultures (as an internal control) were harvested and analyzed for mRNA expression of full length NKG2D (FL_NKG2D), truncated NKG2D (Tr_NKG2D), MICA, MICB, and ULBP-2/5/6. mRNA levels were calculated as relative expression values compared to the mean Ct value of the housekeeping genes (β-actin, β2-microglobulin and 18S rRNA). Graphs represent mean values ± S.E. of three independent experiments with *p*-values < 0.01, 0.05 as **, *. **(B)** Based on the relative expression level, the heatmap illustrates unsupervised hierarchical clustering of respective mRNA for NKG2D receptor-ligands using Euclidian distance method with average linkage rule. Though the graph of relative expression for sequences covering both the full length and truncated NKG2D gene (Both_NKG2D, as an experimental control which includes both truncated and full-length variants) is not shown in the **Figure 4A**, the expression values were used for the hierarchical clustering. The color and intensity of the boxes is used to represent changes of gene expression as indicated in the color legend.

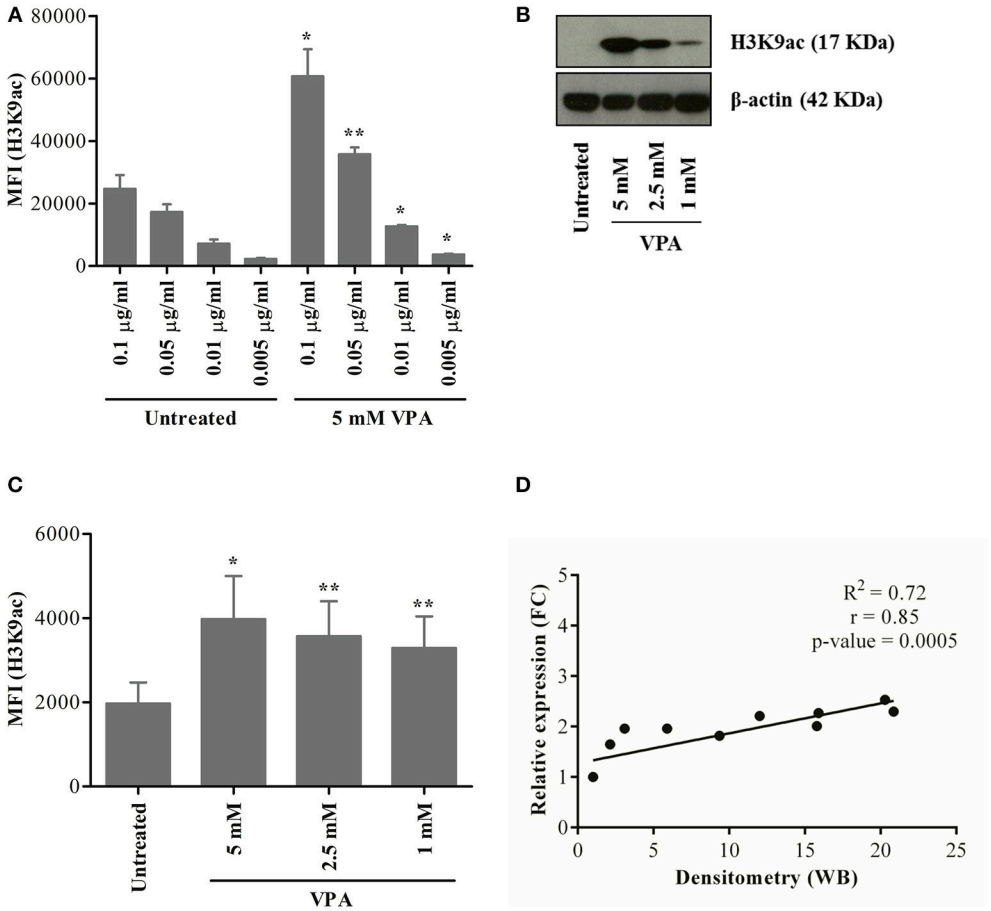


**FIGURE 5 |** Establishment of a flow cytometry-based analysis of H3K9 acetylation. γδ T cells were derived from PBMC stimulated with zoledronic acid and IL-2 for 12 days. **(A)** γδ T cells treated or not with 5 mM VPA were stained with titrated concentration of H3K9ac antibodies. γδ T cells were also treated with 5, 2.5, and 1 mM VPA for 24 h and harvested for analysis by western blot **(B)** or flow cytometry **(C)**. For western blotting, β-actin was used as a loading control. For flow cytometry-based analysis, appropriate isotype control was used. **(D)** The ratio was first calculated between H3K9ac protein expression and loading control from western blot **(B)** and then the relative density was calculated by dividing the ratio of untreated condition by respective VPA treatment. Similarly, median fluorescence intensity (MFI) was first calculated by subtracting the MFI of isotype control from H3K9ac antibody **(C)** and then the relative expression was calculated by dividing MFI of untreated condition by respective VPA treatment. A correlation between relative density from western blot and relative expression from flow cytometry was calculated for statistical significance (*p*-value), correlation co-efficient (r), the coefficient of determination ($R^2$) and represented using the scatter plot. Plots represented in the figure are based on values from three to five independent experiments. *P*-values of significance for <0.05 and 0.01 are indicated by * and **, respectively.

for further analysis of NKG2D ligand shedding. When we targeted proteins involved in histone and DNA modifications, we found that only inhibitors of HDACs (from the family of epigenetic "erasers") modulated NKG2D ligand expression and/or release. Heterogeneity in the release of NKG2D ligands based on the tumor origin was also observed after treatment with the HDAC inhibitor VPA. Interestingly, PC-3 cells released MICA directly into the culture supernatant after VPA treatment at the concentrations tested, suggesting that PC-3 cells are not exclusively dependent on exosome release. This substantial change in the mechanism of MICA release might be due to the MICA heterozygosity of PC-3 cells, exhibiting MICA*008:01:01 (A5.1) and MICA*012:01 (A4) allelic polymorphism (6). However, previous reports have not described such a switch in the release of MICA after treatment with VPA and its possible association with heterozygous MICA expression (33, 34). In human ovarian and cervical cancer cell lines, Trichostatin A (TSA) and sodium butyrate had a similar effect on MICA/B induction, while VPA was less effective (33). But as in human hepatoma cells (34), the effect of VPA was consistent with what we observed in our study using pancreatic and prostate cancer cells. Chitadze et al. demonstrated that shedding of MICA/B from Panc89 cells was mediated by metalloproteases (6). But, at this point, it is not clear whether VPA-induced NKG2D ligand release observed in the present study is due to ADAM-10/17- or MMP-mediated proteolysis. In addition to the heterozygosity of MICA and the possible mechanism of proteolytic cleavage, the origin of tumor cells might also play an important role in the response to VPA.

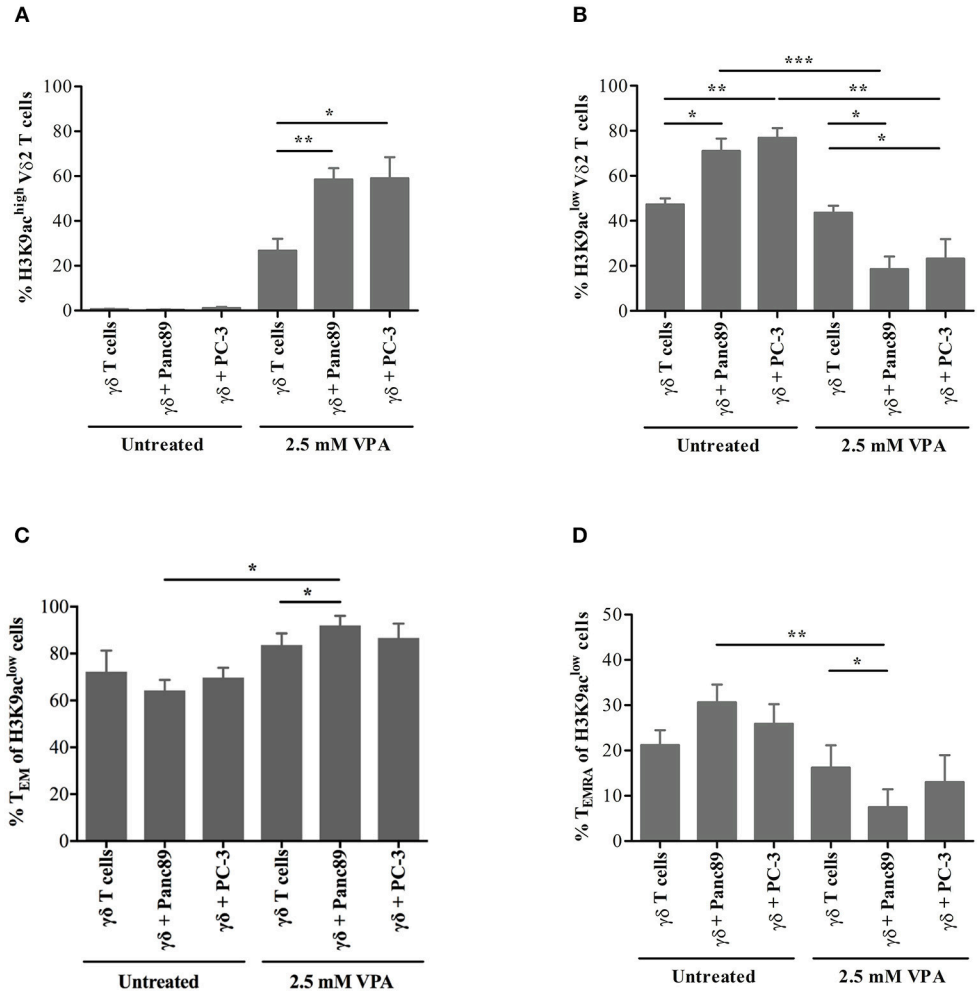


**FIGURE 6 |** H3K9 acetylation-associated memory phenotype of γδ T cells upon *in vitro* co-culture with tumor cells. γδ T cell lines generated from 12 days zoledronic acid-stimulated PBMC were co-cultured with 2.5 mM VPA pre-treated Panc89 and PC-3 cells. After 24 h of co-culture, cells were harvested and stained with FITC-conjugated Vδ2, APC-H7-conjugated CD27, PE-Cy7-conjugated CD45RA and Pacific Blue-conjugated H3K9ac antibodies. Based on flow cytometry gating strategy described in the **Supplementary Figure 5**, Vδ2-gated cells were defined as **(A)** H3K9ac$^{high}$ and **(B)** H3K9ac$^{low}$ in solo- or co-culture with Panc89 and PC3 cells in the presence and absence of 2.5 mM VPA. The proportions of H3K9ac$^{low}$ cells within Vδ2 T cells with **(C)** $T_{EM}$ and **(D)** $T_{EMRA}$ phenotype from the co-culture with Panc89 or PC-3 with or without 2.5 mM VPA treatment are shown. Data represented as mean ± S.E. of four independent experiments. Statistical significance is shown by *, **, *** for *p*-values < 0.05, 0.01 and 0.005, respectively.

VPA has been used *in vitro* at concentrations ranging from 0.5 to 10 mM (35–37). To study the modulation of MICA expression and release from tumor cells, Armeanu et al. used 1 mM VPA for human hepatoma cells. Their results showed a differential induction of NKG2D ligands by VPA on malignant and non-malignant cells (34). Yamanegi et al. also used VPA at 0.5- and 1-mM concentration for human osteosarcoma cells revealing increase in the cell-surface but not soluble form of MICA/B (38). In contrast, our study revealed only minor changes in the shedding of MICA from Panc89 cells and no changes at all in PC-3 cells after treatment with 1 mM VPA. In fact, NKG2D ligand (MICA/B and ULBP-2) gene expression was remarkably induced with 2.5 mM VPA both on malignant (pancreatic carcinoma cells Panc89 and prostate carcinoma cells PC-3) and non-malignant cells (PBMC and γδ T cells). The use of 2.5 mM VPA concentration to induce MICA protein expression is consistent with a previous study performed with Hodgkin lymphoma cells (39). The ULBP-1 expression is known to be associated with proteasome regulation (40). Remarkably, VPA failed to induce ULBP-1 expression on Panc89 and PC-3 cells. Nevertheless, the treatment of melanoma cells with 1 mM VPA has been shown to induce only MICA, MICB and ULBP-2, mediated by the ERK pathway (41), which is also consistent with our results using Panc89 and PC-3 tumor cells. The role of specific signaling pathways like ERK1/2 and AKT in cellular responses of Panc89 and PC-3 to VPA treatment remains to be investigated.

Another important aspect of our study is the direct evidence linking functional cellular responses of γδ T cells to epigenetic mechanisms. In this regard, we first showed down-regulation of the NKG2D receptor on γδ T cells at the protein and gene expression level upon VPA treatment. It has been noted that NKG2D ligand shedding down-modulates NKG2D receptor expression on effector cells, such as NK cells (8). In contrast, however, Deng and coworkers showed increased NKG2D receptor expression (9). At the protein level, we observed down-regulation of the NKG2D receptor on γδ T cells in tumor cell co-cultures, but the expression was maintained on lymphocyte-gated freshly isolated PBMC (including NK cells), indicative of a primarily γδ T-cell-specific response. Down-regulation of the full-length form of the NKG2D receptor and of NKG2D ligands was corroborated at the level of gene expression. The truncated NKG2D receptor plays an important role in down-regulating the expression of the full-length NKG2D receptor (23), which is also a characteristic feature of NKG2D ligand-mediated shedding. Of note, this functional similarity is further strongly supported by the cluster analysis, which grouped the truncated form of the NKG2D receptor together with all NKG2D ligands (MICA/B and ULBP2), though a slight increase in truncated NKG2D receptor in γδ T cells due to VPA treatment was observed. But such increase was not found in co-culture with Panc89 upon VPA treatment. This observation supports the view that in addition to other possible mechanisms, the truncated NKG2D form may be involved in the modulation of VPA-induced NKG2D receptor expression in the co-culture with γδ T cells. Additionally, a decrease in the gene expression of NKG2D receptor (both full-length and truncated form) after VPA treatment was observed during the interaction with Panc89, but not with PC-3. The differences in the mechanisms of NKG2D ligand release are likely affected by VPA-induced epigenetic modification or *vice versa*. This, however, needs to be studied further in detail. But it is clear from our current and previous other studies that the protein/gene expression of NKG2D ligands is heterogenous, cell type-dependent and most likely regulated at the prost-transcriptional level (6, 19, 20, 42, 43).

Furthermore, we provided evidence for a direct link between γδ T-cell memory subset distribution and epigenome-wide H3K9ac (an activating marker) expression. Although the distribution of memory subsets was not altered within the γδ T-cell population with inducible expression of H3K9ac (i.e., H3K9ac$^{high}$), we are the first to show that the memory phenotypes of γδ T cells are most likely modulated through the constitutive H3K9ac expression (i.e., within the H3K9ac$^{low}$ subset). We previously reported that H3K9ac expression induced by VPA (which most likely corresponds to the H3K9ac$^{high}$ population defined in this study) leads to the expression of a non-secreted isoform of IL-4 and the regulation of c-Jun transcription factor in γδ T cells, but not in αβ T cells (18). Thus, this is a very important finding with respect to the effect of the HDAC inhibitor under these *in vitro* conditions mimicking the tumor microenvironment, specifically in the case of pancreatic ductal adenocarcinoma (PDAC). The reversion of the memory phenotype distribution from $T_{EMRA}$ to $T_{EM}$ in γδ T cells supports the view that the γδ $T_{EM}$ subset is mainly associated with the secretion of pro-inflammatory cytokines, cytotoxicity and the expression of homing receptors for inflamed tissues (32, 44, 45). It has already been described that γδ T cells have the capacity to elicit anti-tumor response mediated through the perforin-granzyme pathway, not solely depending on NKG2D receptor-ligand signaling, by the $T_{EM}$ phenotypic subset (45, 46). This seems to hold true based on the analysis of lysosomal degranulation using the marker CD107a in our study. In the present experimental model, γδ T cells exhibited degranulation in response to Panc89 (pancreatic adenocarcinoma cells), but not to PC-3 (prostate carcinoma cells), which corroborates the functional role of γδ $T_{EM}$ subset. In the case of oral-, colon-carcinoma cells and B lymphoblastic cell line (47) and osteosarcoma (48), γδ T cells exerted this cytotoxicity mainly via TCRγδ- and Fas-/perforin-mediated mechanisms, or by blocking PD-1 signal after VPA and Zol treatment (with a minor role of NKG2D and TRAIL), respectively. In the future, we will analyze the expression of perforin and granzymes, PD-1 and NKG2D to explore the mediators of cytotoxicity, exerted by $T_{EM}$ γδ T cells under such *in vitro* conditions mimicking the tumor microenvironment.

The results presented here on the effects of HDACi VPA may have implications to further improve γδ T-cell-based immunotherapy (49, 50). A clinical phase I study with multiple myeloma patients has already proved its safety and emerged as a promising approach for the adoptive transfer of zoledronic acid-activated Vγ9Vδ2 T cells (51). This study specifically revealed the significant increase in the $CD27^{-}CD45RA^{+}$ $T_{EM}$ subpopulation within Vγ9Vδ2 T cells. A combined approach with epigenetic modifiers, such as VPA might further increase the efficacy but this needs to be tested in preclinical studies using suitable *in vivo* models. The combination of γδ T cell based immunotherapy with VPA treatment might be a particularly promising translational strategy for the treatment of patients with pancreatic ductal adenocarcinoma (PDAC).

# MATERIALS AND METHODS

## Tumor Cell Culture and Reagents

Panc89, pancreatic ductal adenocarcinoma has been previously characterized (52) and PC-3, prostate cancer cell line was obtained from ATCC. Both tumor cell lines and effector cells were cultured in RPMI-1640 medium (Gibco®) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS; Gibco), 1% Penicillin/Streptomycin (PS; Biochrom), hereafter referred to as growth medium. Tumor cell lines were maintained in 175 $cm^2$ Cellstar® cell culture flasks (Greiner Bio-One). All cell cultures were kept at 37 °C in a humidified atmosphere with 5% $CO^2$. Valproic acid (VPA; #P4543), Trichostatin A (TSA; #T8552), 4-Phenylbutyric acid (4-PBA; #P21005), Epigallocatechin gallate (EGCG; #1236700), 5-Aza-2′-deoxycytidine (Decitabine; #A3656), Curcumin (#C1386) were used at indicated concentrations; all reagents were purchased from Sigma-Aldrich.

## Effector Cell Cultures

PBMC were isolated from healthy donors (Department of Transfusion Medicine, UKSH, Kiel, Germany) with approval by the local Ethics Committee (D405/10). PBMC were either used freshly or stimulated with 2.5 μM zoledronic acid (kindly provided by Novartis) in the presence of 50 IU/ml IL-2 (Novartis). Furthermore, IL-2 was added every other day until day 12. Day 12 cultures routinely contained >90% Vγ9Vδ2-expressing γδ T cells.

## Analysis of NKG2D Ligand Expression on Tumor Cells by Flow Cytometry

Epigenetic inhibitors VPA, TSA, 4-PBA, EGCG, Decitabine or Curcumin were used at indicated concentrations to study the modulation of NKG2D ligand expression and release from tumor cells. $0.8 \times 10^6$ tumor cells in growth medium were seeded in 12-well plates, either treated with respective epigenetic inhibitors, DMSO as solvent control for TSA, Decitabine and Curcumin treatment or left untreated. Cells were harvested, washed and surface stained with the following anti-human antibodies: PE-conjugated anti-human MICA (clone 159227; #FAB1300P), Alexa Fluor 700-conjugated anti-human MICB (clone 236511, #FAB1599N), PerCp-conjugated anti-human ULBP-1 (clone 170818, #FAB1380C) and APC-conjugated anti-human ULBP2/5/6 (clone 165903, #FAB1298A) together with respective isotype controls (all from R&D Systems). After this cell surface staining, cells were immediately measured on a LSR Fortessa flow cytometer (BD Biosciences). Data was analyzed using the FlowJo software (FlowJo LLC). Median fluorescence intensity was calculated by subtracting the median intensity of the isotype control antibody from the respective test antibody fluorescence.

## NKG2D Ligand Shedding (ELISA)

Culture supernatants were spun down to remove debris. Flat-bottom 96-well Maxisorp Nunc Immunoplates (Thermo Fisher Scientific) were used for coating with antibodies to quantitate soluble MICA, MICB, ULBP-1, ULBP-2 (#DY1300, #DY1599, #DY1380, #DY1298, respectively; all from R&D Systems) by ELISA according to manufacturer's protocol.

## Co-culture Assays

For co-culture assays, $0.8 \times 10^6$ tumor cells per ml of growth medium were treated with 2.5 mM VPA for 24 h in 12 well cell culture plates (Greiner Bio-One). Afterwards, $1 \times 10^6$ effector cells (PBMC or γδ T cells) were added to the culture for another 24 h without changing medium. Thereafter, non-adherent cells (i.e., PBMC or γδ T cells) were carefully resuspended and harvested for cytotoxicity, gene expression and flow cytometric analysis.

## Flow Cytometric Analysis of Effector Cells

As described above, effector cells (i.e., γδ T cells or PBMC) were harvested after 24 h of co-culture with Panc89 and PC-3 cells. Cells were washed once with cold Dulbecco's PBS (Cell Concepts) and surface stained with the following mAb: FITC anti-Vδ2 (clone IMMU389, #IM1464, Beckman Coulter), PE anti-NKG2D (clone 149810, #FAB139P, R&D Systems), APC-Cy7 anti-CD27 (clone M-T271, #560222, BD Horizon), PE-Cy7 anti-CD45RA (clone L48, #337167, BD Biosciences). The effector cells were stained with respective antibodies for the NKG2D receptor and ligands by gating on Vδ2 T cells or on lymphocytes (based on forward-side scatter properties) of PBMC co-culture. Afterwards, cells were either measured immediately or otherwise processed further for intranuclear staining. For intranuclear staining, surface stained cells were permeabilized and fixed using BD Pharmingen Transcription Factor Buffer set (#562574, BD Biosciences). Staining was performed using Pacific Blue-conjugated anti-human H3K9ac rabbit monoclonal antibody (clone C5B11, #11857S) or Pacific Blue-conjugated anti-rabbit isotype control monoclonal antibody (clone DA1E, #9078S; both from Cell Signaling Technology). 10,000 Vδ2 T cells or lymphocyte-gated PBMC were acquired on a LSR Fortessa flow cytometer (BD Biosciences). FACSDiva software was used for acquisition, while data analysis was done using FlowJo software (FlowJo LLC).

## Degranulation Assay

As described in the methods section Co-culture Assays and in the **Supplementary Figure 1**, γδ T cells were co-cultured with Panc89 or PC-3 cells with or without 2.5 mM VPA and/or 2.5 μM Zoledronate (Zol). During the last 4 h of solo-/co-culture, 50

**TABLE 1** | PCR primers used in this study.

| Gene name | Primer | Sequence |
|---|---|---|
| **NKG2D RECEPTOR** | | |
| FL_NKG2D | Forward | GCTGTATTCCTAAACTCATTATTCAACC |
| | Reverse | CTGCCAAGATCCATTTGTTG |
| Tr_NKG2D | Forward | TTCTGCTGCTTCATCGCTGT |
| | Reverse | TGGACTAATAGCAAAAATGTGACAA |
| Both_NKG2D | Forward | CCTCTCTGCGGTAGACGTG |
| | Reverse | GACATCTTTGCTTTTGCCATC |
| **NKG2D LIGANDS** | | |
| MICA | Forward | AGGGTCTGTGAGATCCATGAAGAC |
| | Reverse | CCTGACGTTCATGGCCAAGG |
| MICB | Forward | ACCTTGGCTATGAACGTCACA |
| | Reverse | CCCTCTGAGACCTCGC |
| ULBP-2 | Forward | GCAAGGATGTCTTGTGAGCA |
| | Reverse | GGCCACAACCTTGTCATTCT |
| **HOUSEKEEPING GENES** | | |
| β2-microglobulin | Forward | GGGTTTCATCCATCCATCCGACA |
| | Reverse | ACACGGCAGGCATACTCATC |
| β-actin | Forward | CTGAACCCCAAGGCCAAC |
| | Reverse | CAGAGGCGTACAGGGATAGC |
| 18S RNA | Forward | GACTCAACACGGGAAACCTC |
| | Reverse | AGACAAATCGCTCCACCAAC |

*To analyze the mRNA expression of NKG2D receptor and ligand following PCR primers were used. Housekeeping genes were used as internal control. PCR primers were designed using the Web-based primer3 software (http://primer3.wi.mit.edu/) and purchased from TIB MOLBIOL.*

μl PE anti-CD107a mAb (50 ng/ml, clone: H4A3, #555801, BD Biosciences) was added directly, and after 1 h i.e., during the last 3 h, monensin (3 μM, EMD Millipore/Calbiochem) was added. γδ T cells were harvested from the solo-/co-cultures and were stained for FITC anti-Vδ2 mAb. 10,000 Vδ2 T cells were acquired on a LSR Fortessa flow cytometer (BD Biosciences) and FlowJo software (FlowJo LLC) was used for the data analysis.

## Gene Expression Analysis of NKG2D Receptor and Ligands

Human PBMC, γδ T cells, Panc89, and PC-3 cells were harvested from co-culture experiments as described above. Effector or target tumor cells were washed once with cold Dulbecco's PBS, resuspended in peqGOLD TriFast solution (#30-2010, Peqlab) and stored at −80 °C until further use. RNA was extracted and transcribed into cDNA using the cDNA synthesis kit (#8994-A30, AmpTec). For PCR amplification, respective PCR primers for NKG2D receptor and ligands were used at the annealing temperature 60 °C (details in **Table 1**). qPCR data were analyzed using relative quantitation method by normalizing with the mean Ct value of the housekeeping genes (β2-microglobulin, β-actin, and 18S RNA). The calculated relative expression values of NKG2D receptor and ligand genes were imported and visualized as heatmap based clustering using CIMminer (https://discover.nci.nih.gov/cimminer/home.do). The Euclidian distance method with average linkage rule was used (28, 29).

## Western Blot

Total H3K9ac in human γδ T cells was analyzed by western blot as described previously (17, 18). Briefly, $1 \times 10^6$ per ml of γδ T cells were either treated with 5 mM, 2.5 mM, 1 mM VPA or left untreated. After 24 h of treatment, cells were harvested and lysed. 10 μg of protein was separated by SDS-PAGE, transferred to nitrocellulose and analyzed with a primary rabbit monoclonal antibody against H3K9ac (clone C5B11, #9649, Cell Signaling Technology) and β-actin (clone AC-15, #A5441, Sigma-Aldrich). HRP-conjugated secondary anti-rabbit antibodies (#NA9340V, GE Healthcare) were used for detection and visualized by the enhanced chemiluminescence system (#RPN2106, GE Healthcare).

## ImageStream Analysis

The ImageStream analysis was performed to study co-localization between H3K9ac and nuclear staining as published previously (18). Shortly, $1 \times 10^6$ per ml of γδ T cells were treated with 5 mM VPA or left untreated as a control. After 24 h of treatment, γδ T cells were harvested and stained with Pacific Blue-labeled anti-human H3K9ac antibody. At the end of intracellular H3K9ac staining, 1 μM DRAQ5™ (#ab108410, Abcam plc) was added to the cell suspension. After incubation for 15 min at 37 °C, cells were washed once with cold PBS. Unstained or stained γδ T cells with or without 5 mM VPA treatment were acquired immediately on an ImageStreamX Mark II imaging flow cytometer (Merck Millipore). IDEAS software (v6.0, Amnis) was used to acquire images (60×) and statistical calculations. Special co-localization wizard of the IDEAS software was applied for single cell analysis.

## Statistical Analysis

Statistical analysis was performed using PrismGraph software. Statistical significance was calculated using Student's *t*-test to evaluate the significant differences between two experimental conditions (53). *p*-Value $<0.05$ was considered significant and were displayed as * for $<0.05$, ** for $<0.01$ and *** for $<0.001$.

# AUTHOR CONTRIBUTIONS

JB and DK conceptualized and designed the experiments. JB, SD, and AD developed methodology. JB, ESQ, JF, CMD, GC, and ML acquired the data. JB, ESQ, JF, CMD, AS, GC, and ML analyzed and interpreted the data. JB, CMD, AS, GC, ML, and DK wrote the manuscript. ESQ, JF, and ML provided administrative and technical help. DK supervised the study.

# FUNDING

This study was financially supported by Deutsche Forschungsgemeinschaft (DFG) through the Cluster of Excellence Inflammation-at-Interfaces EXC306-PN and EXC306-PM to DK, Werner-und-Klara-Kreitz Foundation, Germany to JB, and DAAD-RISE scholarships to SD and AD (A/14/06525 and KielGermany_BI_1112, respectively).

# ACKNOWLEDGMENTS

We acknowledge the technical assistance by Hoa Ly, Hilke Clasen, Monika Kunz, Ina Martens, and Sandra Ussat. The authors thank Ankush Borlepawar for the help in densitometry measurements and Dr. Ole Helm for providing the DRAQ5™ dye. We also thank Dr. Daniela Wesch, Dr. Hans-Heinrich Oberg, Dr. Christian Peters for discussion. This work forms part of the Ph.D. thesis of Jaydeep Bhat.

# SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00569/full#supplementary-material

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**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: 29 March 2019
doi: 10.3389/fimmu.2019.00661

# NKG2D/NKG2-Ligand Pathway Offers New Opportunities in Cancer Treatment

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The antitumor functions of NK cells are regulated by the integration of positive and negative signals triggered by numerous membrane receptors present on the NK cells themselves. Among the main activating receptors, NKG2D binds several stress-induced molecules on tumor targets. Engagement of NKG2D by its ligands (NKG2D-Ls) induces NK cell activation leading to production of cytokines and target cell lysis. These effects have therapeutic potential as NKG2D-Ls are widely expressed by solid tumors, whereas their expression in healthy cells is limited. Here, we describe the genetic and environmental factors regulating the NKG2D/NKG2D-L pathway in tumors. NKG2D-L expression is linked to cellular stress and cell proliferation, and has been associated with oncogenic mutations. Tumors have been found to alter their to NKG2D-L expression as they progress, which interferes with the antitumor function of the pathway. Nevertheless, this pathway could be advantageously exploited for cancer therapy. Various cancer treatments, including chemotherapy and targeted therapies, indirectly interfere with the cellular and soluble forms of NKG2D-Ls. In addition, NKG2D introduced into chimeric antigen receptors in T- and NK cells is a promising tumor immunotherapy approach.

**Keywords: NKG2D, natural killer cells, NKG2D ligands, tumor immunosurveillance, NKG2D CARs**

OPEN ACCESS

***Edited by:***
*Nadia Guerra, Imperial College London, United Kingdom*

***Reviewed by:***
*Alessandra Zingoni, Sapienza University of Rome, Italy*
*Michele Ardolino, University of Ottawa, Canada*

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

***Received:*** *07 December 2018*
***Accepted:*** *11 March 2019*
***Published:*** *29 March 2019*

***Citation:***
*Frazao A, Rethacker L, Messaoudene M, Avril M-F, Toubert A, Dulphy N and Caignard A (2019) NKG2D/NKG2-Ligand Pathway Offers New Opportunities in Cancer Treatment. Front. Immunol. 10:661. doi: 10.3389/fimmu.2019.00661*

## NKG2D/NKG2D-LS IN TUMOR IMMUNOSURVEILLANCE

The immunosurveillance theory described by Robert Schreiber in 2002 (1) suggested that NK cells are involved in the early control of tumor development, before the successive equilibrium and escape phases when tumor-induced immunosuppression results in the emergence of immune-resistant tumor variants. During the elimination phase, NK cells detect and kill emerging transformed cells. NK cells naturally express receptors detecting stress-induced molecules and altered expression of Major Histocompatibility Complex (MHC) class-I molecules on transformed targets (2). NK cells also potentiate the adaptive immune response through cytokine secretion and by stimulating dendritic cells (DC), notably within lymph nodes (3–8).

Several groups have used samples obtained after curative resection to investigate the role played by NK cells in primary solid tumors. Their results indicated that NK cell infiltrates may correlate with clinical outcome (9–14). In most reports, NK cells were present at low numbers within tumors,

as reviewed in (15). In contrast, in colorectal carcinoma (16), lung cancers (14), and gastrointestinal stromal tumors (GIST) (17) numerous NK cells were present in peritumoral areas. NK cells are generally underrepresented among tumor-infiltrating lymphocytes compared to their circulating proportions, and their effector functions are also altered within the tumor microenvironment, as shown in breast and lung malignancies (18, 19). NK cell dysfunction, based on reduced cytotoxicity and cytokine release, correlates with downregulation of NK-activating receptors (18–21). However, this is a tumor-specific phenomenon, described in renal cell carcinoma (RCC) (22), GIST (17), neuroblastoma (23), melanoma (24, 25), and acute myeloid leukemia (26, 27). Altogether, these data revealed that, more than NK cell numbers, expression of NK cell receptors (including activating NK receptors or inhibitory KIRs) strongly influence prognosis and disease outcome. The only notable exception to this conclusion is chronic myeloid leukemia (CML) patients for whom treatment with imatinib was interrupted; in these patients, NK cell numbers were a significant predictive parameter for relapse (28, 29).

In addition, NK cells are thought to play a role in the emergence of metastases, as high numbers of circulating or tumor-infiltrating NK cells inversely correlated with metastatic disease (30, 31). The mechanisms deployed to limit cancer dissemination could involve activating NK receptors, including NKG2D or NKp46. In murine models of melanoma and prostate cancer, IFN-γ production by NK cells triggered by NKp46 activation was found to induce expression of the extracellular matrix protein fibronectin 1 in tumor cells, altering tumor architecture and controlling metastatic invasion (32).

NK cell activation is regulated by signals from activating receptors and inhibitory NK receptors, which bind to HLA-class-I molecules. In addition to natural cytotoxicity receptors, or Ig-type family receptors—the ligands for which have not yet been clearly identified—NKG2D (Natural Killer Group 2, member D)—a C-type lectin receptor—is a major activating receptor for NK cells. In humans, the gene encoding NKG2D (*KLRK1*) lies amid a cluster of genes referred to as the "NK-complex" (NKC) that includes several genes expressed by NK cells [*KLRD1* (CD94), *KLRC4* (NKG2F), *KLRC3* (NKG2E), *KLRC2* (NKG2C), and *KLRC1* (NKG2A)]. NKG2D was first identified on the surface of NK cells as an immunosurveillance receptor. It is also expressed by most $CD8^+$ and a small subset of $CD4^+$ cytotoxic αβ T cells, as well as innate-like immune cells, such as some iNKT cells and γδ T cells (33, 34). NKG2D is a type II transmembrane protein and, in humans, it associates with the transmembrane domain of the adaptor protein DAP-10 (DNAX-Activating Protein 10). Ligand binding causes dimerization of two NKG2D monomers to form an active receptor which phosphorylates DAP10 and triggers NK cell activation signaling pathways which promote $Ca^{2+}$ influx, actin-based cytoskeleton reorganization, and microtubule polarization (35). This signaling cascade leads to the release of the contents of cytolytic granules, and in some cases elicits the production of cytokines by NK cells (36). NKG2D provides co-stimulatory signals in activated T cells (37). NKG2D expression in NK cells and $CD8^+$ T cells can be upregulated, in particular in response to cytokines, such as interleukin (IL)-2, and IL-15, while transforming growth factor (TGF)-β can decrease NKG2D expression.

Our current understanding of the role played by NKG2D in controlling tumor development through NK cell and cytotoxic T-lymphocyte (CTL) activity was aided by the early characterization of its ligands (38). NKG2D binds to eight molecules, members of the following two families: MHC class-I-related chains A or B (MICA/B), and UL16-binding proteins (ULBP1-6). Seven *MIC* genes (*MICA* to *MICGI*) have been identified, located on chromosome 6p21.33. Only *MICA* and *MICB* genes are translated into proteins. As to ULBP, six protein-coding genes (*ULBP1* to *ULBP6I)* have been described on chromosome 6q24.2–25.3. Among all these proteins, MICA, MICB, ULBP4, and ULBP5 are transmembrane-anchored glycoproteins, whereas ULBP1, ULBP2, ULBP3, and ULBP6 are bound to the cell surface by a glycophosphatidylinositol (GPI) motif (39). All NKG2D-Ls are composed of one alpha 1 and one alpha 2 extracellular immunoglobulin (Ig)-like domain, which share a strong homology to the corresponding domains in classical HLA-class-I molecules (40). MICA and MICB contain a third, additional extracellular Ig-like domain (alpha 3). NKG2D-Ls are not associated with β2-microglobulin and bind no antigenic peptide.

## ROLE OF NKG2D AND NKG2D-L POLYMORPHISMS IN TUMOR IMMUNOSURVEILLANCE

The pioneering work by Imai et al. attributed a major role to NKG2D polymorphisms in cancer immunosurveillance (41), and in the prevention of cancer formation (42). An 11-years follow-up survey of a cohort including >3,500 members indicated that medium and high natural cytotoxic activity of peripheral-blood lymphocytes was associated with a reduced cancer risk, whereas low natural cytotoxicity correlated with a higher incidence of cancer. These findings suggest a role for NK cell-mediated immunity in controlling cancer. Analysis of 25 SNPs reported with an allele frequency of >10% in the NKC gene cluster identified eight SNPs in the NKG2D locus (*KLRK1*) that form two haplotype blocks (NKG2Dhb1 and hb2). Each of these blocks can generate two major alleles linked to low (LNK) or high (HNK) cytotoxic activity. Patients with the HNK1/HNK1 NK2GDhb1 haplotype had a lower incidence of cancer compared to those with the LNK1/LNK1 haplotype (41). In a Japanese population the HNK1/HNK1 genotype was associated with decreased colorectal (43) and aerodigestive tract cancer (44). A recent report indicated that NKG2D gene polymorphisms also correlated with control of CML by dasatinib (45). Thus, patients with the NKG2D HNK1/HNK1 haplotype achieved deep molecular response (MR4.5) more quickly than those with other haplotypes. Interestingly, phosphorylation of VAV1 on Tyr174, which was proposed as a major mechanism by which dasatinib intensifies NK cell activity (46), could also be enhanced by expression of the NKG2D HNK1 allele (45). These data suggest that the NKG2D HNK1/HNK1 haplotype may influence cancer development and modulate treatment response (**Figure 1**).

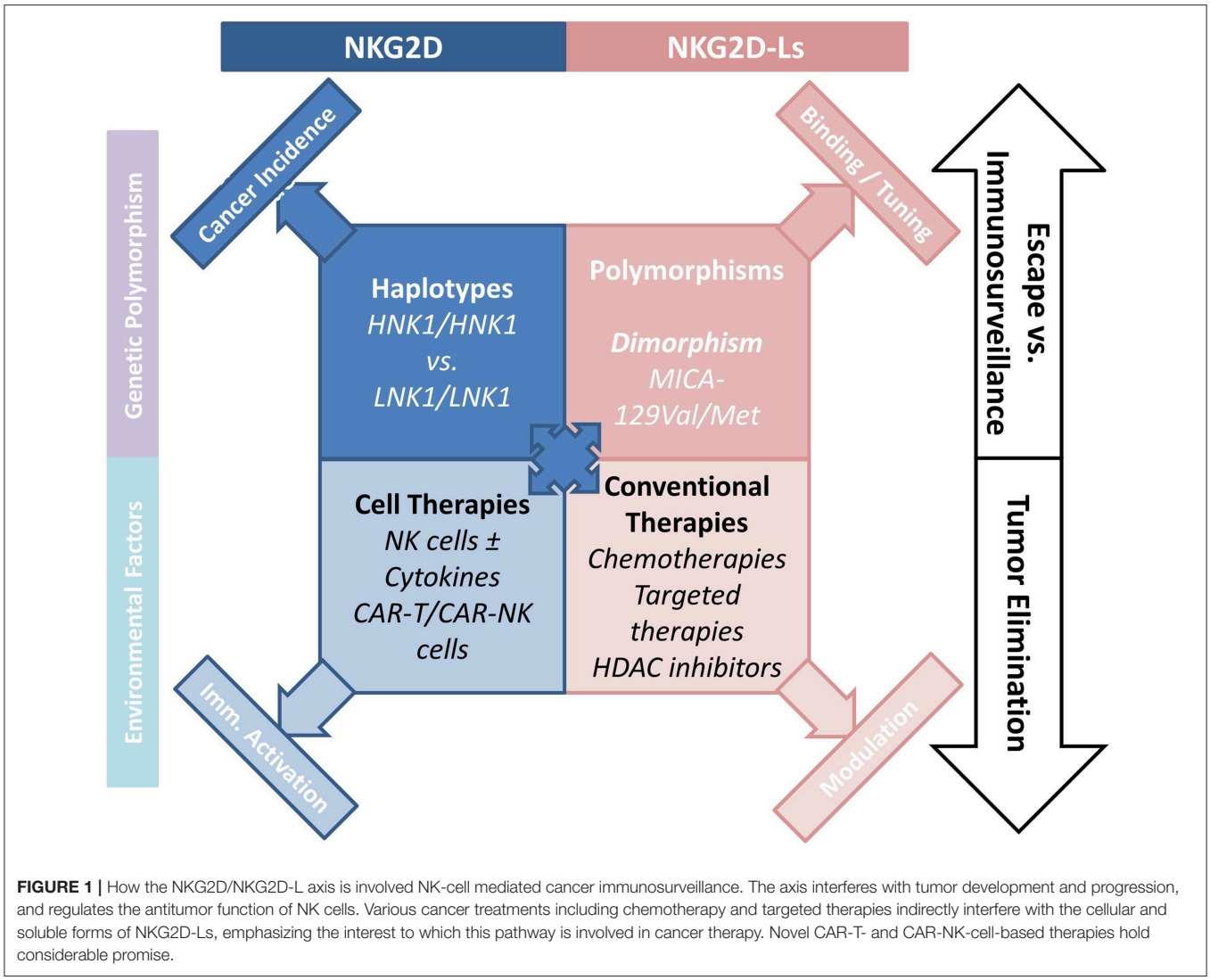


**FIGURE 1 |** How the NKG2D/NKG2D-L axis is involved NK-cell mediated cancer immunosurveillance. The axis interferes with tumor development and progression, and regulates the antitumor function of NK cells. Various cancer treatments including chemotherapy and targeted therapies indirectly interfere with the cellular and soluble forms of NKG2D-Ls, emphasizing the interest to which this pathway is involved in cancer therapy. Novel CAR-T- and CAR-NK-cell-based therapies hold considerable promise.

Importantly, MICA and MICB as well as ULBP molecules are highly polymorphic and allelic variation can alter their expression levels or their affinity for NKG2D. As a result, NKG2D-L polymorphisms may strongly influence NKG2D-mediated NK cell triggering by tumor cells or other stressed targets (47). To date, 107 MICA and 47 MICB alleles have been described (updated allele numbers can be found at http://hla.alleles.org/nomenclature/index.html). SNPs are located within regions encoding the α1 and α2 extracellular domains. These alleles are transcribed to produce a total of 82 MICA and 30 MICB proteins. The impact of MICA polymorphisms on protein expression and function remains only partly characterized, and MICA-129 is the only SNP described so far that affects NKG2D receptor affinity (48). MICA-129 (rs1051792) dimorphism—the substitution of a methionine (Met) for a valine (Val) at position 129—alters MICA affinity for the NKG2D receptor: MICA-129Met has an affinity 10- to 50-fold higher than MICA-129Val. Significantly, expression levels for MICA-129Met isoforms are reduced compared to the MICA-129Val molecule (49), but they nevertheless have a higher capacity to trigger the NKG2D pathway, leading to enhanced NK cell activity (50).

Several studies implicated MICA polymorphisms in viral infections and autoimmunity [reviewed in (51)], but few have investigated the impact of MICA polymorphism in cancers. Initial studies focused on cervical cancer and found no association between MICA polymorphism and disease susceptibility (52). In contrast, MICA polymorphism was found to be a significant risk factor for other tumors. For example, MICA-129Val is associated with poor prognosis in nasopharyngeal carcinoma (53) or breast cancer (54) in a Tunisian population. In melanoma, Isernhagen et al. (49) showed that MICA-129Val-homozygous melanoma cell lines expressed higher surface levels of MICA than cells with the Met/Met genotype, which released more soluble MICA. A group of frequent MICA alleles, named MICA-A5.1 (prototype MICA*008), produce a truncated protein that acquires a GPI anchor allowing it to be recruited to exosomes, from where it can downmodulate NKG2D expression

(55). A GWAS study in cervical cancer patients linked one MICA-adjacent region to the disease and identified a SNP (rs2516448) linked to the MICA-A5.1 frame-shift mutation, suggesting that this allele may cause impaired immune activation resulting in cancer development (56). In colorectal liver metastases, in contrast, the MICA-A5.1 polymorphism was associated with better tumor control and response to treatment (57).

Few studies have investigated ULBP polymorphisms, probably because of the limited number of SNP identified within these genes. However, one ULBP6 dimorphism (two SNP at positions 106 and 147) plays a significant role in determining affinity of the protein for the NKG2D receptor. The ULBP0602 molecule (which contains $Leu^{106}$ and $Thr^{147}$ in contrast to ULPB0601's $Arg^{106}$ and $Ile^{147}$) binds to NKG2D with a 10- to 1,000-fold higher affinity than other ULBPs. This difference in binding could result in decreased interaction of NKG2D with other ligands; it could thus have a negative effect on NK cell function (58).

## REGULATION OF NKG2D AND NKG2D-L EXPRESSION

NKG2D-Ls are rarely expressed by healthy cells, but are induced at the cell surface when the cell is stressed as a result of viral infection or malignant transformation; they are therefore called "induced-self" ligands. NKG2D-L-positive cells are detected and eliminated, mostly by NK cells. Expression of NKG2D-Ls is regulated by several mechanisms, which may be transcriptional, translational or post-translational. Expression of NKG2D-Ls is induced by DNA damage, a characteristic of tumor transformation, which leads to the activation of the ATM-ATR DNA repair pathways (59). In mouse models, hyper-proliferation can also induce NKG2D-L expression through activation of the E2F transcription factor (60). Indeed, following HER2/HER3 or BCR-ABL activation, proliferative signals can induce MICA/B and ULBP expression (61). Cellular stress, such as heat shock, has also been reported to induce heat shock factor 1-mediated MICA/B expression (62, 63).

The NKG2D/NKG2D-L pathway is triggered early in cancer development and participates in the elimination of tumor cells. However, during tumor progression, profound changes occur and the NKG2D and/or NKG2D-Ls are targeted by a range of tumor escape mechanisms. Cancer can sculpt the immune environment by selecting immune-ligand-negative variants (1, 64). Tumor cells expressing high levels of NKG2D-Ls can thus be eliminated as part of the tumor immunoediting process, which involves NK cells and NKG2D, and progressively results in the emergence of NKG2D-resistant variants (65). Persistent NKG2D-L expression by tumor cells may cause systemic immunosuppression as a result of NK exhaustion and perturbation of the immune synapse (26). Epigenetic and transcriptional regulation mechanisms are often perturbed in tumor cells, and NKG2D-L expression may be altered. In melanoma, endoplasmic reticulum stress can reduce MICA transcription by modulating activation of the transcription factor E2F1 (66). Indeed, immature isoforms of MICA are retained in the endoplasmic reticulum, resulting in limited membrane-display of MICA (67). Tumors can also inhibit NKG2D-L expression by altering cell surface glycosylation (68), notably in a hypoxic environment (69). In CML, BCR/ABL controls MICA expression through post-transcriptional mechanisms (70), including MICA glycosylation (71). Finally, histone deacetylases (HDAC) may also regulate NKG2D-L expression (72, 73).

Soluble factors secreted by tumor cells and cells from their microenvironment can also alter NKG2D-L expression levels. Thus, TGF-β and IL-10 secreted by regulatory T cells (Tregs) and myeloid-derived suppressor cells downmodulate the expression of NKG2D-Ls (74, 75). Some tumors were demonstrated to secrete TGF-β, resulting in reduced NK cell-mediated lysis (10). ADAM metallopeptidases can catalyze shedding of NKG2D-Ls from the cell's surface, and the released soluble forms can hamper NKG2D signaling (76). Upregulation of ADAM10 or ADAM17 expression in tumors has been linked to the release of solMICA/B, decreased membrane MICA/B expression, and reduced NKG2D expression on NK cells or CD8 T cells (77, 78). Importantly, high serum levels of soluble MIC associated with poor clinical prognosis and the emergence of metastases in RCC (79) and prostate cancer (80). Most leukemia patients present high levels of at least one solNKG2D-L, associated with reduced NKG2D expression by NK cells and impaired anti-leukemic function. In patients who entered complete remission following treatment, solNKG2D-Ls were no longer detected, and NK function was restored (81). Following publication of these data, a meta-analysis of 19 studies, comprising 2,588 patients with 10 different types of tumor, showed that serum concentrations of solMICA/B represent a potential prognostic marker in human cancer (82). In metastatic melanoma, levels of solULBPs are associated with reduced survival in patients treated with immune checkpoint blockers (83); solNKG2D-Ls could thus be a relevant biomarker to select melanoma patients for immunotherapy.

## CONVENTIONAL CANCER TREATMENT AND THE NKG2D/NKG2D-L PATHWAY

The frequent and high expression of NKG2D-Ls by tumor cells in various human cancers, and the potent antitumor function of the NKG2D/NKG2D-L pathway are now established (**Figure 1**). Cancer therapies aiming to improve or restore NK- and T-cell responses through NKG2D activation have attracted considerable interest. Among the options proposed, conventional cancer treatments can be used to increase NKGD2 expression and signaling. Indeed, a number of cancer treatments increase NKG2D-L expression on tumor cells. Thus, HDAC inhibitors (Valporic acid) acting on epigenetic regulation of NKG2D-Ls can upregulate their membrane expression (84, 85). Cisplatin, Gemcitabine, Oxiplatin, or 5-fluorouracil chemotherapies have all been shown to increase MICA/B expression on tumor cells through modulation of the ATM-ATR pathway. Other

chemotherapies directly kill tumor cells while also inducing NKG2D-L expression, which stimulates tumor elimination by activating immune processes (86–89). Proteasome inhibitors inducing ULBP2 expression and decreasing HLA-I molecules may promote NK cell immunosurveillance of hematologic malignancies (90). ADAM10 or MMP inhibitors could be used to inhibit NKG2D-L-shedding and promote NK cell activity, this approach has been shown to restore NKG2D-Ls in some cancers (91–93). Similarly, antibodies targeting the proteolytic site can prevent shedding of MICA/B proteins, and was shown to limit tumor growth and reduce the formation of metastases in a humanized murine melanoma model (94).

Inhibitors of oncogene-driven mutations are actively being developed to treat various tumor types. Such inhibitors decrease the constitutive activation of kinases involved in tumor cell proliferation and also affect NKG2D-L expression (61, 95). Treatment with imatinib controls the expression of NKG2D-Ls and membrane ganglioside (GM1), and was shown to interfere with NK cell recognition and cytolysis of BCR/ABL cells (70, 71). Treatment of melanoma cells with BRAF and MEK inhibitors modulates the expression of MICA/B and ULBP2, attenuating their recognition by NK cells (96). These effects can be overcome by the simultaneous application of HDAC inhibitors, restoring NKG2D-L expression and stimulating NK cell recognition and function (97). Erk activation was shown to increase NKG2D-L expression (98, 99), but, by acting on MMP, Erk/MEK activation can disrupt the equilibrium between membrane and soluble isoforms of NKG2D-Ls and alter NKG2D function (100, 101). It would therefore be relevant to control membrane and solNKG2D-Ls when performing immunotherapy trials.

## EXPLOITING THE NKG2D/NKG2D-L PATHWAY FOR CELL THERAPY-BASED CANCER TREATMENT

One of the most promising approaches to cancer immunotherapy relies on revisited immune-cell-based therapies, T cells engineered to express chimeric antigen receptors (CARs) can be highly tumor-specific and have a high killing potential (102). CAR-T cells present a major clinical benefit for patients with malignant hemopathies. For instance, CAR-T cells were shown to be potent against CD19-expressing hematologic tumors in several trials (103, 104). However, the safety of CAR-T cells remains a major obstacle, and CARs must be optimized to increase efficacy and limit treatment-related morbidities due to cytokine-release syndrome (105). Up to now, the use of CAR-T cells for treating solid tumors has been limited by the lack of appropriate tumor antigens (106). Production of modified NK cells expressing CARs could represent an alternative for treatment of solid tumors as they recognize numerous tumor cell types. In addition, infusion of large numbers of NK cells is known not to induce autoreactivity. CAR-NK cells could thus be used in complement or as an alternative to CAR-T cells (**Figure 1**). NK cells have recently been engineered with CARs to enhance their killing activity, and trials of these cells for treatment of refractory solid tumors have been initiated (107). NK-92 cells were engineered with tumor antigen-specific CARs (EGFR, EpCAM) and successfully used in xenograft models (108, 109). NK cells have a limited lifespan and do not produce memory cells, as a result the excessive activation observed with CAR-T cells should be avoided. In addition, engineered CAR-NK cell lines could be mass-produced, circumventing the need to generate autologous products for each patient, a process that remains challenging and expensive (110). Another promising option would be to express CARs in human iPSC-derived NK cells. This approach might present several advantages, as it could provide a universal cell therapy product (111).

When considering CAR constructs the NKG2D receptor is worthy of attention. Indeed, as we have seen, NKG2D-Ls are widely expressed by a number of solid human tumors, and their relatively selective expression by transformed cells compared to healthy cells makes them an attractive receptor for CARs constructs in T cells. The extracellular domain of NKG2D is used in different CARs constructs with a view to promoting tumor-reactive T reactions (112–114). However, investigations must be performed with care as NKG2D-ligands can also be expressed by healthy cells (115), potentially leading to significant toxicity (116).

The expression of NKG2D-Ls by myeloid cells, Tregs and endothelial cells in the tumor microenvironment suggests that CAR NKG2D cells could also be used to control *in situ* immunosuppression (117). Thus, CAR-NK cells transduced with NKG2D fused to the TCR CD3z chain could be used to target suppressive myeloid cells and improve infiltration and function of subsequent infusions of tumor-specific CAR-T cells (118).

NKG2D-based CARs with full-length NKG2D or NKG2D-ligand binding domains represent a novel strategy to target several types of solid tumors, and would have the capacity to induce potent antitumor immunity in patients. NKG2D CARs could not only target tumors but also myeloid immunosuppressive cells and Tregs, as well as others cells in the microenvironment that promote tumor progression.

## AUTHOR CONTRIBUTIONS

AF, ND, and AC wrote the manuscript. AT, M-FA, MM, and LR read and corrected the manuscript.

## FUNDING

This work was supported by grants from Institut National du Cancer PAIR Melanome (2013-0662013), la Ligue Nationale contre le Cancer (PhD grant for MM).

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