# THE FUNCTIONAL ROLE OF THE ACTIVATING RECEPTORS TIM-3 AND CD16 IN HUMAN NATURAL KILLER (NK) CELL BIOLOGY

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

ADVISOR: JEFFREY S. MILLER, MD

SEPTEMBER 2012

## ACKNOWLEDGEMENTS

First and foremost I would like to extend my sincerest thanks and appreciation to my advisor Jeffrey Miller for all of his support and guidance. It has been an honor and privilege to work with such a dedicated and talented mentor. His patience, flexibility, enthusiastic encouragement and the very important “sanity checks” always helped me keep perspective and manage the demanding schedule of a graduate student. He has not only inspired a love of NK cell biology, but also a deep passion for science as a whole and I am forever grateful.

I would also like to thank the members of my thesis committee: Steven Jameson, Michael Verneris, and Bryce Binstadt. I truly appreciate their time and effort given in support of my academic progress through the MICaB PhD program and the development of my academic career. A special thank you to my committee chair (who also served as my professor and second rotation advisor) Dr. Jameson, for his generous patience in answering my many questions, insight, direction and ever so kind encouragement. I would also like to give a heartfelt thank you to Dr. Verneris for his collaboration and invaluable contributions to my thesis research as well as his genuine care and concern throughout this process always reminding me “it is not the destination, but the journey that matters”.

In addition, I would like to thank Bruce Blazar, Daniel Vallera, Angela Mortari and Sarah Cooley for their collaborative efforts. Thank you to the Verneris and Kaufman labs for all of their input during group meetings. My gratitude is also extended to the past and present members of the Miller lab, all of whom provided support and made coming to lab

everyday a joy. A special thank you to Valarie McCullar, upon whom I drew daily inspiration through her dedication and enthusiasm. She is not only a colleague, but also a mentor and friend. Her unending support and encouragement helped drive my success and to her I owe my deepest gratitude. Also, thank you to Todd Lenvik for his scientific support and lively science and non-science discussions, Diane Bjork and Louise Shand for so generously coordinating the logistical components of graduate school.

Lastly, I would like to acknowledge Ellen S. Vitetta for generously providing the RFB4 and HD37 hybridoma cell lines used to generate the CD19 and CD22 parent antibodies, which were used in experiments presented in Chapter 2. I would also like to acknowledge the work presented in Chapter 1 was supported in part by the National Institutes of Health Grant P01-CA111412-01 (JSM, SC, MRV), R01 HL55417 (JSM) and R01 CA72669 (BRB) and the work presented in Chapter 2 was supported in part by P01 CA65493, P30 CA77598, the Minnesota Masonic Charities, the US Public Health Service Grants R01-CA36725, the Randy Shaver Foundation, the Lion's Children's Cancer Fund, and the William Lawrence and Blanche Hughes Fund. In addition, the sample procurement and cell processing services from the Translational Therapy Core supported from the National Cancer Institute (P30-CA77598) and Minnesota Masonic Charities through the Cancer Experimental Therapeutics Initiative (CETI) provided support for the work presented in both Chapters 1 and 2.

## DEDICATION

It is a great honor for me to dedicate this work to my loving grandfather Samuel Bragalone. As a child he sparked my curiosity and love of science from his accounts of Albert Einstein and the general theory of relativity. While I chose to study things on a more macroscopic level, he has always been there throughout the years to listen and encourage my growth and development – academically, professionally, and personally. There are no words to express how much his love and support means to me. *Grazie per tutto e ti amo.*

I would also like to express my deepest thanks and appreciation to my family, without whom none of this would be possible: to my parents, Michael and Rebecca Gleason, for their endless love, support and encouragement; to my grandmother, Ethel Bragalone for always brightening my day with kind words and loving thoughts; to my brothers Michael, Nicholas and Tim for always making me laugh and keeping things light; to my dear friend Marta Serafini, *sei come la mia sorella e ti ringrazio per il tuo amore e sostegno*; to my aunt and Godmother Cindy, my uncle Mark, my cousin Nicole and my sister-in-laws Audrey and Carolyn for all of their care and camaraderie. Lastly, to my little loves, my nieces, nephews and Godchildren Rebecca, Nicholas, Grady, Carol and Quinnlyn, who have brought more joy to my life than I could have ever imagined.

## ABSTRACT

Human natural killer (NK) cells are lymphocytes that develop in the bone marrow from hematopoietic progenitor cells (HPCs) and are also found in the lymph nodes, spleen and peripheral blood (PB), where they comprise 10-15% of the mononuclear cell fraction. PB NK cells are phenotypically defined as expressing the surface receptor CD56 (NCAM, neural cell adhesion molecule) and lacking expression of CD3. They mediate their function through the exocytosis of granules that contain lytic enzymes such as perforin and granzymes, the expression of death receptor ligands, the expression of FcRγIIIA (CD16, a mediator of antibody-dependent cell-mediated cytotoxicity or ADCC), and the secretion of cytokines and chemokines. As a result, NK cells take part in both the innate and adaptive immune responses and have critical roles in the control of early viral infection, hematopoietic cell transplantation (HCT) and tumor immunosurveillance.

The ability of NK cells to differentiate normal healthy cells (self) from infected or transformed (non-self) cells is regulated by a sophisticated repertoire of cell surface receptors that control their activation, proliferation and effector functions. The net balance of inhibitory and activating signals transmitted by these receptors determines whether an NK cell will eliminate its target. There are three main inhibitory receptor families, all of which recognize major histocompatibility complex (MHC) class I molecules as their ligands: killer immunoglobulin (Ig)-like receptors (KIRs), the heterodimer CD94/NKG2A receptor and the leukocyte Ig-like receptor-1 (LIR-1). KIRs are a polymorphic set of proteins that belong to the Ig superfamily and recognize with allele specificity the classical MHC class I ligands: human-leukocyte antigen (HLA)-A, HLA-B and HLA-C. CD94/NKG2A is a member of the C-type lectin family of NK

receptors and has binding specificity for the non-classical MHC class I molecule HLA-E. Like KIRs, LIR-1 is also a member of the Ig superfamily, but displays a binding specificity for a broad spectrum of classical (HLA-A, HLA-B and HLA-C) and non-classical (HLA-E, HLA-F and HLA-G) MHC class I molecules. These inhibitory receptors facilitate the "self" versus "non-self" recognition strategy termed the "missing-self" hypothesis initially described by Ljunggren and Kärre, which states that NK cells mediate their function through the recognition of autologous cells that have lost or altered self-MHC class I expression, characteristic of infected or tumor-transformed cells. In addition to inhibitory receptors, NK cells also contain activating receptors in their repertoire, some of which include the following: natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, the heterodimers CD94/NKG2C/E, the homodimer NKG2D and FcγRIIIA (CD16). NK cell activating receptors have multiple ligands with NKG2D recognizing the polymorphic MHC class I-related chain (MIC)A and MICB molecules and UL-16 binding proteins (ULBPs), the NCRs recognizing viral hemagglutinins and membrane associated heparan sulfate proteoglycans, the heterodimeric receptors CD94/NKG2C/E recognizing the non-classical MHC class I molecule HLA-E and the low affinity Fc receptor CD16 recognizing the Fc portion of IgG antibodies. Recently, NK cells have been described to express a novel immune receptor Tim-3 (T cell immunoglobulin and mucin-containing domain-3), which recognizes an S-type β-galactoside binding lectin galectin-9 (Gal-9). The work presented in this manuscript focuses on the modulation of NK cell effector function by two of these activating receptors, namely Tim-3 and CD16, and their potential for enhancing the therapeutic effects of NK cells.

Initially Tim-3 was identified as a specific cell marker of terminally differentiated $CD4^+$ T helper type 1 (Th1) cells, with lower density expression on Th17 cells and no expression

on Th2 cells. Recent work has shown, however, that Tim-3 is also expressed on $CD8^+$ T cells, dendritic cells (DCs) and NK cells. The functional role of the Tim-3/Gal-9 pathway was first described as a mechanism to negatively regulate the Th1 response, inhibiting IFN-γ production and inducing cell death. In contrast, stimulation of $Tim\text{-}3^+$ DCs has been subsequently shown to induce the secretion of pro-inflammatory cytokines, suggesting Tim-3/Gal-9 interactions are capable of mediating both inhibitory and activating immune responses.

While the presence of Tim-3 on NK cells has been identified, the functional relevance of this is poorly understood. We show that Tim-3 functions to increase IL-12 and IL-18 primed NK cell IFN-γ production in the presence of soluble recombinant human Gal-9 (rhGal-9), Raji tumor cells engineered to express Gal-9 and, most importantly, primary acute myelogenous leukemia (AML) human targets that endogenously express Gal-9. We demonstrate this effect is highly specific as Tim-3 antibody blockade significantly decreases IFN-γ production. Furthermore, we definitely show an activating function for Tim-3 as antibody crosslinking induced intracellular $Ca^{2+}$ mobilization, ERK activation and degradation of the NFκB inhibitior IκBα. Consequently, we conclude that Tim-3 functions as a co-activating receptor to enhance IFN-γ production in IL-12 and IL-18 primed NK cells and functions to enhance $Gal\text{-}9^+$ tumor targeting and recognition, which has important implications for the NK cell anti-tumor response.

The ability of CD16 to mediate ADCC is well understood and monoclonal antibody (mAb) therapies for the treatment of cancer have been developed to harness the therapeutic potential of NK cell-mediated tumor cytotoxicity through engagement of this potent

activating receptor. Treatment with the mAb rituximab, which targets the B-cell antigen CD20 on tumor cells, has been shown to be beneficial for the treatment of the most common adult hematologic malignancy, Non-Hodgkin's lymphoma (NHL). Despite success, clinical and pre-clinical studies have highlighted several limitations that decrease the overall efficiency of mAb therapies. Consequently, novel single chain fragment variable (scFv) recombinant reagents termed bispecific and trispecific killer cell engagers (BiKEs and TriKEs) that lack the Fc portion of whole antibodies and specifically target CD16 expressed on effector NK cells and antigens-of-interest on tumor cells are being developed and tested for clinical use. Here, we demonstrate the mechanism by which a CD16/CD19 BiKE and a novel CD16/CD19/CD22 TriKE function to trigger human NK cell activation and their ability to induce cytotoxicity and chemokine/cytokine production against primary B-cell acute lymphoblastic leukemia (ALL) and B-cell chronic lymphocytic leukemia (CLL) leukemia targets overcoming inhibition by MHC class I molecules. Furthermore, we demonstrate that each BiKE and TriKE reagent is capable of eliciting a unique chemokine/cytokine NK cell response against tumor targets. Collectively, our data reveal an important role for CD16-directed bispecific and trispecific scFv reagents in mediating NK cell target recognition and effector signaling, which are crucial for enhancing the specificity of NK cell therapy and ultimately enhanced clinical efficacy.

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- Galileo Galilei

("*All truths are easy to understand once they are discovered; the point is to discover them*.")

# INTRODUCTION

## ***Human NK cell development***

Natural killer (NK) cells are large, granular lymphocytes part of the innate immune system. They comprise 10–15% of all circulating peripheral blood mononuclear cells (PBMC) and are critical for host protection against pathogen-infected or malignantly transformed cells. Mature NK cells are minimally identified by the surface expression of CD56 (NCAM-1) and the lack of expression of CD3(1). Sharing the same developmental precursor as B- and T-cells, human NK cells arise from the common lymphoid progenitor (CLP), as defined by the classical model of hematopoiesis or the lymphoid-myeloid progenitor (LMP) in the revised model of hematopoiesis, found in the bone marrow(2-4). Their presence in SCID (severe combined immunodeficiency), RAG-1- or -2- deficient and athymic nude mice demonstrates that NK cell development does not require the molecular mechanisms necessary for antigen receptor gene rearrangement and suggests the NK cell developmental pathway is unique from that of B- and T-lymphocytes(5-10). Fives stages of human NK cell development have been characterized (**Figure 1**) from bone marrow (BM), umbilical cord blood (UCB) and secondary lymphoid tissue (SLT)-derived $CD34^{+}$ hematopoietic progenitors through analysis of cell surface antigen expression and *ex vivo* culture studies, and are collectively defined as pro-NK (stage 1), pre-NK (stage 2), immature iNK (stage 3), $CD56^{bright}$ NK (stage 4) and $CD56^{dim}$ NK (stage 5) cells(11-16).

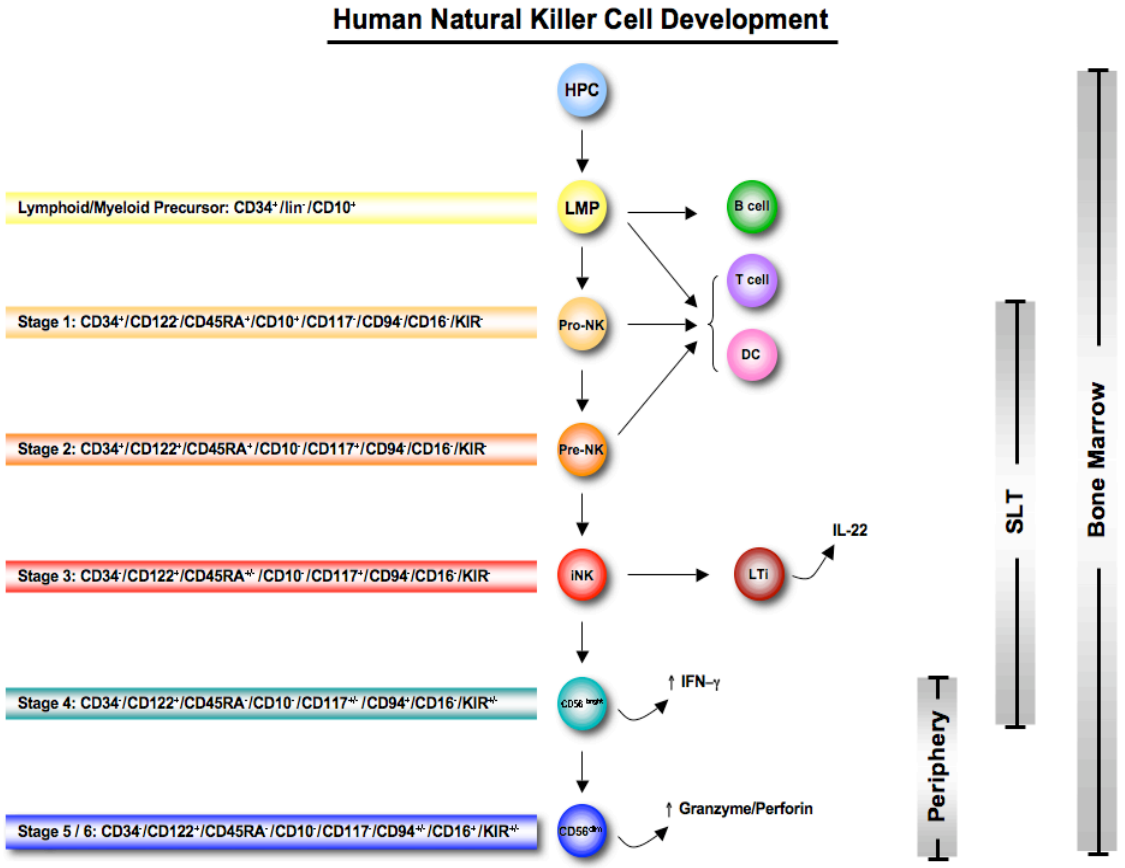


**Figure 1: Schematic representation of human natural killer cell development.** Committed NK cell precursors develop from hematopoietic progenitor cells (HPC) in the bone marrow (BM) and subsequently proceed through distinct developmental stages, which may occur in the BM, secondary lymphoid tissues (SLT) and/or periphery.

*Stage 1: pro-NK cells*

Early studies demonstrated the ability of IL-15 non-responsive $CD34^+$ BM-derived hematopoietic progenitor cells (HPCs) to acquire the expression of the shared IL-2/IL-15 receptor β-chain (CD122) upon *in vitro* culture with flt3 ligand (FL), c-kit ligand (KL), IL-6 and IL-7 and subsequently differentiate into NK cells in response to IL-15(13, 17, 18). A two-step model of early NK cell development was thus established where the

$CD34^+/CD122^-$ population was defined as NK cell progenitors (pro-NK) that were capable of directly generating $CD34^+/CD122^+$ IL-15 responsive pre-NK cells(19). Freud *et al*. later identified a population within human SLT that closely resembled BM HPC-derived pro-NK cells and further defined them as $CD34^+/CD122^-/CD45RA^+/CD117^-/CD94^-$ stage 1 pro-NK cells(15). Upon stimulation with FL, IL-3 and IL-7 the stage 1 pro-NK cells gave rise to stage 2 pre-NK cells, which in response to IL-15 had the capacity for NK cell differentiation(15). Moreover, the pro-NK cells were found to be $CD10^+$ and had the ability to give rise to T-cells and myeloid dendritic cells (DCs), closely resembling the BM-derived CLP(3, 15).

*Stage 2: pre-NK cells*

The acquisition of CD122 and the functional ability to respond to soluble IL-15 and differentiate into stage 3 iNK cells without the addition of other cytokines and in the absence of stroma are defining characteristics of stage 2 pre-NK cells. Examination of the surface antigen expression profile within this population determined a $CD34^+/CD122^+/CD45RA^+/CD117^+/CD94^-$ identifying phenotype with uniform expression of CD33, CD44, HLA-DR, integrin $\alpha_4\beta_7$, CD1a, CD5, CD123 and heterogeneity in the expression of CD2, CD7, CD10, CD56 and CD161(15). Furthermore, *in vitro* cultures of stage 2 pre-NK cells gave rise to T-cells and myeloid DCs, suggesting the acquisition of CD122 is not synonymous with human NK cell lineage commitment and plasticity remains in at least a portion of the pre-NK cell population(15).

*Stage 3: iNK cells*

In contrast to stage 1 and stage 2 populations, *in vitro* differentiation assays revealed stage 3 cells completely lack T- cell and DC lineage potential, but have the capability to

consistently generate stage 4 $CD56^{bright}$ NK cells(15). Phenotypic profile analysis further showed stage 3 cells do not express T-cell (CD1a, CD3, CD4, CD5, T-cell receptor (TCR) $\alpha\beta$, TCR $\gamma\delta$), B-cell (CD19) and DC (CD14, CD123, Blood DC antigen (BDCA)-1, BDCA-2, BDCA-3, DC-SIGN, CD80, CD83, CD86) lineage-associated antigens(15). In addition, they also lack a majority of mature NK cell surface receptors (CD94/NKG2 heterodimeric receptors, NKG2D, NKp46, CD16 and killer cell immunoglobulin-like receptors [KIRs]), but do express NK cell-associated antigens CD2, CD7, CD56, CD161, 2B4 and NKp44 and have high expression of the transcription factors ETS-1 and GATA-3, which are specifically required for NK cell development and maturation(15, 20, 21). Despite NK-cell lineage commitment and the expression of functional receptors, stage 3 cells lack perforin expression and are incapable of mediating perforin-dependent cellular cytotoxicity against the major histocompatibility complex class-I (MHC-I)-negative K562 target cells. Furthermore, stimulation with a combination of cytokines or phorbol myristate acetate (PMA) and ionomycin does not induce the production of IFN-$\gamma$(15). However, *in vivo*-derived iNK cells can produce GM-CSF(15) and *in vitro*-derived iNK cells have the ability to produce type 2 cytokines IL-5 and IL-13, which is subsequently lost upon further maturation to $CD56^{bright}$ NK cells, and lyse MHC-I positive targets via a TRAIL (tumor-necrosis factor-related apoptosis inducing ligand)-dependent mechanism(22, 23). Interestingly, recent studies have further characterized the iNK cell population from SLT and have shown that the majority are $CD127^{+}$/$CD117^{+}$, express the transcription factor RORC and give rise to IL-22 secreting lymphoid tissue–inducer (LTi) cells, which are non-cytotoxic and do not produce IFN-$\gamma$(24-27). These LTi cells are thought to be distinct from conventional NK cells, which arise from the $CD127^{-}$ /$CD117^{+}$/$RORC^{-}$ iNK cell fraction, but developmentally related.

*Stage 4: CD56$^{bright}$ NK cells*

From the time of their initial description, it has been suggested CD56$^{bright}$ NK cells represent a developmental intermediate of terminally mature NK cells(28, 29). This hypothesis is supported by the following observations: (i) only the CD56$^{bright}$ population of peripheral blood (PB) NK cells express CD117, a receptor typically expressed by immature HPCs(30); (ii) *in vitro* culture of CD34$^{+}$ HPCs in IL-2 or IL-15 primarily leads to the generation of CD56$^{bright}$/CD16$^{-}$/KIR$^{-}$ NK cells(31-33); (iii) *in vitro* culture of isolated CD56$^{bright}$/CD16$^{-}$/KIR$^{-}$ cells in the presence of cytokines and stromal cells give rise to CD56$^{dim}$ KIR-expressing cells(34-36); and (iv) donor-derived CD56$^{bright}$ NK cells are the first to appear in the recipient's blood post hematopoietic cell transplantation (HCT) *in vivo*(37-40). Indeed, developmental progression of iNK cells to stage 4 is marked by the acquisition of mature NK cell receptors CD94/NKG2A, NKG2D, NKp46, CD27 and high levels of CD56, the downregulation of CD117, CD33 and CD127 and the induction of the functionally relevant transcription factor T-bet(15, 41-43). Moreover, PB and SLT Stage 4 CD56$^{bright}$ developing NK cells express perforin and are capable of inducing natural cytotoxicity against MHC-I-negative and MHC-I-positive targets in a perforin- and Fas ligand-dependent manner(15, 44). Furthermore, CD56$^{bright}$ NK cells have low expression levels of the phosphatase SHIP-1 and high expression levels of the phosphatase inhibitor SET, which is thought to mediate a lower activation threshold for cytokine secretion(45, 46). Accordingly, CD56$^{bright}$ NK cells are efficient producers of cytokine and chemokines, such as IFN-$\gamma$, TNF-$\alpha$, GM-CSF, MIP-1$\alpha$, MIP-1$\beta$, IL-10, IL-13(40).

*Stage 5: CD56$^{dim}$ NK cells*

Fully mature CD56$^{dim}$ NK cells posses a full repertoire of functional receptors, some of

which include KIR, the low affinity Fc receptor CD16, NKG2D, CD94/NKG2A, CXCR1, CD2 and LFA-1, an abundance of lytic granules that contain perforin and granzymes and are potent mediators of natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC)(47). Early identification of two functionally distinct PB NK cell populations based on the expression of CD56, namely $CD56^{bright}$ and $CD56^{dim}$, suggested the $CD56^{dim}$ population represented the terminally mature cell fraction as the acquisition of CD16 and KIRs are considered late-occurring events both *in vitro* and *in vivo* during NK maturation(11, 29, 36, 39, 48-50). As mentioned above, direct evidence for this has been shown as $CD56^{dim}/CD16^{+}/KIR^{+}$ cells are generated in culture from $CD56^{bright}/CD16^{-}/KIR^{-}$ cells. Moreover, Cooley et al. demonstrated the $CD56^{dim}$ population could be further separated into discrete functional subsets based on the expression of NKG2A and KIR, specifically $NKG2A^{-}/KIR^{-}$, $NKG2A^{+}/KIR^{-}$, $NKG2A^{+}/KIR^{+}$ and $NKG2A^{-}/KIR^{+}$(36). They showed the $NKG2A^{-}/KIR^{-}$ subset makes up ~20% of circulating NK cells in the peripheral blood of healthy individuals with a frequency of ~30% for the $NKG2A^{+}/KIR^{-}$ subset, ~15% for the $NKG2A^{+}/KIR^{+}$ subset and ~35% for the $NKG2A^{-}/KIR^{+}$ subset. Functional analysis of each subset revealed $NKG2A^{-}/KIR^{-}$ cells were hyporesponsive to cytokine and target cell stimulation compared to the other subpopulations, showing reduced proliferation capacity, IFN-$\gamma$ production and cytotoxicity. Furthermore, culture of the $NKG2A^{-}/KIR^{-}$ subpopulation with IL-15 and stromal cells resulted in the acquisition of both NKG2A and KIR as well as effector functions, suggesting $CD56^{dim}/NKG2A^{-}/KIR^{-}$ cells are precursors for the more mature $CD56^{dim}$ NKG2A-expressing and/or KIR-expressing populations. Consequently, the stage 5 $CD56^{dim}$ NK cells described by Freud et al. are suggested to be more appropriately divided into two steps with stage 5 corresponding to the hyporesponsive $CD56^{dim}/NKG2A^{-}/KIR^{-}$ population (immature $CD56^{dim}$) and a stage 6 that is marked by

the acquisition of a functional inhibitory receptor, namely NKG2A and/or KIR (mature $CD56^{dim}$). Moreover, recent studies have further delineated the heterogenous $CD56^{dim}$ population using the markers CD62L and CD57. Juelke and colleagues demonstrated that the $CD56^{dim}/CD62L^{+}/NKG2A^{+}$ subpopulation represents an earlier developmental intermediate of the terminally differentiated mature $CD56^{dim}$ NK cell population as they retain the high potential to produce IFN-γ and proliferate after cytokine stimulation, similar to stage 4 $CD56^{bright}$ NK cells, as well as having the capacity to kill after activating receptor stimulation, reminiscent of mature stage 5 $CD56^{dim}$ NK cells(51). Lopez-Vergès and colleagues revealed that CD57 marks the terminally differentiated $CD56^{dim}/CD16^{+}/KIR^{+}/LIR\text{-}1^{+}$ population with the $CD56^{dim}/CD57^{-}/CD16^{+}$ subpopulation having functional and phenotypic characteristics of earlier developmental intermediates that have high expression of NKp46, NKG2D, NKp30, NKG2A, $CD27^{+}$ and $CD62L^{+}$(52).

*Sites of human NK cell development*

While there are clear differences between mouse and human NK cell biology, murine NK cell studies have led the way in delineating NK cell differentiation, function and regulation and have proven to be an invaluable preclinical model for understanding and establishing biologic paradigms for human translation. There is evidence that NK cells develop and mature at multiple sites. Studies using athymic, nude mice revealed NK cells still exist in normal numbers, demonstrating NK cell development, unlike T-cell development, does not require the thymus(9, 10, 53). However, thymic NK cells have been identified and are generated from bipotent T/NK cell early thymic progenitors (ETP)(54-56). More recently a CD127−expressing NK cell population has been described that arises independently from T-cell precursors in the thymus through a

GATA-binding protein-3 (GATA3) dependent pathway(57). Furthermore, a subpopulation of early human intrathymic $CD34^+CD1a^-$ progenitor cells expressing the type IA bone morphogenetic protein receptor (BMPRIA) has been identified to contain a large proportion of NK cell lineage–committed precursor cells that differentiate into functional NK cells when cultured on stroma and in the presence of KL and IL-15(58). Nevertheless, the primary location for early NK cell development in adults, such as the generation of $CD34^{dim}/CD10^+/CD45RA^+$ pro-NK cells from HPCs, is thought to occur within the BM, the residing location of the $CD34^+/Lin^-/CD10^+$ CLP(3). Indeed, mouse studies have revealed NK cell development is markedly impaired upon bone marrow ablation by β-estradiol or radioactive strontium treatment(59, 60). Moreover, the BM microenvironment is rich in cytokines and growth factors and contains stromal cells that are important for NK cell development(17, 31, 61-64). Interestingly, it was found that pro-NK cells constitute <1% of BM, ~6% of PB and >95% of lymph node (LN) $CD34^+$ HPCs and accordingly express the SLT homing molecules CD62L and integrin β7(12, 15). Furthermore, the pre-NK cell, iNK cell and $CD56^{bright}$ NK cell populations have also been found to be enriched in SLT, while the $CD56^{dim}$ NK cell population is more frequently found in the BM, PB and spleen(15, 65, 66). This suggests early NK cell progenitors migrate from the BM to SLT, where there is an abundance of DCs with membrane bound IL-15 critical for development and maturation. This is further supported by the fact that pro-NK cells found in LN reside in parafollicular T-cell regions, where IL-2 produced during T-cell activation may contribute to NK maturation as stages 2-4 constitutively express high-affinity IL-2Rαβγ complexes(12, 65-68). Moreover, exposure of LN pro-NK cells to exogenous IL-2 or IL-15 generated $CD56^{bright}$ NK cells, which are also found in the T-cell parafollicular regions(12). As developmental intermediates progress to stage

5 $CD56^{dim}$ NK cells, CD62L and CCR7 expression is downregulated and CD69 is upregulated(69). The S1P receptor $S1P_5$ has been shown in mice to be expressed on CD69-expressing NK cells and is important in mediating NK cell egress from BM and LN(70). Therefore, these dynamic changes in receptor expression likely contribute to mature NK cell trafficking out of developmental sites. However, as $CD56^{bright}$ NK cells are also found in PB alongside $CD56^{dim}$ NK cells, the final steps of maturation may not strictly occur in SLT and could also take place in circulation.

*Transcription factors involved in human NK cell development*

Loss-of-function studies using gene-deficient mice and gain-of-function studies performed with human $CD34^+$ HPCs have revealed a number of transcription factors (TFs) are involved in NK cell development and can be placed into three groups: (1) early development/lineage commitment, (2) maturation and (3) function (**Table I**).

The first group of TFs is thought to have determinant roles in NK cell lineage-specification and maintenance of committed NK cell progenitors and includes Ikaros, PU.1, Ets-1, VDUP-1, Id2 and E4BP4. Ikaros was initially demonstrated by Geogopoulos et al. to be important for the generation of lymphoid precursors from HPCs as mice bearing germline mutations in the Ikaros DNA-binding domain lacked NK cells, T- and B-cells, while the erythroid and myeloid lineages remained intact(71). Subsequent studies using Ikaros-deficient NK cell precursors revealed an inability to express c-kit and CD122 when exposed to NK cell differentiation- and proliferation-promoting culture conditions, which resulted in a drastic reduction in mature NK cell numbers(72).

PU.1 is another TF important in NK cell development. It has been demonstrated that

while BM chimeric PU.1$^{-/-}$ Rag2$\gamma$c$^{-/-}$ mice lack T- and B-cells, they are able to generate NK cells(73). However, a markedly reduced number of committed NK cell progenitors, which lacked IL-7R$\alpha$ and c-kit expression, was observed and accordingly there was a large decrease in the peripheral NK cell pool(74, 75). Interestingly, the NK cells that were present in the periphery largely maintained mature NK cell features, both phenotypically and functionally(73). Therefore, while PU.1 is not required for NK cell lineage commitment, it appears to have an important role in mediating NK cell survival and expansion early in development and may be expendable once NK cells have fully matured.

**Table I: Transcription factors involved in NK cell development, maturation and function**

| Group | Transcription Factor | Function |
|---|---|---|
| Group 1 | Ikaros | Early lymphoid precursor factor and promotes early c-kit and CD122 expression |
| | PU.1 | Promotes survival and expansion early in development, IL-7R$\alpha$ and c-kit expression |
| | Ets-1 | Essential early development factor and promotes CD122 expression |
| | VDUP-1 | Promotes early development and CD122 expression |
| | Id2 | Promotes lineage commitment and developmental maturation |
| | E4BP4 | Promotes lineage commitment |
| Group 2 | GATA-3 | Promotes IFN-$\gamma$ production, homing to the liver and developmental maturation |
| | T-bet | Promotes IFN-$\gamma$ production and developmental maturation |
| | IRF-2 | Promotes survival of mature NK cells, cytolytic function and IFN-$\gamma$ production |
| Group 3 | MEF | Promotes developmental maturation, regulates perforin gene expression and promotes IFN-$\gamma$ production |
| | MITF | Promotes IL-12R$\beta$ and IL-18R$\alpha$ expression, IFN-$\gamma$ production and regulates perforin gene expression |
| | CEBP$\gamma$ | Promotes IFN-$\gamma$ production and cytolytic function |

Ets-1 is an essential TF for NK cell development. Early studies using an Ets-1 knockout mouse model demonstrated specific depletion of NK cells in the BM, LN and spleen while T- and B-cell lineages were maintained(21). Furthermore, cytolytic activity and cytokine production of Ets-1-deficient NK cells were defective and could not be rescued by exposure to exogenous cytokines IL-2, IL-12 or IL-15(21). In both humans and mice Ets-1 has been demonstrated to be a transcriptional regulator of the IL-15/IL-2Rβ gene and has a critical role in the induction of CD122 and IL-15/IL-2 responsiveness(76, 77). Moreover, a positive-feedback loop that promotes the NK cell lineage has been established between IL-15 and Ets-1 where Ets-1 induced expression of IL-15Rβ allows signaling via the IL-15R, which further promotes the induction of Ets-1 and hence IL-15Rβ expression and IL-15 signaling(78).

Another TF shown to be important for the generation of NK cells is vitamin D3 upregulated protein-1 (VDUP-1). Lee et al. demonstrated that VDUP-1-deficient mice have a profound reduction in NK cell numbers in the BM, LN and spleen while displaying relatively normal T- and B-cell development(79). Evaluation of NK cell maturation from VDUP-1-deficient developing NK cells revealed an inability to produce phenotypically mature NK cells, which corresponded with a significant impairment in cytotoxicity and IFN-γ production. *In vitro* cultures of VDUP-1-deficient HPCs in NK cell-differentiation conditions generated committed NK cell progenitors that could not progress in maturation due to a lack of CD122 expression. Moreover, ectopic expression of VDUP-1 directly increased CD122 promoter activity, suggesting VDUP-1 plays an important role promoting the developmental progression of NK lineage-committed cells through the regulation of CD122 expression and, thus, IL-15/IL-2 responsiveness.

While Id2 is involved in the regulation of NK cell development, it is not a transcription factor as it belongs to the inhibitors of DNA binding family of proteins that lack a DNA binding domain(80). Early studies showed that targeted disruption of the Id2 gene led to defective NK cell development without disruption of the T- and B-cell compartments(81, 82). Furthermore, Id2-deficient HPCs transferred into irradiated wild-type mice were not able to differentiate into NK cells while wild-type HPCs transferred into irradiated Id2-deficient mice efficiently generated NK cells(81). These results suggested Id2 functions intrinsically within the cell to promote NK cell development. Indeed, Id2 exerts its function by forming heterodimers with transcriptional regulator E-proteins, preventing them from binding to DNA and thus inhibiting their activity(80). It was demonstrated that overexpression of E-proteins E2A and HEB excessively promoted B- and T-cell development(80) while overexpression of Id2 inhibited B- and T- cell development and favored NK cell development(83). Moreover, deletion of E2A from Id2-deficient mice restored the development of mature NK cells in the BM further demonstrating the ability of Id2 to stimulate NK cell development by sequestering NK cell-inhibitory E-proteins(84). Although initial studies have proposed that Id2 is required for NK cell lineage commitment from HPCs(81, 82), a more recent study by Boos et al. found normal numbers of committed NK cell progenitors in the BM of Id2-deficient mice. The ability of these NK cell precursors to develop into mature NK cells, however, was blocked, suggesting Id2 is not essential for NK cell lineage specification, but is required for NK cell maturation(84).

The basic leucine zipper transcription factor E4BP4 (also called NFIL3) was recently suggested to be an NK cell lineage-specifying factor. Studies using E4BP4-deficient

mice showed dramatic decreases in mature NK cell numbers, while T- and B-cell development appeared unaffected(85-87). Adoptive transfer experiments using HPCs from wild-type mice transferred into sub-lethally irradiated E4BP4-deficient mice efficiently generated NK cells, suggesting a cell-intrinsic requirement for E4BP4 in NK cell development(85). Moreover, ectopic expression of E4BP4 in E4BP-4 deficient and wild-type HPCs revealed complete restoration of NK cell development from E4BP4-deficient HPCs and a lack of a proliferation or survival advantage in wild-type HPCs, demonstrating the role of E4BP4 in NK cell development functions to promote NK cell lineage-specification of HPCs rather than survival and expansion of developing NK cells(85). However, E4BP4 expression was shown to remain high throughout NK development, suggesting it may also play a role in maintaining mature NK cell function. Further evidence supporting an early developmental role for E4BP4 demonstrated *ex vivo* addition of IL-15 could not rescue NK cell development in E4BP4-deficient HPCs, showing E4BP4 functions downstream of the IL-15R in immature NK cells(85). In addition, loss of E4BP4 led to decreases in GATA-3 and Id2 expression, which were rescued with E4BP4 overexpression and suggests GATA-3 and Id2 expression is directly or indirectly regulated by E4BP4(85).

The second group of TFs, which includes GATA-3, T-bet and IRF-2, appears to function primarily in NK cell maturation and the migration process of NK cells to the periphery. While both committed NK cell progenitors and mature NK cells express GATA-3(88, 89), GATA-3-deficient hematopoietic chimeras revealed GATA-3 to be dispensable for NK cell development and cytolytic function of mature NK cells(20). However, IFN-$\gamma$ production was severely impaired in GATA-3-deficient NK cells, which corresponded to a decrease in T-bet levels, suggesting T-bet functions downstream of GATA-3. Moreover,

despite intact cytolytic function, GATA-3-deficient NK cells displayed an incomplete repertoire of activating and inhibitory receptors that resembled the phenotype of an immature NK cell and correlated with impaired cytokine production(20). GATA-3-deficient chimeras also revealed a marked reduction in hepatic NK cells and adoptive transfer of GATA-3-deficient HPCs in Rag2$\gamma c^{-/-}$ mice showed defective NK cell homing to the liver(20).

Unlike GATA-3, studies using T-bet knockout mice revealed a global deficiency in peripheral NK cells and an increased number in the BM(42). While T-bet-deficient NK cells expressed markers found on immature NK cells, namely c-kit and B220, they maintained a normal repertoire of activating and inhibitory receptors. Furthermore, there was a block in NK cell terminal maturation in T-bet-deficient mice despite normal expression levels of CD122 and a retained ability to respond to IL-2 and IL-15(42). Interestingly, T-bet-deficient NK cells were able to rapidly secrete IFN-$\gamma$ upon cytokine stimulation. However, after 24-hours of stimulation only T-bet competent NK cells maintained the ability to produce IFN-$\gamma$, suggesting the early and rapid production of IFN-$\gamma$ is T-bet independent while sustained NK cell IFN-$\gamma$ secretion requires T-bet(42, 90). In contrast, NK cell cytotoxicity was minimally affected in T-bet-deficient NK cells(42).

IRF-2 is a transcriptional repressor part of the interferon regulatory factor family of proteins(91). Studies using IRF-2-deficient mice revealed a large decrease in the number of peripheral NK cells while BM NK cell numbers were increased(92, 93). Lohoff et al. showed NK cells developing from IRF-2-deicient HPCs adoptively transferred into irradiated hosts were phenotypically immature and unable to traffic out of the BM to the

periphery. Interestingly, despite normal CD122 expression, IRF-2-deficient developing NK cells were unable to proliferate in response to IL-15(92). Conversely, a more recent study revealed the reduction in peripheral NK cells in IRF-2-deficient mice was due to a selective loss of mature NK cells and not to an arrest in maturation as IRF-2-deficient NK cells in the BM showed a relatively mature phenotype and could proliferate normally, but underwent accelerated apoptosis(93). Consequently, IRF-2 is suggested to play a role in the survival of mature NK cells, preventing premature cell death and allowing for appropriate localization. Furthermore, IRF-2 is thought to also be required for NK cell function as both cytotoxicity and IFN-γ production was severely impaired in IRF-2-deficient NK cells(92).

The third group of TFs includes myeloid elf factor (MEF), microphtalmia-associated transcription factor (MITF) and CCAAT/enhancer binding protein-γ (CEBPγ), all of which play a role in promoting NK cell effector function. MEF is part of the ETS-family of proteins and has been shown to be critical in the development and function of NK cells. MEF-deficient mice displayed reduced numbers of peripheral NK cells that were functionally impaired in both IFN-γ secretion and cytotoxicity(94). Furthermore, MEF was shown to directly regulate perforin gene expression in NK cells and MEF-deficiency resulted in a lack of perforin-containing granules, corresponding to the killing defects.

In mice MITF is encoded by the *mi* locus and early studies examining the effects of abnormal MITF, due to *mi* mutant alleles, demonstrated a profound reduction in NK cell cytotoxicity(59, 95, 96). Analysis of the lytic machinery in NK cells expressing mutant-MITF revealed normal expression of granzyme B, but a lack of perforin within the

cytotoxic granules(97). It was subsequently revealed that mutant-MITF binds to essential transcription factors that drive perforin gene expression and causes their cytoplasmic retention, disrupting nuclear localization and perforin gene expression(97). Furthermore, mutant-MITF expressing NK cells displayed impaired IFN-γ responses to stimulation with IL-12, IL-18, and a combination of IL-12 and IL-18, which was shown to be a result of decreased IL-12Rβ and IL-18Rα expression levels(98). Mutant-MITF has been demonstrated to interact with PEBP2/CBF(99), a transcription factor involved in IL-12Rβ and IL-18Rα gene expression, and thus, it thought that mutant-MITF functions to disrupt IL-12Rβ and IL-18Rα expression by inhibiting PEBP2/CBF transactivation of the gene promoter region.

CEBPγ has been shown to be dispensable for early NK cell development. CEBPγ-deficient mice have normal generation of NK cells in the BM and periphery, but produce mature NK cells that display significant functional defects, suggesting CEBPγ is critical for the late stages of functional maturation(100). Kaisho et al. demonstrated that CEBP-γ-deficient NK cells have reduced cytotoxicity even in the presence of IL-2 cytokine stimulation and impaired IFN-γ production in response to IL-12 and IL-18 stimulation(100). Both IL-12R and IL-18R expression levels were found to be normal and the IL-12 and IL-18 signaling pathways were largely intact in CEBPγ-deficient NK cells. It is, therefore, thought that CEBPγ may play a direct or indirect role in IFN-γ gene regulation.

*Cytokines involved in human NK cell development*

A number of cytokines and cytokine combinations have been identified that can support

human NK cell development *in vitro* from $CD34^+$ HPCs and include FL, KL, IL-2, IL-3 IL-7, IL-12, IL-15 and/or IL-21(101). It has been shown that IL-2 and IL-15 can synergistically act with FL, KL, IL-3 IL-7, IL-12, IL-15 and IL-21 *in vitro* to mediate NK cell lineage commitment, differentiation and expansion of early developing NK cells and promote the survival, proliferation and maturation of late stage developing NK cells(11, 13, 15, 18, 31, 48, 102). While stage 4 $CD56^{bright}$ NK cells can be generated *in vitro* by common gamma ($\gamma_c$) chain-signaling cytokines IL-2, IL-15 and, to a lesser extent, IL-7 without the addition of other cytokines and in the absence of stroma, they have been demonstrated to be phenotypically and functionally immature compared to *in vivo*-derived PB $CD56^{bright}$ NK cells (12, 31, 33, 61, 103, 104). Studies in mice and humans lacking IL-15 or components of its receptor (IL-15R$\alpha$, $\beta$ or $\gamma_c$) show profound NK cell deficiencies that are not seen with the absence of IL-2 or IL-7(105-111). Furthermore, SCID patients that lack the $\gamma_c$ – chain receptor component have a complete absence of NK cells, whereas IL-2-deficient and IL-2R$\alpha$-deficient patients have normal numbers of NK cells(19, 107, 112, 113). Consequently, IL-15 is regarded as the most central and critical cytokine that supports NK cell development as well as homeostasis(114, 115).

While *in vitro* NK cell differentiation and proliferation can be supported by high-dose soluble IL-15(31), *in vivo* IL-15 primarily exists in a complex with membrane bound IL-15R$\alpha$ found on accessory cells(116, 117). The IL-15-IL-15R$\alpha$ complex is thus presented in *trans* by the accessory cell to the recipient NK cell, which expresses CD122 and the $\gamma_c$ chain, and this *trans*-presentation has been shown to be essential for mature NK cell survival(114, 118, 119). Furthermore, a study by Minagawa et al. demonstrated that overexpression of Bcl-2 in CD122-deficient mice could restore the generation of NK

cells, but not their cytolytic functions, suggesting that IL-15 signaling may act on late-stage developing NK cells to induce the acquisition of cytotoxic capabilities(120). In humans, CD122 expression *in vivo* is detectable at the mRNA level in stage 2 pre-NK cells and at the protein level in stage 4 $CD56^{bright}$ NK cells(15). As stage 3 iNK cells have high surface expression levels of CD117 (c-kit) and CD127 (IL-7R$\alpha$), it is thought that other cytokines such as KL and IL-7 may support the survival of early developing human NK cell intermediates while IL-15 may act at a later stage in development to support maturation and function(15). Moreover, it has been demonstrated that uncontrolled signaling through the IL-15/IL-2 receptor *in vivo* leads to the development of T/NK cell leukemia and can override self-tolerance(121-123). Therefore, it is thought that the responsiveness of developing human NK cells to IL-15 trans-presentation is tightly regulated and may be qualitatively different for each intermediate stage functioning to maintain proper NK cell development and functional maturation.

### ***Regulation of function – The receptor repertoire of human NK cells***

In the 1970s NK cells were first identified and distinguished from T-cells by their ability to spontaneously kill tumor targets without prior sensitization(9, 10, 124). Subsequent studies revealed this natural cytotoxicity occurred in an MHC-unrestricted manner and led to the proposal of the “missing-self” hypothesis, which states a lack of or downregulation of MHC class I proteins, which may be induced by a pathological event such as infection or transformation, renders a target preferentially susceptible to NK cell killing(125-130). Accordingly, it was determined that an inverse relationship exists between the expression of surface MHC class I molecules on target cells and vulnerability to NK cell cytotoxicity. As MHC class I proteins are ubiquitously expressed

under homeostatic conditions, this hypothesis also provided a basis for an NK cell self-tolerance mechanism, which was further supported upon discovery of inhibitory receptors for which MHC class I molecules served as ligands(131-133). An early model of NK cell tolerance to self based on the missing-self hypothesis was thus defined as follows: every mature NK cell expresses at least one inhibitory receptor that recognizes self-MHC class I molecules allowing the clonally distributed NK cell receptor repertoire of a given individual to sense the loss of even a single MHC class I allele on autologous cells distinguishing missing-self from self(50, 130, 134). Under certain circumstances, however, it was shown that the presence of MHC class I is neither necessary nor sufficient to protect target cells from NK cell lysis(135, 136). This subsequently led to the identification and description of NK cell activating receptors and the notion that NK cell effector function is regulated by a balance of opposing signals delivered by a sophisticated repertoire of inhibitory and activating cell surface receptors. Furthermore, it was shown that NK cells from MHC-deficient hosts as well as NK cells that lack the expression of inhibitory receptors were not autoreactive as predicted by the missing-self hypothesis, but instead were hyporesponsive(36, 123, 137-141). Consequently, different models of how NK cell self-tolerance and functional acquisition is achieved have been proposed based on the current understanding of NK cell function and the receptor repertoire.

*Inhibitory receptors*

In humans, three main types of inhibitory receptors are found that recognize MHC class I molecules: KIRs, the heterodimer CD94/NKG2A receptor and the leukocyte Ig-like receptor-1 (LIR-1). KIRs are a polymorphic set of type I transmembrane proteins that belong to the Ig superfamily and are encoded by 15 genes (*KIR2DL1* to *KIR2DL5A/B*,

*KIR3DL1* to *KIR3DL3*, *KIR2DS1* to *KIR2DS5*, and *KIR3DS1*) and two pseudogenes (*KIR2DP1* and *KIR3DP1*) on human chromosome 19(142-144). The KIR family of receptors contains both inhibitory and activating receptors that are distinguished by the length (and content) of their cytoplasmic domain where inhibitory KIRs display long (L) cytoplasmic tails, which contain two immunoreceptor tyrosine-based inhibition motifs (ITIMs), and activating KIRs display short (S) cytoplasmic tails, which associate with the DAP12 signaling adaptor protein via a charged amino acid residue in their transmembrane domain (**Figure 2A**)(142). KIR2DL4, however, is an exception to this short/long-tailed rule as it is a unique long-tailed activating KIR that associates with the immunoreceptor tyrosine-based activation motif (ITAM)-containing FcεRI-γ adaptor instead of DAP12(145, 146). Each KIR has two (2D) or three (3D) C2-type Ig-domains in its extracellular region that recognizes with allele specificity a subgroup of human leukocyte antigen (HLA) class I allotypes, namely HLA-C or HLA-A/B, with inhibitory KIR having higher avidity for MHC class I than activating KIR (**Table II**)(147). Inhibitory receptors KIR2DL1 and KIR2DL2/3 recognize distinct HLA-C allotypes based on polymorphisms in the α1-domain of the HLA heavy chain at amino acid positions 77 (Ser or Asn) and 80 (Asn or Lys), with KIR2DL1 binding to C2 allotypes (HLA-Cw2, HLA-Cw4, HLA-Cw5 and HLA-Cw6) and KIR2DL2/3 binding to C1 allotypes (HLA-Cw1, HLA-Cw3, HLA-Cw7 and HLA-Cw8)(142, 148). The KIR3DL1 inhibitory receptor recognizes HLA molecules that share the Bw4 epitope (defined by amino acid residues 77–83 in the α1 domain), which represents ~50% of human HLA-B alleles, and KIR3DL2 recognizes and binds HLA-A3 and HLA-A11. KIR haplotypes at the population level can be divided into 2 groups, namely haplotype A and haplotype B(144). Both haplotypes have four framework genes, which are present in every individual with very few exceptions, and

consist of *KIR3DL3* at the centromeric end, *KIR3DL2* at the telomeric end and *KIR3DP1* and *KIR2DL4* in the middle(149-151).

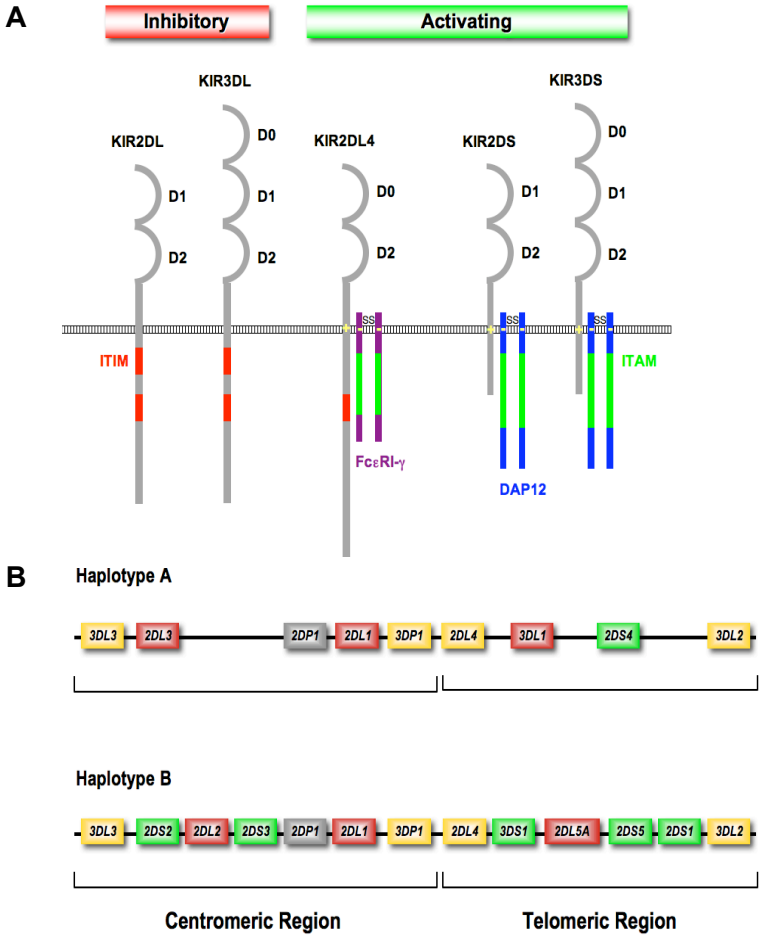


**Figure 2: Schematic diagram of human NK cell KIR genes and receptors.** A) Structures of inhibitory and activating KIR receptors expressed on human NK cells are diagrammed with the Ig-like domains designated D0, D1, and D2 (D0 being the most N-terminal followed by D1 and D2). A short stalk region separates the Ig-like domains from the transmembrane segment and the cytoplasmic domains are variable in length. Inhibitory receptors are characterized by long (L) cytoplasmic domains with one or two ITIMs, while activating KIR have short (S) cytoplasmic domains that do not contain ITAMs. Instead, activating KIRs have a positively charged Lys residue centrally located within their transmembrane region that associates with the DAP12 adapter protein via a negatively charged amino acid residue located in its transmembrane region. The KIR2DL4 activating receptor is an exception to these general defining characteristics. The extracellular region is composed of two Ig-like domains, the first designated D0 and the second designated D2. The KIR2DL4 transmembrane region has an Arg residue rather than a Lys residue in its transmembrane region that is more membrane proximal near the extracellular region rather than centrally located. KIR2DL4 also has a long cytoplasmic tail and associates with the FcεRIγ adapter protein instead of the DAP12 adaptor protein like other activating KIRs. Interestingly, KIR2DL4 contains a functional ITIM in its cytoplasmic domain. However, despite the presence of an ITIM, triggering of KIR2DL4 induces IFN-γ production and cytolytic function. B) A representation of KIR haplotypes A and B examples at the KIR locus is diagrammed with yellow boxes depicting framework genes (can be activating, inhibitory or pseudogenes), red boxes - inhibitory KIR genes, green boxes - activating KIR genes and grey boxes - pseudogenes. There is high diversity of KIR haplotypes due to the fact that each centromeric region can combine with any telomeric region.

Haplotypes A and B share in their repertoire inhibitory KIR for HLA-C1 and HLA-C2 molecules, but drastically differ in their activating KIR content.

**Table II: KIR molecules and their HLA ligands**

| Gene Name[1] | CD Designation | Allelic Variation (# of Alleles)[2] | HLA Ligand Recognition Motifs | Common Recognition Alleles of HLA Ligands |
|---|---|---|---|---|
| *KIR2DL1* | CD158a | 43 | HLA-C Group 2 | HLA-C2: C*02, C*04, C*05, C*06 |
| *KIRD2L2* | CD158b1 | 28 | HLA-C Group 1; some HLA-C Group 2 and HLA-B | HLA-C1: C*01, C*03, C*07, C*08; HLA-C2: C*0501, C*0202, C*0401; HLA-B: B*4601, B*7301 |
| *KIR2DL3* | CD158b2 | 34 | HLA-C Group 1; some HLA-C Group 2 and HLA-B | HLA-C1: C*01, C*03, C*07, C*08; some HLA-C2: C*0501, C*0202; some HLA-B: B*4601, B*7301 |
| *KIR3DL1* | CD158e1 | 73 | Bw4 eoitopes among HLA-B allotypes; some HLA-A | HLA-B: B*08, B*27, B*57, B*58; HLA-A: A*24, A*23, A*32 |
| *KIR3DL2* | CD158k | 84 | Certain HLA-A allotypes | HLA-A: A*03, A*11 |
| *KIR3DL3* | CD158z | 107 | Unknown | Unknown |
| *KIR2DL5A*[3] | CD158f | 10 | Unknown | Unknown |
| *KIR2DL5B*[3] | CD158f | 21 | Unknown | Unknown |
| *KIR2DL4* | CD158d | 46 | HLA-G | HLA-G |
| *KIR2DS1* | CD158h | 15 | HLA-C Group 2[4] | HLA-C2: C*02, C*04, C*05, C*06 |
| *KIR2DS2* | CD158j | 22 | HLA-C Group 1[4] | HLA-C1: C*01, C*03, C*07, C*08 |
| *KIR2DS3* | No CD assigned | 14 | HLA-C Group 1[4] | HLA-C1: C*01, C*03, C*07, C*08 |
| *KIR2DS4* | CD158i | 30 | HLA-C (some of Group 1 and 2); some HLA-A | HLA-C: C*0501, C*1601, C*0202; HLA-A: A*1102 |
| *KIR2DS5* | CD158g | 16 | Unknown[5] | Unknown |
| *KIR3DS1* | CD158e2 | 16 | Unknown[5] | Unknown |

[1]Inhibitory receptors are designated by the red background and activating receptors are designated by the green background

[2]A database serving as a centralized repository for human KIR sequences can be accessed at http://www.ebi.ac.uk/ipd/kir/ (updated 04/15/2011)

[3]The *KIR2DL5* gene is duplicated and encoded by two separate loci

[4]Activating KIR bind classical HLA molecules with low affinity

[5]Although the ligand specificity for inhibitory KIRs has been extensively characterized, less is known for activating KIR molecules

The gene organization of haplotype A is generally consistent and contains all four framework genes present plus *KIR2DL1*, *KIR2DL3*, *KIR3DL1*, *KIR2DS4* and *KIR2DP1*. On the other hand, haplotype B has greater variation in the number and combination of the KIR gene content and is characterized by the presence of one to five activating KIR (i.e. *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1*) and may include inhibitory KIR genes known to be absent in haplotype A (i.e. *KIR2DL2* and *KIR2DL5*) (**Figure 2B**) (152). The frequencies of haplotypes A and B vary among populations with, for example,

Caucasians displaying a relatively even distribution(144), whereas haplotype A is predominant in Japanese populations(153) and haplotype B is predominant in Aboriginal Australian populations(154).

CD94/NKG2 receptors are type II transmembrane proteins and members of the C-type lectin family of NK receptors encoded on human chromosome 12(155). CD94 is expressed on the cell surface as a disulfide-linked heterodimer with NKG2A, NKG2C, or NKG2E(156). The CD94/NKG2A heterodimer serves as the only inhibitory receptor of this family as NKG2A contains an ITIM in its cytoplasmic domain(157-159). Conversely, the CD94/NKG2C and CD94/NKG2E heterodimers associate with the ITAM-containing DAP12 adaptor protein via a charged amino acid residue in the transmembrane region and serve as activating receptors (**Figure 3A**)(160, 161). CD94/NKG2A and the other family members recognize and bind the non-classical MHC class I molecule HLA-E and, unlike KIRs, these receptors do not demonstrate extensive polymorphism(162-164). The peptides bound in the groove of HLA-E are typically derived from the leader segments of other MHC class I proteins and notably the leader peptides of HLA-E do not bind their own antigen-binding pockets(155, 162). The CD94/NKG2 receptors, therefore, function as sensors of the net overall expression of the classical and certain non-classical (i.e. HLA-G) MHC class I proteins, as the expression of HLA-E on the cell surface is dependent upon the availability of leader peptides provided by HLA-A, HLA-B, HLA-C, or HLA-G, respectively. Similar to activating and inhibitory KIR, the inhibitory CD94/NKG2A receptor has a higher affinity for HLA-E ligands than the activating CD94/NKG2C receptors(165). However, it has been shown that the peptide bound to HLA-E can differentially affect the ability of inhibitory and activating CD94/NKG2 receptors to recognize and bind the ligand, demonstrating the ability of these receptors to distinguish

abnormal cells from normal cells. One such example demonstrated peptides derived from heat shock protein 60 (hsp60) bound to HLA-E were not recognized by the inhibitory CD94/NKG2A receptor, suggesting HLA-E on “stressed” cells may preferentially bind to CD94/NKG2 activating receptors promoting NK cell targeting and elimination of the abnormal target cells(166).

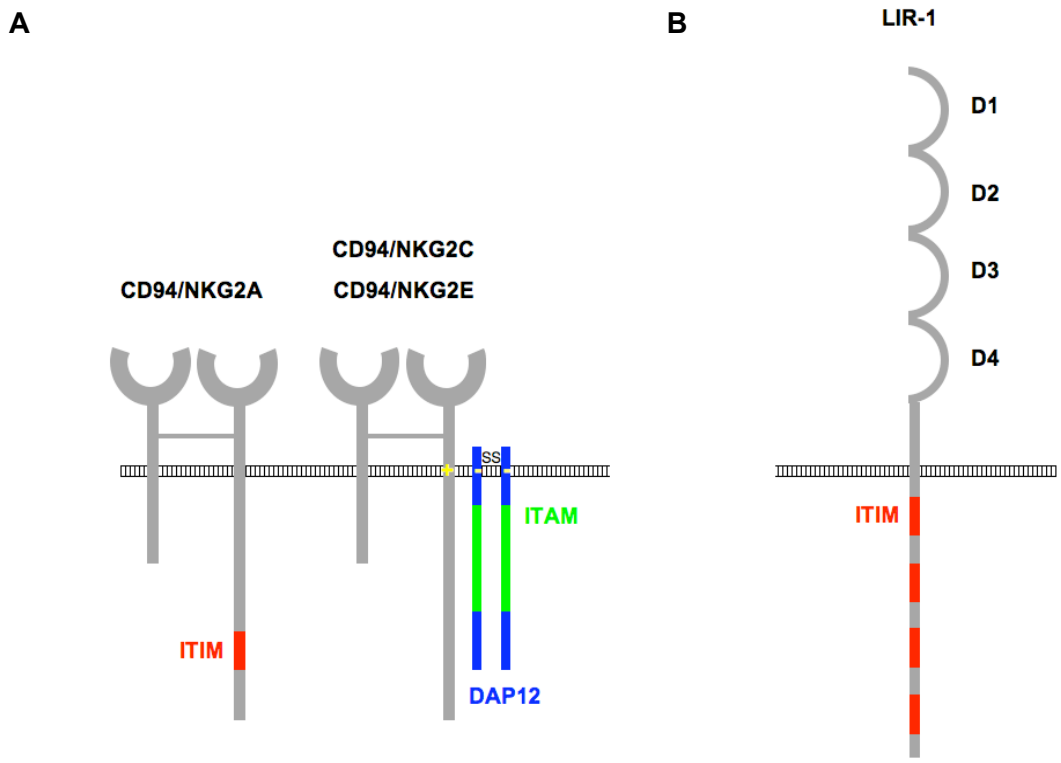


**Figure 3: Schematic diagram of CD94/NKG2 and LIR-1 human NK cell receptors.** A) CD94/NKG2 receptors are type II glycoproteins and members of the C-lectin family of receptors. CD94 can be expressed on the cell surface as a disulfide-linked heterodimer with NKG2A, NKG2C or NKG2E. The CD94/NKG2A heterodimeric receptor contains an ITIM in its cytoplasmic domain and functions as an inhibitory receptor. Conversely, CD94/NKG2C, E heterodimers associate with the ITAM-containing DAP12 adapter protein via charged amino acid residues in the transmembrane region and serve as activating receptors. B) LIR-1 belongs to the IgSF and contains four C-type Ig domains in the extracellualr region and four ITIMs in the cytoplasmic tail. Extracellular domains D1-D4 have an elongated structure with minimal interdomain interactions. LIR-1 binds to the $\alpha$3 domain of MHC class I proteins using the N-terminal D1 domain. In contrast to KIRs, LIR-1 responds to a region on MHC class I molecules that does not involve the peptide-binding grooves.

The third group of NK cell inhibitory receptors is encoded by the *LILR* family of genes

(also termed LIR, ILT, and CD85) located on human chromosome 19 centromeric of the *KIR* genes(155). There are 13 *LILR* genes, two of which encode inhibitory receptors that bind MHC class I molecules, specifically *LILRB1* (ILT2/LIR-1) and *LILRB2* (ILT4/LIR-2)(167). Human NK cells do not express LIR-2 and have variable expression of LIR-1 that ranges from undetectable to ~75% on PB NK cells(168). The structure of LIR-1 consists of four Ig-like domains in the extracellular region and four ITIMs in the cytoplasmic domain (**Figure 3B**)(167). LIR-1 binds with low affinity to a conserved region in the $\alpha$3 domain of both classical (HLA-A, HLA-B and HLA-C) and non-classical (HLA-E, HLA-F and HLA-G) MHC class I molecules, which differs from the other HLA class I binding receptors that bind residues in the $\alpha$1 domain(169, 170). Despite NK cell expression of LIR-1, inhibitory KIRs and CD94/NKG2A are generally thought to have a more dominant role in the suppression of NK cell function(171). Conversely, B-cells and monocytes, both of which lack the KIR and CD94/NKG2 receptor systems, have uniformly high expression of LIR-1 and it is suggested this receptor plays a significant role in regulating the function of these leukocytes(167, 168).

*Activating receptors*

As inhibitory receptors provide a mechanism to turn “off” NK cell effector function and prevent attack of normal autologous cells, activation receptors function to generate an “on” signal upon interaction with their ligands on potential target cells. A number of structurally distinct receptors expressed on NK cells have been described that mediate the activation of effector functions and can be grouped into three categories: receptors that signal through ITAM–containing adaptor subunits, the DAP10-associated receptor NKG2D, and receptors that directly signal through their cytoplasmic domain (**Table III**).

Within the NK cell activating receptor repertoire, CD16 (FcγRIII) is a potent inducer of NK cell function. As a member of the immunoglobulin superfamily, CD16 is a 50-70kD integral membrane protein that is encoded by two genes, *FCGR3A* (FcγRIIIA/CD16A)

**Table III: Activating receptors expressed by human NK cells[1]**

| Receptor | Class | Signaling Motif/Adaptor[2] | Ligand |
|---|---|---|---|
| CD16 (FcγRIII) | IgSF | ITAM/FcεRγ, CD3ζ | IgG |
| CD94/NKG2C, E | C-lectin | ITAM/DAP12 | HLA-E |
| NKp46 | IgSF | ITAM/FcεRγ, CD3ζ | Viral hemagglutinins, ? |
| NKp30 | IgSF | ITAM/FcεRγ, CD3ζ | BAT3 (HLA-B-Associated Transcript 3) |
| NKp44 | IgSF | ITAM/DAP12 | Viral hemagglutinins, ? |
| NKp80 | C-lectin | ? | AICL (Activation-Induced C-type Lectin) |
| NKG2D | C-lectin | YxNM/DAP10-PI3K | MICA, MICB, ULBP1-ULBP4 |
| 2B4 (CD244) | SLAM | ITSM | CD48 |
| CRACC (CD319) | SLAM | ITSM | CRACC (CD319) |
| DNAM (CD226) | IgSF | PKC,? | CD155, Nectin-2 (CD112) |
| LFA-2 (CD2) | IgSF | Proline-rich domain, CD2AP | LFA-3 (CD58) |
| LFA-1 (CD11a) | IgSF | Src family kinases, PI3K | ICAM (CD54) |

[1]This table provides a non-exhaustive list of activating receptors expressed by human NK cells

[2]The yellow background denotes activating receptors that utilize ITAM-containing adaptor proteins, the green background denotes receptors that utilize the DAP10 adaptor protein, the purple background denotes receptors that directly signal through their cytoplasmic domains and the grey background denotes receptors for which the signaling motif/adaptor molecules are not well understood.

and *FCGR3B* (FcγRIIIB/CD16B), on human chromosome 1(172). CD16A is predominantly expressed on the $CD56^{dim}$ subpopulation of NK cells and structurally consists of two extracellular C-type Ig-domains, a single transmembrane segment and a carboxy terminal cytoplasmic tail that associates with disulfide-linked hetero- or homodimers of the ITAM-containing FcεRI γ chain (FcεRIγ) and/or CD3 ζ chain (CD3ζ) (**Figure 4**). In contrast, CD16B is mainly expressed on neutrophils, anchored in the

membrane through a glycosylphosphatidyl inositol (GPI) linkage and does not associate with ITAM-containing subunits(173, 174). CD16 recognizes and binds the Fc portion of immunoglobulins and has low affinity ($K_d$=$10^{-6}$ M) specificity for the IgG1 and IgG3 subclasses(175). Antigen-bound immunoglobulin binds and cross-links the ligand binding extracellular domain (D1) of CD16, resulting in Src kinase activation, tyrosine phosphorylation of the associated ITAMs and subsequent NK cell activation and destruction of the antibody-coated target, a process termed antibody-dependent cell-mediated cytotoxicity (ADCC)(176).

The receptors responsible for NK cell activation in the process of natural cytotoxicity include NKp30, NKp46 and NKp44 and are collectively termed natural cytotoxicity receptors (NCRs). NCRs are Ig-like transmembrane glycoproteins with *NCR1*, the gene encoding NKp46, located on human chromosome 19 and *NCR2* and *NCR3*, the genes encoding NKp44 and NKp30, located on chromosome 6(177-179). In the extracellular region, NCRs contain two, C-type (NKp46) or a single V-type Ig-domain (NKp30 and NKp44) and have cytoplasmic tails that lack ITAMs (**Figure 4**). Like activating KIRs and the CD94/NKG2C heterodimer, NCRs contain positively charged amino acids in their transmembrane regions and associate with DAP12 homodimers (for NKp44) or ITAM-containing CD3ζ and FcεRIγ subunits (for NKp46 and NKp30) similar to CD16(180, 181). Although specific ligands of NCRs are still unknown, engagement and clustering of NCRs result in Src kinase activation and tyrosine phosphorylation of the associated ITAM-containing subunits, leading to $Ca^{2+}$ mobilization, cytokine production and activation of NK cell cytotoxicity(182). Furthermore, it has been shown that engagement of a single NCR is sufficient to activate the signal transduction machinery of other NCRs, which suggests these receptors may form and optimally function as a receptor complex

instead of separate, single receptor units(182). Expression of NKp46 and NKp30 occurs early during NK cell maturation and can be found on essentially all mature NK cells independent of their activation state. In contrast, acquisition of NKp44 occurs only upon activation, contributing to the increased effector function displayed by activated NK cells, and serves as a marker of NK cell activation.

Another activating receptor that contributes to NK cell function is NKG2D, encoded by the gene *KLRK1* on human chromosome 12 and, unlike KIR, demonstrates essentially no polymorphism(183, 184). While NKG2A, NKG2C and NKG2E are highly related to each other, NKG2D shares very little homology with these receptors and does not form heterodimers with CD94, but rather is expressed as a disulfide-linked homodimer (**Figure 4**)(185). Like NKG2C and NKG2E, NKG2D does not contain ITAMs in its cytoplasmic domain and requires an adaptor protein to mediate a signal. However, instead associating with DAP12, NKG2D utilizes the adaptor protein DAP10 that contains a phosphatidylinositol-3 kinase (PI3K) and Grb adaptor protein-binding motif (Tyr-x-x-Met, where x denotes any amino acid)(185-188). Association of NKG2D with DAP10 is required for surface expression of NKG2D, as it has been demonstrated that NKG2D is retained in the cytoplasm and degraded in the absence of DAP10(185, 186, 189). The ligands for NKG2D have structural homology to MHC class I molecules and include MICA, MICB, ULBP1, ULBP2, ULBP3, and ULBP4(190-192). The MICA and MICB ligands are encoded by genes located on human chromosome 6 within the MHC locus, while the ULBP ligands are encoded by 10 related genes or pseudogenes found in a cluster on the opposite end of the human chromosome 6 relative to the MHC complex(193, 194). Crystal structures of NKG2D ligands have determined all have $\alpha$1

and α2 domains forming an MHC class I–like fold without open peptide-binding grooves(195-198). Only MICA and MICB have an α3 domain, but these proteins do not bind β2-microglobulin like HLA class I molecules(199). The binding affinity of both MIC and ULBP ligands to NKG2D has been determined to be relatively high despite low homology between the two groups and the use of completely different residues for binding interaction(196-198).

The triggering receptor 2B4 (CD244) is expressed by essentially all NK cells and is unique in its ability to mediate both activating and inhibitory signals. It is thought to function as a co-receptor that amplifies signals induced by other receptors, thus, promoting different functional outcomes depending on the state of the cell. 2B4 is a member of the SLAM family of Ig-related proteins and is encoded by a cluster of related genes located on human chromosome 1(200, 201). Its extracellular domain consists of one membrane distal Ig-V-type domain and one membrane proximal Ig-C-type domain. The transmembrane region lacks charged amino acids and thus does not associate with signal adaptor proteins. Instead, 2B4 has a long cytoplasmic tail that contains four tyrosine-based motifs (TxYxxI/V), referred to as immunoreceptor tyrosine-based switch motifs (ITSMs) (**Figure 4**). In humans, two cytoplasmic SH2 domain–containing adaptor proteins, namely SAP and EAT-2, recognize and bind this motif to mediate signaling(200, 201). Cross-linking of 2B4 by its ligand CD48, a cell surface protein expressed broadly on hematopoietic cells, results in tyrosine phosphorylation of the ITSMs and recruitment of either SAP or EAT-2 via binding through their SH2 domains(201). It has been hypothesized that 2B4-SAP receptor complexes activate NK cells, whereas 2B4-EAT2 receptor complexes inhibit NK cell activation. This proposal is

supported by the finding that NK cells from patients with the X-linked lymphoproliferative disease (XLP) genetic disorder, characterized by the presence of a loss-of-function mutation in the SAP gene (*SH2D1A*), are unable to lyse $CD48^{+}$ targets(202-204). Furthermore, EAT-2 deficient mice displayed enhanced natural cytotoxicity and IFN-γ production while mice overexpressing EAT-2 had significant impairments in these

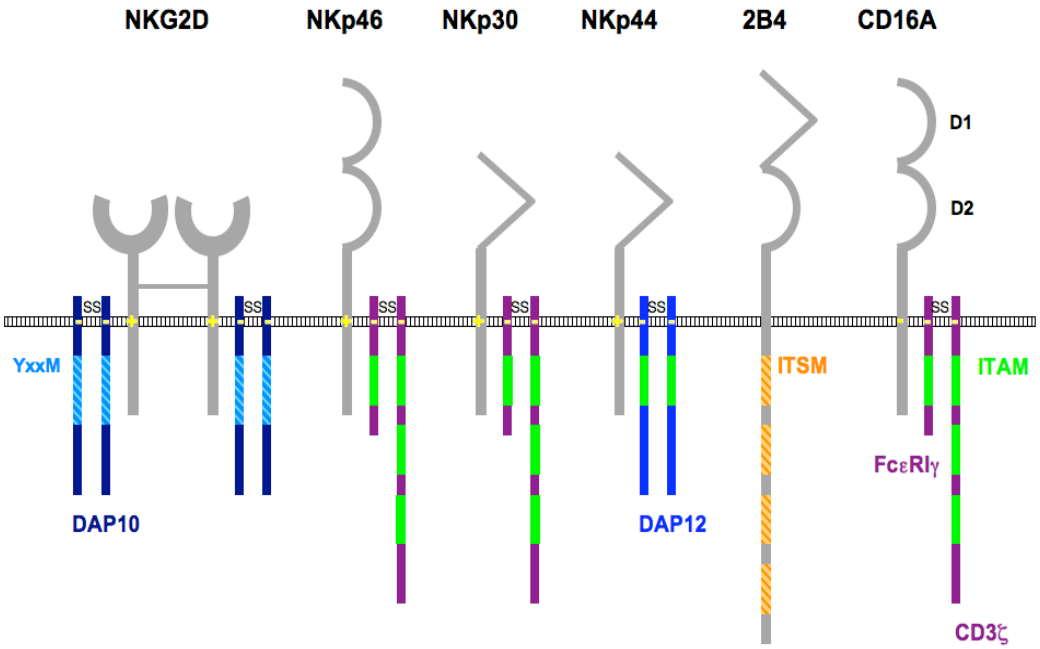


**Figure 4: Schematic diagram of human NK cell activating receptor structures.** NKG2D is a type II glycoprotein belonging to the C-type lectin receptor family. The NKG2D receptor complex is a hexamer, with one NKG2D homodimer associating with two DAP10 homodimers that contain a PI3K-binding tyrosine based motif (YxxM) via centrally located arginine residues in the NKG2D transmembrane domain and aspartate residues in the transmembrane region of DAP10. NCRs NKp46, NKp30 and NKp44 are type I glycoproteins belonging to the IgSF. NKp46 contains two C-type Ig domains and NKp30 contains one V-type Ig domain in the extracellular region and both associate with ITAM-containing hetero- or homodimers of FcεRIγ and/or CD3ζ signal adaptors via a positively charged arginine residue located in the NKp46 transmembrane region and a negatively charged aspartate residue located in the transmembrane region of the signaling chain. NKp44 displays one V-type Ig domain in the extracellular region and associates with the ITAM-containing signal adaptor DAP12 via charged amino acid residues in the transmembrane region as with NKp46, however, the aspartate residue in more centrally located in the transmembrane domain of DAP12 compared to the FcεRIγ and CD3ζ chains, in which the aspartate residue is located closer to the extracellular region. 2B4 is a member of the SLAM family of receptors and is considered to be a multifunctional receptor as it can mediate both activating and inhibitory signals. It possesses one V-type and one C-type Ig domain in the extracellular region and is characterized by a long cytoplasmic tail that contains four ITSMs. CD16A is a potent activating receptor that is part of the IgSF. It contains two Ig extracellular domains and associates with ITAM-containing hetero- or homodimers of FcεRIγ and/or CD3ζ signal adaptors via an aspartate residue located in the CD16 transmembrane domain and an aspartate residue located in the ITAM-signaling chains, contrasting the interactions of NKp46 with the signaling adaptors. Further studies are needed to understand the basis of the CD16-signaling chain interactions. Ligand recognition and binding occurs through domain 1 (D1) of the extracellular portion of CD16A.

effector functions(205). Studies have also shown that of the four cytoplasmic ITSMs, the third ITSM when phosphorylated can recruit the phosphatases SHP-1, SHP-2, and SHIP and the inhibitory kinase Csk(206). Consequently, it has been suggested that SAP competes with SHP-1, SHP-2, and SHIP for ITSM binding and can competitively displace these phosphatases preventing their suppressive activity and promoting NK cell activation. Therefore, in the absence of SAP, a scenario represented by patients with XLP, engagement of 2B4 would lead to inhibition, as there would be unobstructed access to the phosphorylated ITSM for phosphatase binding. Further evidence supporting this model for the mechanism by which 2B4 serves dual, opposing roles demonstrated that immature human NK cells generated *in vitro* from $CD34^+$ progenitors express 2B4, but not SAP, and when triggered 2B4 repressed NK cell function(207). Moreover, the tyrosine motifs in the cytoplasmic domain of 2B4 have lower binding affinities for the SH2 domains of SHP-1, SHP-2 and SHIP compared to those for the SH2 domains of SAP(208).

*NK cell tolerance – An "educational" process*

Regulation of NK cell effector responses, determined by the functions and specificities of activating and inhibitory receptors, has been well studied. However, their role in NK cell "education", namely the ability to establish self-tolerance while conferring functional competency, is less clear. Current models for NK cell education include MHC-dependent processes, such as licensing, arming and disarming, and MHC-independent processes that may involve non-MHC-specific receptors and/or cytokine stimulation. While there is a general consensus that MHC-dependent education occurs through a self-MHC-specific receptor, the mechanisms of this process and the contributing roles of inhibitory and activating receptors remain a subject of debate.

It has been long established that NK cells are regulated by the host MHC class I environment. This was first noted by Cudkowicz et al. in experiments demonstrating the phenomenon of "hybrid resistance", where $F_1$ hybrid mice reject BM grafts from either inbred parent, but not from an $F_1$ hybrid(209). Studies using β2-microglobulin (β2m)-deficient mice, which consequently lack MHC class I molecules, showed NK cells in MHC-sufficient mice reject β2m-deficient BM, whereas NK cells in β2m-deficient mice allowed engraftment, demonstrating the host MHC class I environment influences host NK cells that are ultimately responsible for graft rejection(210-212). While the results of these studies fit well with the missing-self hypothesis, they pointed to a contradiction in a main prediction of this proposal, which states that MHC-deficient hosts should display uninhibited NK cell function that causes autoreactivity(130). As NK cells from MHC-deficient hosts did not reject MHC-deficient targets (i.e. β2m-deficient BM), NK cells were not hyperactive as predicted and in fact were subsequently shown to be defective in their ability to kill MHC-deficient targets(138, 213, 214). Furthermore, the hypothesis that the expression of at least one self-specific inhibitory receptor on every NK cell mediated self-tolerance was also contradicted with evidence that revealed the presence of self-specific inhibitory receptor negative NK cells in MHC-sufficient hosts that were hypofunctional despite the lack of MHC-specific inhibitory receptor engagement(137). Moreover, self-MHC inhibitory receptor positive NK cells were shown to be functionally competent when stimulated through activating receptors, whereas self-MHC inhibitory receptor negative NK cells were not able to respond to the activating stimuli(215-217). It was also demonstrated that an intact ITIM was required for self-MHC inhibitory receptor positive NK cell function induced through activating receptors(216). Consequently, the

basis for MHC-dependent NK cell education was formed and the terms “licensed” and “unlicensed” were coined for describing the mechanisms by which NK cells acquire functional competence and achieve tolerance to self.

Licensed NK cells are defined as functionally competent cells with regard to their activation receptors and are tolerant to self due to the presence of an inhibitory receptor specific for self-MHC. Conversely, unlicensed NK cells do not express a self-MHC-specific inhibitory receptor, but are tolerant to self as they are not functionally competent (**Figure 5A**). Several mechanisms have been proposed to explain how NK cells become licensed through self-MHC-specific inhibitory receptors. Two models described by Raulet and colleagues are termed “arming” and “disarming”(218). The arming model postulates that self-MHC-specific inhibitory receptors deliver signals necessary to confer functional competence (i.e. a licensed phenotype) analogous to an activating receptor (**Figure 5B**). Although the concept of an ITIM-containing inhibitory receptor providing a positive signal seems counterintuitive, there is precedence for the ability of receptors to differentially signal in a context-dependent manner. The T-cell receptor (TCR), for example, traditionally serves as an activating receptor; however, under certain circumstances it can mediate signals that lead to apoptosis, positive or negative selection or anergy(219, 220). In the case of NK cell MHC-specific inhibitory receptors, their function was initially studied and described in the context of an effector response against targets. However, during licensing the signal delivered by the inhibitory receptors may indeed be qualitatively different. Recent evidence demonstrated by Long and colleagues suggests such a possibility(221); they show that MHC-specific inhibitory receptors exploit the properties of the small adaptor protein Crk to achieve their dual function. Interaction of inhibitory receptors with their ligand induces phosphorylation of Crk, which subsequently

dissociates from scaffolding proteins that contribute to cytoskeleton organization and Vav1 activation, disrupting the actin network, microclustering of activation receptors and

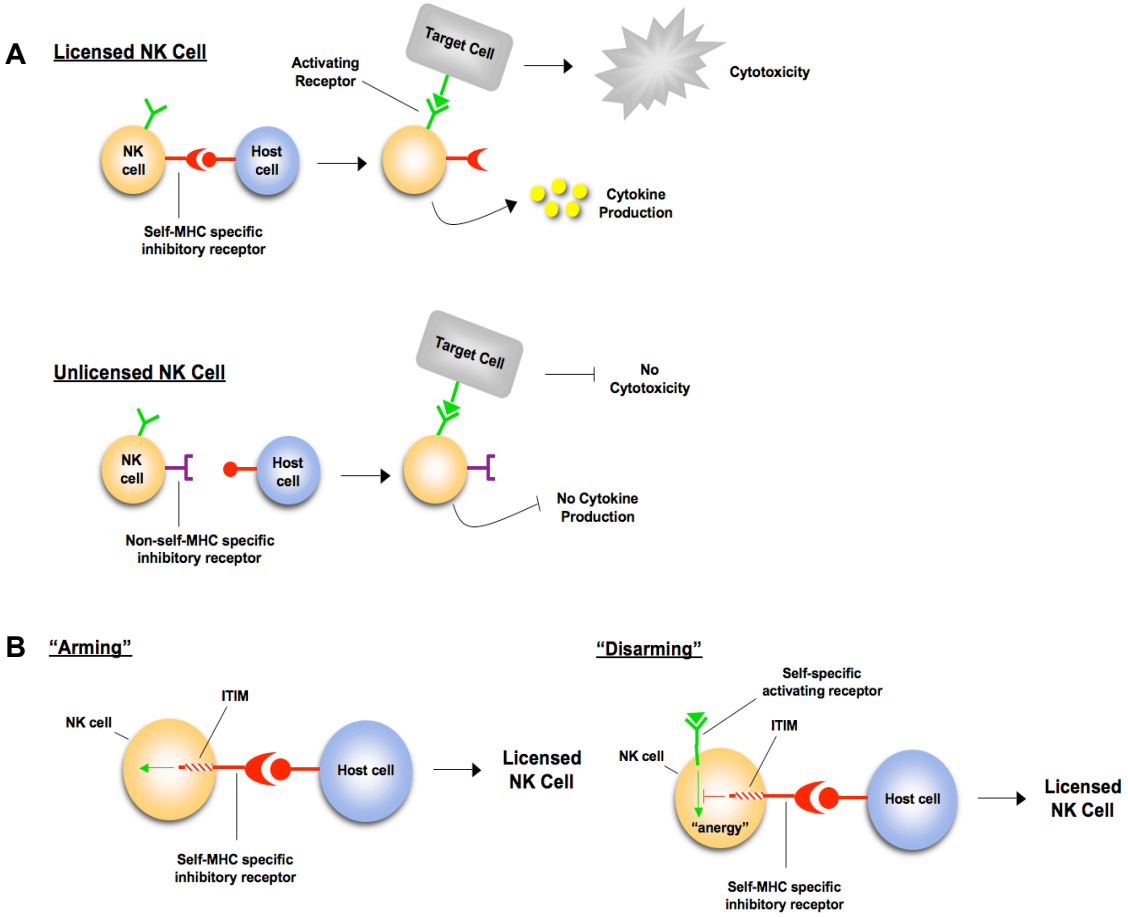


**Figure 5: NK cell education - Licensing, Arming and Disarming.** A) NK cells expressing a self-MHC specific inhibitory receptor recognize self-MHC ligands on host cells (indicated by the red receptor/ligand pair), which results in licensing of the NK cells. When activation receptors on the licensed NK cells are crosslinked by their ligands (indicated by the green receptor/ligand pair), cytokines are readily produced and target cells lacking self-MHC ligands are lysed. Licensed NK cells are also tolerant to self as they are inhibited by self-MHC ligands through the same receptor that confers licensing. Alternatively, NK cells that lack self-specific MHC inhibitory receptors do not recognize and are not licensed through self-MHC-ligands (indicated by the purple receptor and red ligand) despite expressing another inhibitory receptor. Upon crosslinking of activating receptors on unlicensed NK cells, function is not induced and they are tolerant to self even in the absence of self-MHC specific inhibitory receptors due to their unlicensed status. Therefore, in this situation inhibition by MHC class I is not required to achieve self-tolerance. B) Under the "arming" model of NK cell licensing, engagement of self-MHC-specific inhibitory receptors signals a licensed phenotype, with the "inhibitory" receptor having a "positive" effect on the NK cell. The "disarming" model of NK cell licensing postulates in the absence of self-MHC-specific inhibitory receptors, chronic engagement of self-specific activating receptors constitutively activates NK cells and leads to an anergic-like (or hyporesponsive) state. Licensing occurs when NK cells express self-MHC-specific inhibitory receptors that counter the activating signals upon engagement with self-ligand establishing a balance between activating and inhibitory signals.

actin-dependent signaling. However, once the inhibitory receptor is released from its ligand (removing the inhibitory stimulus) more efficient activation receptor engagement may occur(222), as there is greater lateral mobility resulting from Crk phosphorylation and a less constrained F-actin network, and the NK cell is subsequently licensed with function.

In the disarming model, the absence of self-MHC-specific inhibitory receptors and the expression of a putative self-specific activation receptor that chronically stimulates the NK cell lead to an anergic state that leaves the NK cell hyporesponsive (**Figure 5B**). Expression of a self-MHC-specific inhibitory receptor functions to impede the chronic stimulation by delivering opposing inhibitory signals upon engagement with its ligand, resulting in NK cell responsiveness to subsequent stimuli(218). Early studies demonstrating the ability of NK cells from wild-type mice to kill MHC-deficient cells provided indirect evidence for a self-specific activation receptor as these results suggested MHC-deficient cells express a ligand for an unknown activating receptor(138). Subsequent studies demonstrated that constitutive expression of ligands for the NK cell activating receptor NKG2D in a transgenic mouse model results in a hyporesponsive state for all NK cells(223, 224). In humans, it was demonstrated that KIR2DS1 expressing NK cells derived from HLA-C2-positive donors display a hyporesponsive functional phenotype(225). Both of these observations endorse the concept that chronic engagement of an activating receptor can lead to an anergic-like state and provide further support for the disarming model of education.

While the arming and disarming models describe licensing as an all or none phenomenon, Höglund and colleagues propose a rheostat model in which NK cell

responsiveness is tuned along a continuous scale by the net input from inhibitory receptors (**Figure 6**)(226). More specifically, the greater the number of self-MHC-specific inhibitory receptors capable of interacting with different self-MHC class I ligands an NK cell has, the more responsive it is to activating receptor stimulation thus ensuring self-tolerance while optimizing sensitivity for detecting changes in normal cells. In mice it has been shown that NK cell function is greater in hosts expressing multiple MHC class I alleles demonstrating a positive correlation between MHC class I allele expression and NK cell education or licensing(227, 228). Furthermore, in addition to number, the type of MHC class I alleles present influence the frequency and strength of responding NK cell effector functions, with certain MHC class I alleles having a greater "educating impact" than others(229). Interestingly, lower tuning is required to potentiate degranulation than IFN-$\gamma$ production, suggesting different educational thresholds for distinct effector functions(229). Along these lines, the affinity with which a self-MHC-specific receptor binds its MHC class I ligand also affects NK cell licensing as mouse studies have revealed differential licensing of a single NK cell in a MHC haplotype-dependent manner, which correlated with the degree to which the given haplotype inhibited NK cell cytotoxicity(230). Moreover, it was determined that the strength of licensing was saturated by a relatively low level of MHC class I binding affinity, which interestingly was a higher threshold than that of effector inhibition by MHC-specific receptors(230, 231). These results suggest a safeguard mechanism that precludes the education of NK cells that cannot be inhibited thereby preventing autoimmunity.

The models described above all consider licensing of NK cells in the context of self-tolerance and steady-state function. However, in an inflammatory environment it has been shown that cytokine stimulation induces effector function in unlicensed NK cells

bypassing the MHC-dependent licensing effect(137, 216, 232). Various studies have demonstrated the ability of unlicensed NK cells to function in the context of viral infection(137, 216, 233-235). It is, therefore, thought that the need for the host to quickly eradicate an infection overrules the strict requirement for MHC-dependent licensing and education takes places via an alternative MHC-independent pathway.

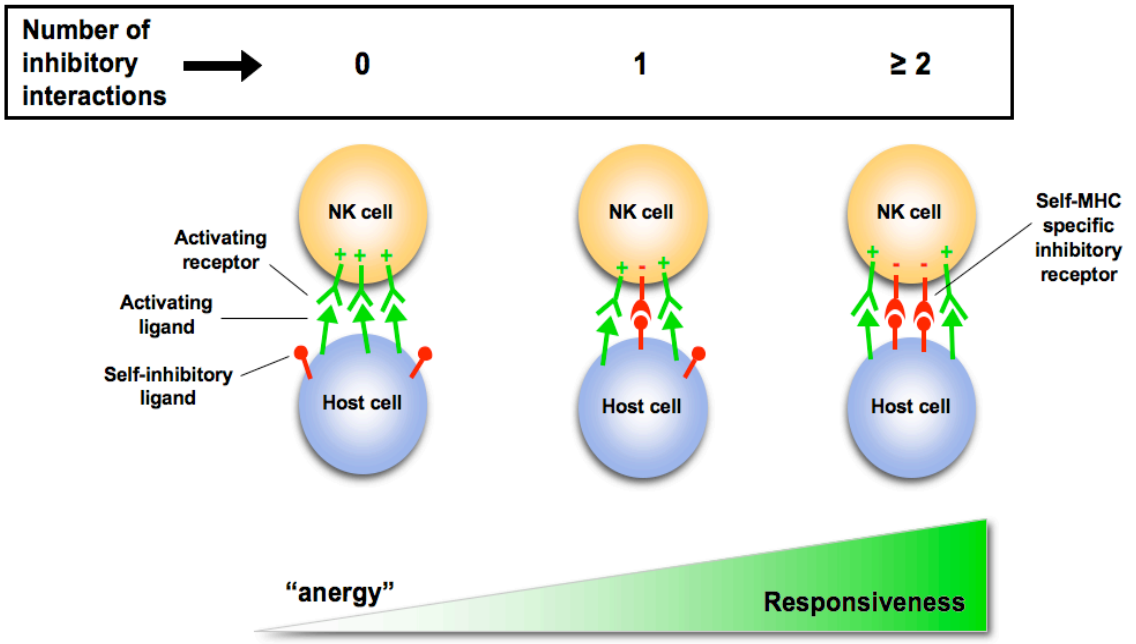


**Figure 6: Receptor tuning - the rheostat model of NK cell education.** The rheostat model of NK cell education proposes that the balance of signals upon encounter with activating and inhibitory ligands sets the responsiveness state of NK cells. With the lack of self-MHC-specific inhibitory receptor expression, strong persistent stimulation by activating receptors leads to an anergic like state and the NK cells are functionally hyporesponsive. Expression of one self-MHC-specific inhibitory receptor partially opposes the activating signals and results in partial restoration of functional responsiveness. Expression of two or more self-MHC-specific inhibitory receptors allows NK cells to reach a state of high responsiveness, as there is greater opposition of the persistent activating signals. Therefore, according to this model the number of receptor-ligand interactions quantitatively tunes NK cell function.

## ***Mechanisms of NK cell activation and effector function***

NK cells serve as important arsenals for defense against intracellular microbial infections and tumors and have also been implicated in autoimmunity and hypersensitivity

reactions(236-238). IFN-γ secretion and perforin/granzyme-dependent target cell elimination are two critical mediators of NK cell resistance to intracellular pathogens and immunosurvelliance of tumors(239, 240). In addition, NK cell cytokine release and accessory cell interactions serve to initiate, shape and instruct the adaptive immune response(241). NK cells have also been shown to play an important role in killing allogeneic target cells in the context of HCT and are considered to be highly beneficial for cancer immunotherapies(242, 243). The mechanisms that drive these functions are regulated by the heterogenous receptor repertoires of the various NK cell populations, in which hierarchies of activating strength stimuli have been demonstrated for the induction of specific functional responses(244-247); a low activation threshold is required for inside-out signals for leukocyte functional activation (LFA)-1-mediated adhesion while chemokines such as macrophage inflammatory protein (MIP)-1β require greater activating stimuli for induction and production of tumor necrosis factor (TNF)-α and IFN-γ is even further restricted and display the most stringent requirements for induction. Furthermore, these activating thresholds that regulate NK cell effector function have been demonstrated to be highly dynamic and use different molecular pathways depending on the type of activation(248). Consequently, mechanisms of NK cell activation are driven by the dynamic integration of signals derived from multiple receptors that are influenced by environmental cues and ultimately result in differential, graded and highly targeted NK cell responses.

*Steps in NK cell activation*

Target cell-mediated activation of NK cell effector functions involves several distinct molecular events that occur within an NK cell upon encountering an NK cell-sensitive

target and result in target cell adhesion, lytic granule polarization, degranulation and cytokine production (**Figure 7**). A number of receptors have been shown to mediate initial contact between NK cells and target cells, promoting adhesion and formation of an immunological synapse. In resting human NK cells, activating receptors CD16, 2B4,

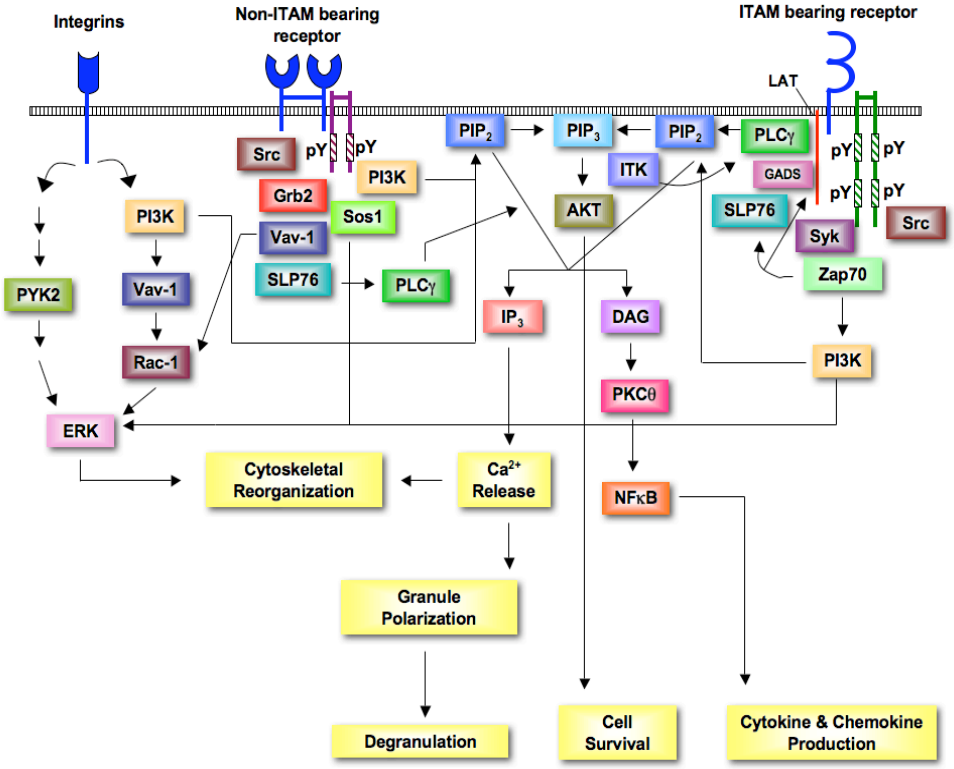


**Figure 7: Schematic representation outlining the molecular events involved in inducing NK cell effector responses.** Conjugate formation is mediated by integrin interactions that initiate cytoskeletal reorganization via outside-in signals. This promotes recruitment of engaged-activating receptors (non-ITAM and ITAM-bearing receptors) to the immunological synapse. Non-ITAM bearing receptors mediate NK cell activation via DAP10 and involves the recruitment of PI3K and recruitment of the Grb2-Vav1-Sos1 complex to the phosphorylated YxxM motif in the cytoplasmic domain of DAP10. These events trigger distal signaling cascades as depicted. ITAM-bearing receptors utilize signaling subunits, such as DAP12, CD3ζ and FcεRIγ, and are phosphorylated by Src family kinases after receptor engagement. Syk and/or ZAP-70 are recruited to the phosphorylated ITAMs, initiating a cascade of downstream signaling as depicted. These molecular events ultimately result in intracellular $Ca^{2+}$ mobilization, lytic granule polarization and exocytosis, and chemokine and cytokine production. DAG, diacylglycerol; $IP_3$, inositol-3,4,5-trisphosphate; $PIP_2$, phosphatidylinositol-3,4-bisphosphate; $PIP_3$, phosphatidylinositol-3,4,5-trisphosphate; pY, phosphotyrosine; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells (transcription factor); PYK2, protein tyrosine kinase 2; PKC-θ, protein kinase C-θ; Vav-1, guanine nucleotide exchange factor; Rac-1, Ras-related C3 botulinum toxin substrate 1; ERK, extracellular-signal-regulated kinase; GADS, adaptor protein; LAT, linker of activated T cells (scaffold protein); SLP-76, scaffold protein; ITK, Tec kinase; ZAP-70, ζ-chain-associated protein (tyrosine kinase); PLCγ, phospholipase C-γ; Grb2, Growth factor receptor-bound protein 2 (adaptor protein); SOS, son of sevenless (Ras activating protein); AKT, serine/threonine-specific protein kinase; Src, src tyrosine kinase; Syk, spleen tyrosine kinase.

NKG2D and DNAM-1 demonstrated the ability to rapidly induce inside-out signals that lead to adhesion(245). These signals are overridden by engagement of an inhibitory receptor with its ligand, such as NKG2A and HLA-E, thus ensuring appropriate action against the contacted target cell(245, 249).

Adhesion is a prerequisite for NK cell effector function as it provides stable contact and allows for the formation of an immunological synapse with the target cell. The integrin LFA-1 has been demonstrated to be essential in the adhesion process as LFA-1-deficient NK cells have profound defects in target cell adhesion(250). Inside-out signals from activating receptors making initial contact lead to increases in LFA-1 affinity through conformational changes and LFA-1 avidity through induction of receptor clustering. Once in the open, ligand-binding conformation, LFA-1 can transduce outside-in signals that further promote target cell adhesion and NK cell activation(244). Staining of resting human NK cells with an LFA-1 conformation-specific monoclonal antibody (mAb) notably revealed a subset of NK cells that express LFA-1 in an active, ligand-binding, open conformation, which was subsequently abrogated upon treatment with pharmacological inhibitors of Src family kinases, PI3K or phospholipase C (PLC)-γ(245). This demonstrated that tonic signals dynamically maintain LFA-1 in an active conformation and is consistent with the signaling-dependent ability of resting NK cells to bind intracellular adhesion molecule (ICAM)-1, a ligand of LFA-1(251). While signals for inside-out activation of LFA-1 and adhesion conferred by the contact-initiating activating receptors are not well defined, phosphorylation and activation of the guanine exchange factor and actin regulator Vav-1 have been proposed to be involved in downstream signaling pathways of activating receptors and LFA-1(252). Moreover, phosphorylated Vav-1 is a substrate for SHP-1 associated with inhibitory receptors and is thought to

serve as a proximal point where inhibitory receptor signals oppose activating receptor signals(253). Recent data from various studies implicate important roles for talin, Cdc42, Wiskott-Aldrich syndrome protein (WASP), Csk, Rap1 and hematopoietic cell-specific homolog of cortactin (HS)-1 in the pathways leading to Vav-1 phosphorylation and LFA-1-dependent adhesion, ultimately regulating immunological synapse formation and NK cell effector function(254-258).

Once a stable synapse has been established, granule polarization towards the point of target cell contact begins, which involves two molecular processes. In the first step, granules quickly congregate in a dynein-dependent, minus end-directed motion to the microtubule-organizing center (MTOC) followed by the second step where the MTOC and granules polarize towards the site of contact(244, 259). Both of these processes have been demonstrated to be dependent on LFA-1 mediated signals(259). Several studies have investigated the molecular components that mediate granule polarization. In human NK cells, it was shown that silencing of WASP leads to impaired lytic granule polarization despite efficient effector-target cell conjugate formation(260). While inhibition of phosphatidylinositol 4-phosphate 5-kinase type I (PI5KI)-$\alpha$ or PI5KI$\gamma$, the product of which is phosphatidylinositol (4,5)-bisphosphate (PIP2), does not affect the ability of granules to polarize, it impedes their exocytosis(261). In contrast, pharmalogical inhibition of PLC-$\gamma$, a downstream target of LFA-1 signaling, was shown to inhibit granule polarization(262). Blockade of the tyrosine kinase PYK2 impaired the ability of paxillin to be recruited to the immunological synapse and inhibited MTOC and granule polarization(263). This was also observed with the inhibition of the mitogen-activated protein kinase (MAPK) JNK(264). Furthermore, LFA-1 engagement in NK cells

with the actin nucleator Arp2/3 silenced led to decreases in Vav-1 activation and recruitment of active PYK2, which resulted in diminished target cell adhesion, reduced actin assembly at the synapse and impaired cytotoxicity(265). Interestingly, knockdown of hDia1, also an actin nucleator, did not disrupt actin assembly at the immunological synapse, but nevertheless resulted in impaired granule polarization and reduced cytotoxicity(265).

Following polarization of lytic granules to the immunological synapse, degranulation (or exocytosis of secretory lysosomes) is required to mediate NK cell cytotoxicity. Degranulation can be measured at the single cell level by quantifying the expression of CD107a (lysosomal-associated membrane protein-1 or LAMP-1) on the NK cell surface upon activation. CD107a colocalizes with perforin and granzymes in secretory granules and upon lysosome fusion with the plasma membrane redistributes on the surface of the cell(244, 266). Resting NK cell degranulation occurs rapidly and parallels intracellular $Ca^{2+}$ mobilization after activating receptor crosslinking or target cell interaction(244, 247, 267). While LFA-1-mediated signals play a prominent role the early steps of NK cell activation, namely contact, adhesion and granule polarization, they have been demonstrated to be dispensable for degranulation(244). Long and colleagues showed that mAb crosslinking of the activating receptor CD16 in the absence of LFA-1 engagement was sufficient to induce NK cell degranulation(247). Notably, independent ligation of NKp46, NKG2D, 2B4, DNAM-1 or CD2 did not produce the same results. Instead, degranulation was only induced when distinct, synergistic combinations of the activating receptors were triggered(247). These results suggested the existence of a critical activation threshold that must be surpassed in order for NK cells to mount a productive response and this is only achieved by simultaneous engagement of

synergistic co-activating receptor pairs, with the exception of CD16. Indeed, individual analysis of NK cell receptor signal strength of has revealed qualitative differences; engagement of CD16 is sufficient to induce robust $Ca^{2+}$ release from the endoplasmic reticulum (ER), a requirement for NK cell degranulation, whereas other receptors require specific pair-wise combinations to trigger intracellular $Ca^{2+}$ mobilization(247, 268, 269). A recent study by Kim et al. revealed the molecular basis for NK cell activation by synergizing receptors involves a requirement for strong Vav-1 signals that must overcome inhibition by the E3 ubiquitin ligase c-Cbl(270). They demonstrated that Vav-1 overexpression or c-Cbl knockdown each abrogated the need for synergistic co-receptor activation, as individual engagement of either NKG2D or 2B4 became sufficient to induce NK cell effector function. Investigation of the molecular components involved in NK cell $Ca^{2+}$ mobilization upon activation revealed a role for the ER $Ca^{2+}$ sensor STIM1 and the plasma membrane $Ca^{2+}$ release-activated $Ca^{2+}$ channel ORAI1. In patients with mutations in either STIM1 or ORAI1, NK cell degranulation was profoundly impaired while target cell adhesion and granule polarization remained intact(271). While the $Ca^{2+}$-binding proteins that facilitate exocytosis of lytic granules are not fully defined, they are thought to include synaptotagmin VII (proposed to be a candidate $Ca^{2+}$ sensor for lytic granule release)(272), Munc13-4 (proposed to be a candidate $Ca^{2+}$ sensor for lytic granule release and mediate priming of granules for vesicle fusion)(248, 273, 274), the small GTPase Rab27a (shown to bind to synaptotagmin-like proteins SLP1 and SLP2 and proposed to mediate tethering of lytic granules to the plasma membrane)(248, 275-277), syntaxin-11 (proposed to interact with SNAP23, VAMP7 and VAMP8 and facilitate granule fusion with the plasma membrane)(248, 272) and Munc18-2 (binding partner and regulator of syntaxin-11)(278, 279). Interestingly, Rab27a and Munc13-4 were found to colocalize with perforin-containing granules only upon NK cell activation through

engagement of LFA-1 or activating receptors such as CD16, suggesting that distinct endosomal compartments converge prior to lytic granule fusion with the plasma membrane(280, 281).

In addition to $Ca^{2+}$ mobilization, the Ras-ERK pathway has been implicated in regulating NK cell granule exocytosis. The Ras guanyl nucleotide-releasing protein-1 (RasGRP-1) was shown to act downstream of PLC-$\gamma$ integrating $Ca^{2+}$ and diacylglycerol (DAG) signals for activation of the Ras-ERK pathway and mediate NK cell cytotoxicity in IL-15 expanded NK cells(282). Furthermore, the MAPK scaffolding protein KSR1 involved in ERK activation was demonstrated to be dispensable for target cell adhesion, but required for lytic granule polarization and target cell lysis(283). Navigating the dense intracellular actin network located at the immunological synapse is another process that precedes NK cell exocytosis of lytic granules and has been shown to involve the ATP-dependent actin motor protein myosin IIA. NK cells from patients with the May-Hegglin disorder caused by autosomal dominant mutations in the myosin IIA encoding gene display impaired NK cell degranulation and cyctotoxicity, suggesting an important role for this motor protein in facilitating granule movement through the actin network for subsequent exocytosis(284). Taken together, it is clear that lytic granule exocytosis is a multi-step process, which may represent a mechanism for preventing the inappropriate release of the cytotoxic protein stores found within the NK cell arsenal.

Another important effector function of human NK cells is the production and release of chemokines and cytokines. Upon target cell recognition, resting NK cells have been shown to secrete chemokines such as MIP-1$\alpha$, MIP-1$\beta$ and RANTES, as well as

cytokines such as TNF-α and IFN-γ(246). The kinetics and signal strength requirement for chemokine secretion substantially differs from that of cytokine release. Bryceson and colleagues demonstrated that individual engagement of the activating receptors CD16, 2B4, or NKG2D induced rapid secretion of chemokines after stimulation with co-engagement of certain activating receptor pairs accelerating and increasing chemokine secretion(246). In contrast, IFN-γ secretion required the engagement of multiple receptors and occurred much later. These data revealed a hierarchy in requirements for induction of NK cell responses and is defined kinetically and quantitatively as follows: upon stimulation degranulation occurs immediately, followed by chemokine secretion (MIP-1α and MIP-1β, 1 hour), TNF-α secretion (3 hours) and IFN-γ secretion (5-6 hours). The activating signal strength required for chemokine secretion, however, is relatively weak, with degranulation requiring intermediate levels of activating stimuli and cytokine production requiring the strongest activating stimuli. This hierarchy is reflected in the molecular components required for induction of these different effector functions. While proximal signals from PLC-γ are required for all responses, deficiency in store-operated $Ca2^{+}$ entry (SOCE), resulting from mutations in STIM1 and ORAI1, lead to defective degranulation and cytokine production, but only partial impairment of chemokine production(268, 269, 271). Selective impairment of NK cell cytokine production was observed in PI3K p110δ-deficient mice, whereas both p110δ- and p110γ-deficiency was required to diminish cytotoxicity(285, 286). In NK cells from PKCθ-deficient mice, defective IFN-γ production and secretion was observed and found to be due to impaired JNK, AP-1 and NFAT activation(287). Notably, NK-cell mediated cytotoxicity was not impaired in these mice. Similarly, Rap1b-deficiency was shown to selectively impair chemokine and cytokine production without affecting NK cell cytotoxicity, suggesting a

divergence in signaling pathways that regulate different effector functions(257). This, in fact, has been demonstrated by various groups. Huntington et al. demonstrated that stimulation of CD45-deficient NK cells via activation receptors, such as CD16 and NKG2D, resulted in the activation of PI3K and induced cytotoxicity, but failed to elicit a cytokine and chemokine response. This defect in cytokine and chemokine secretion was associated with impaired activation of Src family kinases(288). Rajagopalan et al. showed that IFN-γ production, but not cytotoxicity, was specifically induced upon activation of resting NK cells through KIR2DL4, whereas CD16 and 2B4 induced cytotoxicity, but not IFN-γ production(289). Furthermore, they revealed that KIR2DL4 induction of IFN-γ production was dependent on the p38 MAPK signaling pathway, in contrast to the IL-2-induced IFN-γ secretion that requires the MAPK ERK1/2 pathway. IFN-γ production induced through the activating receptor 2B4 was also demonstrated by Chuang et al. to be selectively dependent on the p38 MAPK pathway(290). Together, these results show a functional dichotomy in the signals required for the cytokine versus cytotoxicity response of resting NK cells.

## ***NK cell cancer immunotherapies – Manipulation of the receptor repertoire***

Advances in our knowledge of the molecular mechanisms that govern NK cell function have driven the development of NK cell-based therapeutic strategies shown to be essential for tumor immunosurvelliance and eradication of hematological malignancies. Interest in the graft-versus-leukemia (GvL) effects of NK cells was first established by studies in which transplantation across the HLA class I barriers triggered alloreactive NK cell responses and were based on the KIR-ligand incompatibility model first described by the Perugia group(291). This model proposes, in agreement with the “missing self-

hypothesis", that during HCT donor-derived NK cells will be autoreactive when recipients lack donor-specific KIR ligands, or are in other words "KIR ligand mismatched". For example, individuals who express group 2 HLA-C alleles and possess KIR specific NK cells for group 2 HLA-C alleles (KIR2DL1) are alloreactive towards cells from individuals who lack group 2 HLA-C allele expression. The clinical efficacy and therapeutic benefits of KIR ligand mismatched donor/recipient pairs has been tested(292-297) and demonstrate that alloreactive NK cells are capable of: (i) decreasing rates of graft-versus-host-disease (GvHD) by killing host antigen-presenting cells (APCs) that present host alloantigens to donor T-cells(298, 299), (ii) decreasing rates of graft rejection through the lysis of host T-cells(291), (iii) increasing the GvL effect(300), (iv) improving engraftment and hematopoiesis via the secretion of hematopoietic cytokines(301, 302), and (v) decreasing transplant-related infections due to enhanced immune reconstitution and NK cell anti-viral activity(303). However, recent data indicate there are further complexities in what drives NK cell effector function against malignant targets as specific donor KIR genotypes are shown to be more beneficial to transplanted acute myeloid leukemia (AML) patients in both HLA-matched and HLA-mismatched settings(304). It was found that the KIR B haplotype, which contains a greater activating KIR content, conferred greater protection against relapse with the centromeric region of the KIR locus providing the most benefit. While the therapeutic potential for these alloreactive NK cells has been demonstrated in controlling the relapse of myeloid leukemias (i.e. AML), it has not been shown to translate into improved outcomes for lymphoid leukemias (i.e. acute lymphoblastic leukemia, ALL)(292, 304). Therefore, along with KIR ligand incompatibility, these data indicate the GvL effect is mediated by additional mechanisms influenced by the activating receptor repertoire. Studies examining the factors that facilitate leukemia resistance to NK cell cytotoxicity have revealed the importance of adhesion molecules

and NK cell activating ligand expression on tumor cells(305). As discussed earlier, the LFA-1/ICAM-1 ligand/receptor pair is a dominant adhesion pathway for NK cells and a lack of ICAM expression on target cells has been shown to partially contribute to the resistance of tumor cell lysis by alloreactive NK cells(306-309). Additional studies have also demonstrated that resistance of ALL to NK cell cytotoxicity may involve deficient engagement of activating NCRs, such as NKG2D, NKp30, NKp44 and NKp46, due to a lack of NCR ligand expression on leukemia blasts(309-311). Therefore, further investigation of the essential receptor/ligand interactions that convey a favorable activation status is critical for enhancing the therapeutic effects of alloreactive NK cells for the treatment of cancer.

In addition to cell-based immunotherapies, new drugs targeting NK cells are in development and designed to exploit the basic properties of NK cell effector function. A compound relatively advanced in its development is a human IgG4 blocking monoclonal KIR antibody IPH2101, which targets inhibitory KIR2DL-1, KIR2DL-2, and KIR2DL-3 and has been shown to enhance NK cell function against malignant cells by preventing inhibitory KIR-ligand interaction and subsequent inhibitory signaling(312). Studies using wild-type and KIR/HLA transgenic mice demonstrated that antibody blockade of MHC-specific inhibitory receptors did not abolish developing NK cell reactivity, indicating this type of treatment does not break tolerance to self or interfere with NK cell education(312-314). Furthermore, a recent study demonstrated combination therapy of IPH2101 and lenalidomide, an approved drug for treatment of multiple myeloma (MM) with immunostimulatory effects on NK cells(315), resulted in an additive anti-tumor effect where IPH2101 provided release from inhibition while lenalidomide simultaneously promoted NK-cell expansion and activation via induction of activating ligands on MM

targets(316). Different treatment combinations, such as chemotherapy, which induces the DNA damage pathway shown to upregulate NK cell activating ligands on tumor cells(317), or bortezomib, which upregulates tumor cell expression of tumor necrosis factor–related apoptosis-inducing ligand receptor 2/death receptor 5 (TRAIL-R2/DR5)(318), could also function to synergize with KIR blocking mAbs further enhancing the NK cell anti-tumor response.

Other therapeutic reagents developed to exploit NK cell effector functions target the potent activating receptor CD16 and its ability to mediate ADCC. Rituximab, a chimeric mouse anti-human mAb that targets the B-cell antigen CD20, was the first mAb approved by the Food and Drug Administration (FDA) for the treatment of relapsed or refractory, low-grade (indolent) or follicular, $CD20^+$ non-Hodgkin's lymphoma (NHL) and has shown to improve clinical outcomes when used alone or in combination with chemotherapy(319-321). There are several other antibodies currently being evaluated in the clinic that target B-cell malignancies and show therapeutic promise(322), some of which include: ofatumumab, a fully human IgG1κ mAb that targets a novel epitope of CD20(323); alemtuzumab, a humanized anti-CD52 mAb(324); galiximab, a macaque–human chimeric anti-CD80 mAb(325); dacetuzumab and lucatumumab, humanized antagonistic anti-CD40 mAbs(326, 327); epratuzumab, a humanized IgG1 anti-CD22 mAb(328); and lumiliximab, a macaque–human chimeric anti-CD23 mAb(329). An important mechanism by which these antibodies elicit a therapeutic immune response is via the interaction of the Fc domain with the potent NK cell activating receptor CD16 (FcγRIIIA) inducing ADCC(330). Unfortunately, the triggering of ADCC by therapeutic mAbs faces several limitations. First, several studies have examined the relationship

between FcγR polymorphisms and clinical responses to mAb therapy and have demonstrated a fivefold higher affinity between CD16 with a valine in position 158 (CD16-V158, represented in only ~20% of the population) and the Fc portion of IgG compared to CD16 with a phenylalanine in position 158 (CD16-F158, represented in ~80% of the population), with expression of the higher affinity CD16-V158 resulting in more efficient *in vivo* ADCC(331, 332). Second, differential glycosylation of the CH2 domain of the Fc region modulates the affinity between CD16 and the Fc region and thus affects the *in vivo* efficacy of the mAbs. It has been shown that the presence of fucose residues decreases ADCC efficiency and this is dependent on the enzymes expressed by the cell line used for antibody production(333, 334). Shinkawa et al. demonstrated that an anti-CD20 chimeric IgG1 produced by the rat hybridoma YB2/0 cell line displayed more than 50-fold higher ADCC than the same antibody produced by the Chinese hamster ovary (CHO) cell line, which has elevated expression of *FUT8*, the gene coding for 1,6-fucosyltransferase, and is the cell line traditionally used for the production of therapeutic proteins(333). Third, IgG serum concentration is 8–17 mg/mL and ~66% is of the IgG1 isotype capable of interacting with CD16 and competing with the therapeutic mAbs. Consequently, most mAbs have to be injected at high doses, reaching a serum concentration between 10-100 mg/mL, in order to mediate a therapeutic effect, whereas 10 ng/mL has been shown to be a saturating concentration for ADCC *in vitro* in the absence of competing IgG molecules(335). Lastly, the Fc portion of the mAb can also interact with inhibitory Fc receptors, such as FcγRIIb, which is expressed by B-cells, macrophages, DCs and neutrophils, decreasing their overall therapeutic efficiency(330).

To overcome these limitations, related reagents utilizing modified structural forms with bivalent effector/target specificities have been generated and are being clinically tested. One such reagent is a bispecific antibody altered in structure and initially developed targeting the effector functions of T-cells. This recombinant reagent is called a BiTE (bispecific T-cell engager) and, as discussed later, serves as model for the development of NK cell-directed reagents. BiTEs lacks an Fc domain and has both T-cell specificity and tumor target specificity by fusion of an anti-CD3 single chain variable fragment (scFv) to a tumor specific scFv via a short, flexible peptide linker, generating a tandem scFv that is small in size ~60kD (**Figure 8A**). Kufer and colleagues engineered one of the first tandem scFvs targeting EpCAM (epithelial 17-1A antigen) and human CD3 using the CHO cell line and demonstrated this reagent to be highly cytotoxic at nanomolar concentrations against various cell lines, using unstimulated human PBMCs in the absence of co-signaling(336). In 2000, Bargou and colleagues engineered a tandem scFv targeting murine anti-CD19 scFv and murine anti-CD3 and demonstrated *in vitro* that primary human T cells could be redirected against $CD19^+$ lymphoma cells inducing strong cytotoxicity at very low concentrations (10-100 pg/mL) and effector:target (E:T) ratios (2:1), which occurred rapidly and surprisingly without the need for T-cell co-stimulation(337).

As a result of these promising pre-clinical studies, the physical and functional properties of BiTEs have been thoroughly evaluated and many impressive qualities have been revealed for this class of molecule. First, the ability of these reagents to mediate strong anti-tumor activity in the absence of T-cell pre- or co-stimulation is thought to be due to its small size which facilitates the formation of a tight immunological synapse and provokes efficient effector cell activation overriding the need for reagent induced co-

activation signals(338). Second, the concentration of reagent required to achieve half maximal tumor cell lysis *in vitro* was shown to be in the range of 0.2-2pM and in some cases as low as 18fM was sufficient to elicit anti-tumor activity(339). Finally, BiTEs were observed to induce target cell cytotoxicity at E:T ratios as low as 1:10 and were demonstrated to mediate T-cell serial killing of multiple target cells(340). Remarkably, this reagent-mediated killing was target cell dependent as effector cells were not activated in the absence of targets, even at concentrations exceeding half maximal effective concentration ($EC_{50}$) values by several thousand fold(341).

The first Phase I clinical trial testing a murine anti-human CD3/anti-human CD19 BiTE, called blinatumomab (MT103), in patients with relapsed NHL also showed encouraging results; doses as low as 5 µg per square meter per day ($\mu g/m^2/d$) led to an elimination of target cells in blood and patients treated at a dose level of 60 µg had tumor regression(342). While blinatumomab was found to have a short serum half-life due to its small size and, as a result, required continuous intravenous infusion by a portable mini-pump, remarkably cumulative doses of several milligrams were sufficient to induce notable responses in patients, in contrast to conventional antibody treatments, such as rituximab, which require gram amounts per treatment cycle. Furthermore, a Phase II clinical trial with patients being treated for B-precursor acute lymphoblastic leukemia (B-ALL) who were minimal residual disease positive ($MRD^+$) demonstrated a complete molecular response rate of 80% to blinatumomab monotherapy, converting patients into a $MRD^-$ status and showing a corresponding Kaplan-Meier survival estimate for relapse-free survival probability of 78%(343). In this trial, blinatumomab was continuously infused to patients at a dose level of 15 $\mu g/m^2/d$ for 4 weeks and then followed by

subsequent treatment cycles that were spaced by 2-week treatment-free intervals. Analysis of the pharmacokinetics, response of B- and T-cells, and cytokine release during blinatumomab treatment revealed that (i) a steady-state serum concentration of 13pM was achieved after continuous IV infusion of blinatumomab, which mediated a durable depletion of $CD19^+$ target B-cells in patients by redirected lysis; (ii) treatment induced T-cell expansion above baseline counts with the majority upregulating CD69; (iii) cytokines IL-2, IL-6, IL-10, IFN-$\gamma$, and TNF-$\alpha$ were transiently detectable after the first infusion and no longer detected on start of a second treatment cycle; and (iv) long term, continuous exposure to the global T-cell-activating BiTE blinatumomab did not lead to uncontrolled T-cell activation or T-cell anergy, further demonstrating the therapeutic efficacy of BiTE molecules(344).

Tandem scFvs being developed to target the potent NK cell activating receptor CD16 are still in the pre-clinical phases of development and are termed bispecific killer cell engagers (BiKEs) (**Figure 8B**). In 1999, McCall et al. generated a bispecific scFv that co-targets CD16 on human effector cells and the Her2/neu tumor associated antigen expressed on human tumors(345). They showed that peripheral blood lymphocytes treated with the bispecific reagent significantly enhanced *in vitro* lysis of human SKOV3 ovarian cancer cells overexpressing the Her2/neu tumor antigen and *in vivo* biodistribution analysis revealed localization and retention of the bispecific reagent in the tumor, demonstrating the tumor specific nature of the CD16/Her2/neu BiKE. In 2005, Bruenke et al. demonstrated the *in vitro* efficacy of a CD16/CD19 BiKE targeting $CD19^+$ B-cell malignanices(346). Purified human NK cells or PBMCs treated with the bispecific reagent efficiently lysed human B-lymphoid cell lines and primary B-cell chronic lymphocytic leukemia (CLL) or B-cell ALL tumors (**Figure 8C**). For the treatment of acute

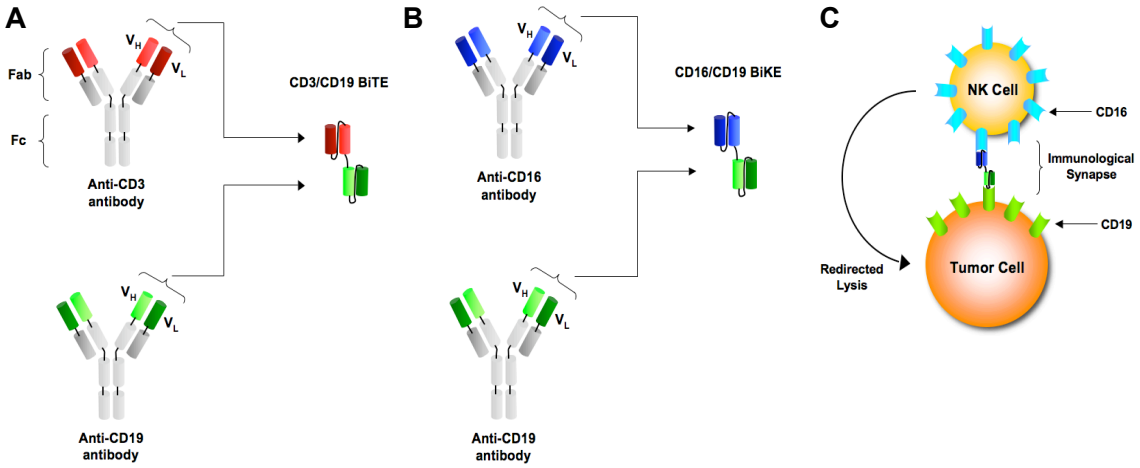


**Figure 8: Schematic representation of BiTEs and BiKEs structures.** The generation of BiTEs (A) and BiKEs (B) results from the genetic fusion of variable domains from two distinct monoclonal antibodies. In a BiTE, the anti-CD3 single chain variable domain is specific for the effector T-cell (red) and in a BiKE, the anti-CD16 single chain domain is specific for the effector NK cell (blue), noting that other CD16 expressing cells, such as macrophages, may also be targeted by this domain. The target antigen single chain domain is specific for the tumor antigen of interest (green), such as CD19 (as seen in the BiTE blinatumomab or MT103). As shown, both BiTEs and BiKEs lack an Fc domain and directly target the effector cell of interest making them more specific and less likely to induce an excessive immune response via the engagement of various Fc receptors. Moreover, the single polypeptide chain that is produced is flexibly linked and, as a result, the two binding sites of the bispecific scFv reagents can bend, twist and rotate freely about their axes. This flexibility facilitates the simultaneous binding of two antigen epitopes on two different cell surfaces enhancing effector-tumor cell interactions. Once bound, these reagents transiently connect an effector cell and a tumor cell (C, represented in the diagram by a BiKE) and trigger effector cell activation, which leads to the redirected lysis of the associated tumor cell.

myeloid leukemia (AML), Singer et al. recently developed a bispecific scFv specific for CD16 and the tumor-associated antigen CD33 and demonstrated enhanced *in vitro* cytotoxicity against primary human tumor cells when PBMCs were treated with the reagent(347). Together, these data indicate antibody derivatives that specifically target and exploit the effector functions of human NK cells, such as BiKEs, may be clinically beneficial for cancer treatment and warrant further testing in clinical trials. This may especially be the case in targeting MRD after HCT as recipient recovery of donor immune cells after HCT is slow resulting in a diminished capacity for NK cells to recognize and kill residual tumor cells, limiting the anti-tumor control mechanisms of the

GvL effect. Therefore, CD16-directed recombinant reagents have great therapeutic potential for inducing optimal NK cell effector functions post-HCT by enhancing NK cell-mediated innate immune responses.

## ***Tim-3 in immune regulation***

T-cell immunoglobulin and mucin-containing domain (Tim)-3 was originally identified in 2002 as a molecule specifically expressed on IFN-γ producing $CD4^+$ T helper type 1 (Th1) and $CD8^+$ T cytotoxic type 1 (Tc1) cells in mice and in 2004 it was found to also be expressed on IFN-γ producing human T-cells(348, 349). The specific expression of Tim-3 on Th1 T-cells thus focused initial investigations of Tim-3 immunoregulatory roles on this lymphocyte population. Early studies demonstrated Tim-3 functions to negatively regulate the Th1 immune response in various disease states triggering cell death upon engagement with its ligand(350-355). However, it has been subsequently shown that other cell types, such as DCs(356), monocytes(356), mast cells(357) and NK cells(349), also express Tim-3 where it functions to promote distinct immune responses that include both negative and positive regulation. How these dual roles of Tim-3 in different cell types are regulated and which one predominates in different disease states is not yet fully understood and remains a focus of investigation.

### *The Tim family of proteins*

In humans, the Tim family is composed of three members (Tim-1, Tim-3, and Tim-4) encoded by genes *hepatitis A virus cellular receptor (HAVCR)1*, *HAVCR2* and *TIMD4* located on human chromosome 5. Expression, function, and structural studies reveal that human Tim-1, Tim-3, and Tim-4 are orthologs of murine Tim-1, Tim-3, and Tim-4,

with human Tim-3 sharing ~70% homology with murine Tim-3(358). The Tim family of proteins are type I cell-surface glycoproteins that share common structural features consisting of an N-terminal Ig-like domain followed by a mucin domain with O-linked and N-linked glycosylations, a single transmembrane domain and a cytoplasmic region that contains tyrosine phosphorylation motif(s), except in TIM-4 where the cytoplasmic tail lacks such a region (**Figure 9A**)(359). Despite these similarities, there are differences in their molecular structures as well as in their expression patterns indicating their potential for distinct regulatory functions of cellular responses. Indeed, TIM-1 is preferentially expressed on Th2 T-cells and has been shown to function as a potent co-stimulatory molecule for T-cell activation(360). As mentioned earlier, TIM-3 is expressed on specific subsets of T-cells as well as cells of the innate immune system and has various roles in immune regulation. In contrast, TIM-4 is exclusively expressed on APCs and has been demonstrated to function in the phagocytosis of apoptotic cells and also plays an important role in maintaining tolerance(361, 362). The Tim family thus represents a diverse functional repertoire of proteins that have a variety of immunoregulatory roles.

*Tim-3 protein structure*

Crystal structures of the Tim-Ig-like domains determined they belong to the immunoglobulin variable (IgV) set and are composed of two, anti-parallel β-sheets that contain six conserved cysteine residues(363-365). Four of these cysteine residues form a unique binding cleft, termed FG-CC', that is conserved in all Tim family members and not seen in the Ig domain of any other Ig superfamily members. This distinctive binding pocket is used for the recognition of ligands, such as phosphatidylserine (PS)(363-366). In the IgV domain of Tim-3 two, N-linked sugars serve to facilitate interactions with carbohydrate recognizing proteins, such as galectin-9 (Gal-9), and are located opposite

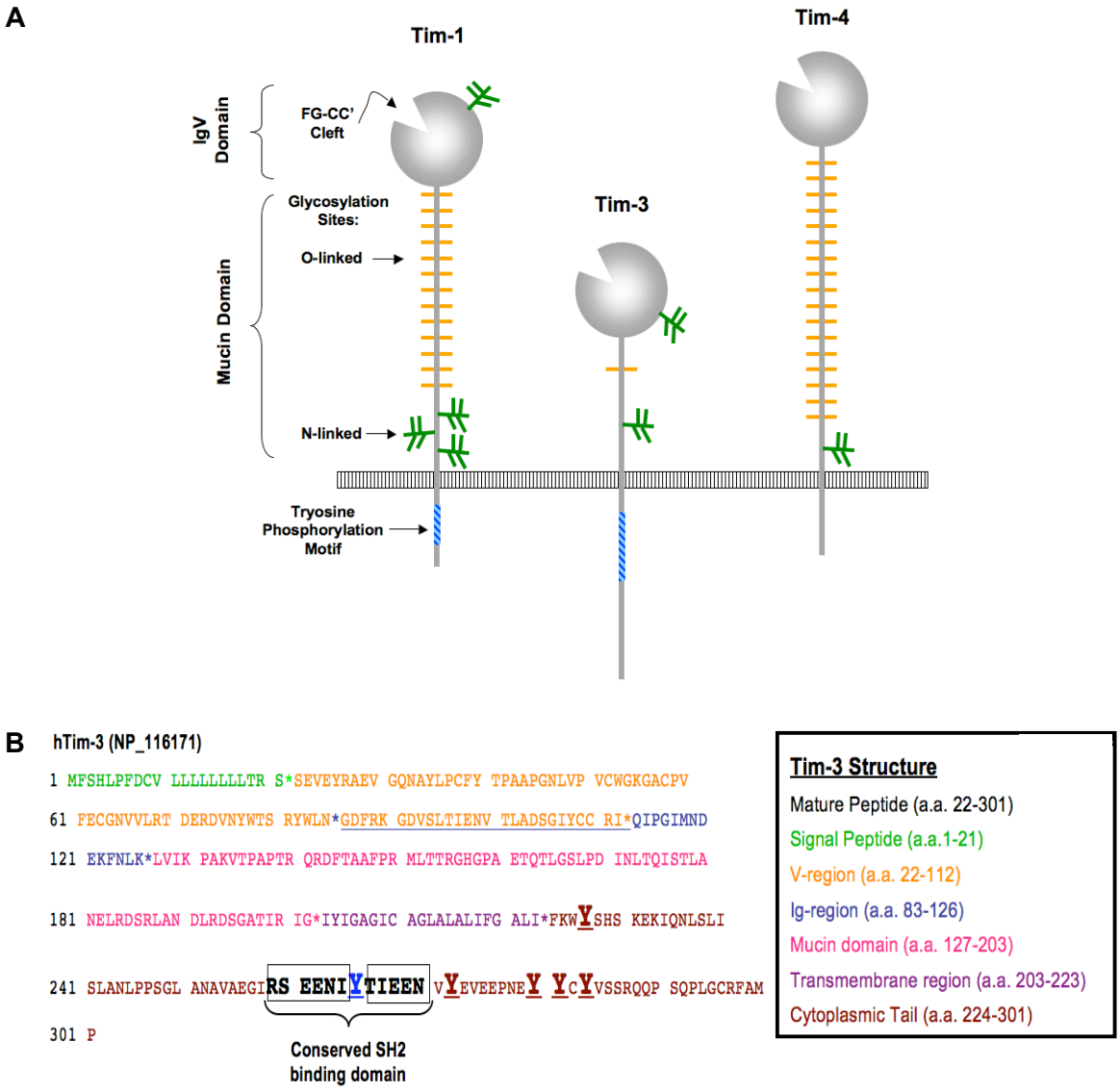


**Figure 9: Schematic representation of human Tim protein structures.** A) The Tim family of proteins are type 1 membrane proteins that all possess a similar structure, which consists of an N-terminal cysteine-rich IgV domain, a mucin domain, which is heavily glycosylated by N- and O-linked sugars in the Tim-1 and Tim-4 receptors, a transmembrane domain and an intracellular tail that contains tyrosine phosphorylation motifs (except in the Tim-4 receptor) that are involved in receptor signaling. Notably, Tim family members contain a unique FG-CC' binding cleft, also termed metal-ion ligand binding site (MILIBS), in the IgV domain formed by six conserved cysteine residues. B) The human Tim-3 amino acid sequence with the distinct regions color-coded. The asterisks indicate the end of a region, noting that the Ig-region overlaps with the V-region and the beginning is indicated by the first purple asterisk and underlined portion. There are six conserved tyrosine residues in the cytoplasmic domain as depicted, one of which resides in the SH2 binding domain.

of the FG-CC' cleft. While Tim-3 is thought to typically exist in monomeric form, a recent study demonstrated the ability of Tim-3 to form heterotypic interactions with Tim-1 and

Tim-4 that was highly dependent on the presence of the glycosylated mucin domains, indicating an adhesion role for the mucin stalks of Tim proteins(367). The Tim-3 cytoplasmic tail contains six well-conserved tyrosine residues and an Src homology 2 (SH2) binding domain (**Figure 9B**). In a series of experiments, Kane and colleagues demonstrated distinct roles for five of the cytoplasmic tail tyrosine residues with the three most C-terminal tyrosine residues exhibiting dominant inhibitory activity and with two of the more N-terminal membrane proximal tyrosine residues displaying an activating function, which ultimately serve to mediate both the positive and negative immunoregulatory roles of Tim-3(368).

*Tim-3 ligands*

In 2005, Kuchroo and colleagues demonstrated Gal-9 to be a ligand for Tim-3, interacting with the N-linked sugars located in the IgV domain of Tim-3 via its carbohydrate recognizing domains (CRDs)(350). Gal-9 is a 40-kD S-type β-galactoside binding tandem-repeat-type lectin that contains two non-identical CRDs joined by a flexible linker peptide sequence of 60 amino acids(369, 370). While the mechanisms of interaction between Gal-9 CRDs and target carbohydrates is not fully clear, it has been demonstrated that Gal-9 preferentially binds larger poly-N-acetyllactosamine containing structures with the N-terminal CRD displaying greater affinity compared to the C-terminal CRD(371). Furthermore, it was shown that the two CRDs can bind simultaneously to one carbohydrate residue or engage two separate molecules crosslinking engaged targets and generating Gal-9-glycan lattices, which function to modulate cell surface receptor organization and signal duration(372). While Gal-9 lacks a signal sequence, it can localize to the plasma cell membrane or be secreted, the mechanism of which remains poorly understood(369). Gal-9 is broadly expressed and highly prevalent in tissues of the

immune system, such as the bone marrow, lymph nodes, thymus and spleen(369). Gal-9 expression is particularly high in mast cells and also found in T-cells, B-cells, macrophages, endothelial cells, and fibroblasts. Consequently, Gal-9 has been linked to the regulation of immune homeostasis and inflammation(373). In fact, the expression of Gal-9 has been shown to be regulated by IFN-$\gamma$ and a negative feedback loop has been proposed where IFN-$\gamma$ produced by Th1 T-cells upregulates Gal-9 expression, which in turn functions to downregulate the Th1 inflammatory response inducing apoptosis in Tim-3-positive Th1 T-cells(356, 374). As Gal-9 recognizes and binds carbohydrates, it is not surprising that in addition to Tim-3, Gal-9 interacts with multiple target molecules. This is supported by findings that showed only a 40% reduction in Gal-9-mediated Th1 cell death in Tim-3 deficient mice(350). Moreover, in murine models of allergic asthma Gal-9 has been demonstrated to interact with CD44 and IgE providing an anti-allergic effect(375, 376).

In addition to Gal-9, PS has been demonstrated to be a ligand for Tim-3 and interacts via the FG-CC' cleft in a $Ca^{2+}$-dependent manner(366). This unique binding site contains several conserved residues that coordinate with metal ions, such as calcium, and thus has been termed the metal ion-dependent ligand-binding site (MILIBS). PS is normally localized to the inner leaflet of the plasma membrane and upon induction of apoptosis it is redistributed and exposed on the outer membrane, thus providing a key signal that triggers phagocytosis of injured cells(377, 378). As noted earlier, the FG-CC' cleft and N-linked sugars are located on opposite sides of the IgV domain; therefore, binding of one ligand does not preclude binding of the other. Interaction of Tim-3 with PS has been shown to be important for clearance of apoptotic cells and maintenance of peripheral

immune tolerance. Nakayama et al. demonstrated an increase in apoptotic cells, auto-antibodies and inhibition of apoptotic cell phagocytosis and reduced cross-presentation of dying cell-associated antigens by $CD8^+$/Tim-$3^+$ DCs in mice treated with a Tim-3 mAb(379). Furthermore, while Tim-3-positive APCs phagocytized apoptotic cells upon interaction with PS, Tim-$3^+$ T- and B-cells were capable of forming conjugates via PS, but incapable of engulfing apoptotic cells, thought to be due to a lack of cellular machinery required for phagocytosis or the expression of SIRP$\alpha$, which actively signals for the inhibition of phagocytosis(366, 380). Although the functional relevance of Tim-3/PS interactions on these lymphocytes is unclear, it is hypothesized they may provide pro-apoptotic signals similar to Tim-3/Gal-9 interactions or binding of both Gal-9 and PS to Tim-3 may synergize to induce crosslinking of Tim-3.

*Immunoregulatory roles of Tim-3 in disease*

The role of Tim-3 *in vivo* has been most rigorously tested in experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS). MS pathogenesis involves chronic inflammation associated with dysregulated Th1 responses and the production of IFN-$\gamma$ and TNF-$\alpha$ proinflammatory cytokines, widespread primary demyelination and glial scarring(381). Monney et al. demonstrated accelerated disease progression during induction of EAE with the blockade of Tim-3-Tim-3 ligand interaction *in vivo* using a Tim-3 mAb resulting in a severe form of demyelinating disease characterized by higher numbers of inflammatory foci in the central nervous system (CNS) and higher numbers of activated macrophages in demyelinating lesions(348). In contrast, when treated with soluble Gal-9 there was a selective reduction in the number of IFN-$\gamma$ producing cells and overall disease severity(350). Human T cell clones derived

from the cerebrospinal fluid of patients with MS displayed lower Tim-3 expression and increased IFN-γ production compared to those from healthy control subjects, suggesting downregulation of Tim-3 may serve as a mechanism by which autopathogenic cells escape immune regulation(352). In fact, MS patients being treated with glatiramer acetate or IFN-β had restoration of Tim-3 expression in $CD4^+$ T cells and displayed negative regulation of the Th1 inflammatory response, which was abrogated with Tim-3 blockade, further demonstrating the physiological relevance of the negative regulatory functions of Tim-3(382). Moreover, it has been shown that infiltrating monocytes that contribute to CNS inflammation also express Tim-3(383). Studies revealed that Tim-3 was upregulated on CNS infiltrating $CD11b^+$ monocytes and resident microglia during the induction of EAE while activated peripheral $CD11b^+$ monocytes lacked Tim-3 expression(356). In humans, higher Tim-3 expression was found in $CD11b^+$ microglia in CNS white matter from the active border regions of MS lesions compared to the non-inflamed tissues. Correspondingly, Gal-9 expression in MS lesion astrocytes was elevated relative to normal human CNS tissue, suggesting a potential immunoregulatory system that could terminate detrimental inflammatory responses by Tim-3 expressing cells(356). However, while Tim-3/Gal-9 interactions terminate Th1 responses, it has been shown that Gal-9 stimulates TNF-α production of $Tim\text{-}3^+$ monocytes, which would contribute to the pathogenic inflammatory process(356). Furthermore, a role for $Tim\text{-}3^+$ microglia in phagocytosis of apoptotic cells and cross-presentation of antigen has been demonstrated in tolergenic pathways and is suggested to contribute to the phagocytosis of apoptotic myelin and cross-presentation of myelin antigens to T cells as myelin is a PS-containing membrane(379, 384, 385). Consequently, whether Tim-3 expressing microglial cells function to improve or exacerbate disease remains unclear and further

investigation is needed to fully understand the role of Gal-9, PS and their interactions with Tim-$3^+$ microglial cells in the pathogenesis of this disease.

Tim-3 is also involved in the induction of peripheral tolerance. Sabatos et al. demonstrated that administration of a Tim-3-Ig fusion protein upon restimulation of mice tolerized with high dose aqueous antigen resulted in abrogation of tolerance induction as Th1 cells displayed hyperproliferation and cytokine release(386). Furthermore, they showed Tim-3-deficient mice were refractory to the induction of high-dose tolerance. In another study, nonobese diabetic (NOD) mice treated with Tim-3-Ig displayed accelerated diabetes onset and despite costimulatory blockade with either a cytotoxic T lymphocyte antigen 4 chimeric fusion protein (CTLA4-Ig) or combined treatment with donor-specific transfusion (DST) and an antibody to CD154 (CD40L), were incapable of acquiring transplantation tolerance as MHC-mismatched allografts were rejected(387). In a murine models of acute GvHD (aGvHD) Tim-3-deficient mice or administration of Tim-3-Ig resulted in Tim-3 expressing $CD4^+$ and $CD8^+$ T-cells displaying increased IFN-$\gamma$ production, proliferation and enhancement of donor $CD8^+$ T-cell cytotoxicity against host alloantigen, which ultimately accelerated aGvHD lethality(388, 389). Moreover, there is evidence suggesting the mechanism by which Tim-3 regulates auto- and alloimmunity occurs by modulating the ability of regulatory T cells ($T_{regs}$) to dampen inflammatory responses(387, 388).

Recent studies have also demonstrated a role for Tim-3 in mediating T-cell exhaustion during chronic viral infections and anti-tumor responses. In human immunodeficiency virus (HIV) infection, Tim-3 has been identified to mark a population of exhausted T-cells in infected patients distinct from the population expressing the inhibitory molecule

programmed cell death 1 (PD-1)(354). The frequency of this population was significantly upregulated in chronically HIV-1-infected individuals compared to uninfected individuals and levels of Tim-3 expression positively correlated with HIV-1 viral load and inversely with $CD4^+$ T-cell counts. Moreover, during progressive infection Tim-3 expression was upregulated on HIV-1-specific $CD8^+$ T-cells, which were functionally hyporesponsive in response to antigen. Blockade of Tim-3 in HIV-1 specific T-cells with Tim-3-Ig or Tim-3 mAbs restored proliferation and enhanced cytokine production. Furthermore, Tim-3 expressing $CD8^+$ HIV-specific T-cells from chronically infected HIV patients displayed reduced cytotoxicity resulting from a block in degranulation, which was rescued upon interruption of the Tim-3 pathway(390). In hepatitis C virus (HCV) infection, Tim-3 expression was found to be specifically upregulated on liver-resident HCV-specific T-cells, which correlated with an exhausted phenotype displaying reduced proliferation and cytokine production by Th1 and Tc1 T-cells(353). Blocking the Tim-3 pathway restored the ability of Tim-3 expressing HCV-specific T-cells to proliferation and produce IFN-$\gamma$ in response to HCV-specific antigens. Furthermore, Tim-3 pathway has also been linked to disease progression in hepatitis B virus (HBV) infection and HBV-associated hepatocellular carcinoma (HCC), with hepatic T-cells displaying increased Tim-3 expression levels that induced T-cell proliferative senescence and decreased cytkoine production, which was shown to be mediated through interactions with Gal-9-expressing Kupffer cells in HBV-associated HCC(391-393). Tim-3 expression and function has also been investigated in lymphocytic choriomeningitis virus (LCMV) and herpes simplex virus (HSV) infection. Sehrawat et al. demonstrated that during HSV infection Tim-3 expression on $CD8^+$ T-cells was transient and found only on a small fraction of cells, but blockade of Tim-3-Gal-9 interactions nonetheless resulted in increased effector and memory $CD8^+$ responses and more efficient viral clearance(394). Furthermore, it was

shown that viral reactivation was delayed in Gal-9-deficient mice, suggesting a mechanism by which Tim-3/Gal-9 interactions may influence the outcome of HSV latency(395). In contrast to HSV, during chronic LCMV and Friend virus infection $CD8^+$ T-cells displayed sustained, high-levels of Tim-3 co-expressed with PD-1 on a large fraction of cells and functioned to impair $CD8^+$ T cell cytokine production(396, 397). These results all indicate that Tim-3 is upregulated during the course of T-cell activation and serves as a negative regulator to limit immunopathology during viral infections.

In solid tumor models, such as melanoma, colon and mammary adenocarcinoma, tumor-infiltrating lymphocytes (TILs) have been shown to express both Tim-3 and PD-1 regulatory molecules with Tim-3/PD-1 double positive populations representing a major fraction of the $CD8^+$ TILs in all three models(355, 398). This double positive population exhibits significant defects in proliferation, IL-2, IFN-$\gamma$ and TNF-$\alpha$ production and were shown to represent the most functionally impaired cells. By contrast, PD-1 single positive cells produced the most IFN-$\gamma$ among all the TILs in the colon adenocarcinoma tumor model and had fewer defects in proliferation, IL-2 and TNF-$\alpha$ production, suggesting the double positive Tim-3/PD-1 population represents the most exhausted TILs(355). Furthermore, combined blockade of Tim-3 and PD-1 resulted in significant decreases in tumor growth in a murine model of melanoma and significant increases in proliferation, IFN-$\gamma$, IL-2 and TNF-$\alpha$ production in tumor-antigen-specific $CD8^+$ T-cells isolated from patients with melanoma when stimulated *in vitro* with specific antigen(355, 398).

A recent study demonstrated exhausted $CD8^+$ T-cells that coexpress Tim-3 and PD-1 could also be found in a mouse model of acute myelogenous leukemia (AML)(399). It

was shown that PD-1 and Tim-3 co-expression increased during AML progression and that Tim-3/PD-1 double positive cells had defects in their ability to produce IFN-γ, TNF-α, and IL-2 in response to PD-1 ligand (PD-L1) and Gal-9 expressing AML cells. Interestingly, PD-1-deficient cells, which displayed partial resistance to AML, up-regulated Tim-3 expression during AML progression and the Tim-$3^+$/PD-$1^-$/$CD8^+$ T-cells had significant reductions in cytokine production. Moreover, as shown in the solid tumor models, blockade of the PD-1/PD-L1 and Tim-3/Gal-9 pathways resulted in significant reductions in tumor burden and enhanced survival. Together, these studies demonstrate a role for Tim-3 in T-cell exhaustion in both solid and non-solid tumors and have implications for combined targeted therapies that aim to restore antigen-specific $CD8^+$ T-cell function in disease.

*The role of Tim-3 in innate immune responses*

In addition to providing an immunoregulatory role for T-cells, Tim-3 is also expressed on cells of the innate immune system and has been shown to induce diverse functions. Anderson et al. demonstrated that stimulation of DCs with lipopolysaccharide (LPS) and Gal-9 resulted in enhanced TNF-α production synergizing with toll-like receptor (TLR) signaling, in contrast to Tim-3-deficient DCs that displayed a considerably reduced response(356). Moreover, Gal-9-mediated TNF-α production by Tim-$3^+$ human monocytes was blocked when treated with a blocking Tim-3 mAb while stimulation of DCs with an agonistic Tim-3 mAb led to the activation of NFκB, demonstrating an immunopotentiating role for Tim-3 in DCs. In a mouse model of fibrosarcoma, Gal-9 administration increased the number of Tim-$3^+$/$CD86^+$ mature DCs as well as Tim-$3^+$/$CD8^+$ T-cells that displayed enhanced IFN-γ production and granzyme B and perforin

expression, while inducing apoptosis of $CD4^+$ T-cells(400). Furthermore, depletion of Tim-$3^+$ DCs from Gal-9-treated tumor-bearing mice decreased $CD8^+$ T-cell IFN-$\gamma$ production, suggesting Tim-3/Gal-9 interactions on DCs and $CD8^+$ T-cells enhance antitumor immunity that may occur through a positive feedback loop where activation of DCs through Tim-3 leads to the secretion of proinflammatory cytokines that in turn activate and upregulate Tim-3 expression in $CD8^+$ T-cells.

In mast cells, Tim-3 expression was found to be upregulated upon stimulation with antigen and IgE and Tim-3 crosslinking led to the production of Th2 cytokines without promoting mast cell degranulation, suggesting a more prominent role for Tim-3 in enhancing the cytokine response(357). Moreover, treatment with a Tim-3 polyclonal antibody (pAb) induced IL-3 production and countered IL-3 withdraw-induced mast cell apoptosis. These results demonstrate a positive immunoregulatory role for Tim-3 in mast cells and suggest Tim-3 may influence mast cell modulation of the T-cell response. However, in a model of coxsackievirus B3 (CVB3)-induced myocarditis, Tim-3 blockade on mast cells and macrophages led to increased cardiac inflammation, indicating a negative immunoregulatory role in the context of heart disease(401, 402). In a recent study evaluating the role of Tim-3 in anti-microbial immunity, Gal-9 expressing macrophages infected with *Mycobacterium tuberculosis* were found to be activated by Tim-3-expressing Th1 T-cells producing IL-1$\beta$ via caspase-1 activation, demonstrating bi-directionality in the Tim-3/Gal-9 signaling pathway(403). As Gal-9 lacks an intracellular signaling domain, it is proposed that Tim-3 and Gal-9 form a signal-transduction complex on the macrophage cell surface with other transmembrane proteins capable of signaling and facilitating activation of the macrophage. Furthermore,

in a mouse model of melanoma administration of Gal-9 led to increased numbers of NK cells and macrophages and enhanced NK cell effector function, which was shown to be dependent on Gal-9 treated macrophages(404). While the exact mechanism underlying the induced NK cell function was not evaluated, it is possible Tim-3/Gal-9 interactions play a role as activated macrophages express Gal-9 and NK cells express Tim-3. In contrast to a potential activating role for Tim-3 in NK cells in the context of tumor immunity, a study investigating the effects of Tim-3 on NK cell function during chronic HBV infection demonstrated enhanced cytotoxicity and elevated IFN-γ production in NK cells isolated from patients with HBV infection upon *ex vivo* Tim-3 blockade(405). Collectively, these data clearly indicate a dual role for Tim-3 in immune regulation that is influenced by cell type and microenvironment.

# CHAPTER 1

## TIM-3 IS AN INDUCIBLE HUMAN NATURAL KILLER CELL RECEPTOR THAT ENHANCES INTERFERON GAMMA PRODUCTION IN RESPONSE TO GALECTIN-9

Originally published by the American Society of Hematology in the journal *Blood*. Volume: 119(13), Pages: 3064-3072

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Natural killer (NK) cell function is regulated by the integration of signals received from activating and inhibitory receptors. Here we show that a novel immune receptor, Tim-3, is expressed on resting human NK cells and is upregulated upon activation. The NK92 NK cell line engineered to overexpress Tim-3 showed a marked increase in IFN-γ production in the presence of soluble rhGal-9 or Raji tumor cells engineered to express Gal-9. The Tim-$3^{+}$ population of low dose IL-12/IL-18-activated primary NK cells significantly increased IFN-γ production in response to soluble rhGal-9, Gal-9 presented by cell lines, and primary acute myelogenous leukemia (AML) targets that endogenously express Gal-9. This effect is highly specific as Tim-3 antibody blockade significantly decreased IFN-γ production and Tim-3 crosslinking induced $Ca^{2+}$ mobilization, ERK activation and degradation of IκBα. Exposure to Gal-9 expressing target cells had little effect on CD107a degranulation. Reconstituted NK cells obtained from patients after hematopoietic cell transplant had diminished expression of Tim-3 compared to paired donors. This observation correlates with the known IFN-γ defect seen early post-transplant. In conclusion, we show that Tim-3 functions as a human NK cell co-receptor to enhance IFN-γ production, which has important implications for control of infectious disease and cancer.

## Introduction

Human NK cells are lymphocytes that develop from hematopoietic progenitor cells in the bone marrow and secondary lymphoid tissues(69). Peripheral blood NK cells are phenotypically defined as expressing the surface receptor CD56 (NCAM, neural cell adhesion molecule) and lacking expression of CD3(406). They mediate their function through the exocytosis of lytic granules that contain perforin and granzymes(407), the expression of death receptor ligands(44), the expression of FcRγIII (CD16A), which mediates antibody-dependent cell-mediated cytotoxicity(408), and the secretion of cytokines and chemokines(409). As a result, NK cells take part in both the innate and adaptive immune systems and play important roles in the control of viral infections, pregnancy, tumor immunosurveillance, and hematopoietic cell transplantation (HCT)(294, 410).

The ability of NK cells to differentiate normal healthy cells (self) from virally infected or malignantly transformed cells (non-self) is regulated by a sophisticated repertoire of cell surface receptors that control their activation, proliferation and effector functions(155, 246, 411). Recently, a novel receptor, T cell immunoglobulin and mucin-containing domain-3 (Tim-3), has been described to have various roles in immune regulation and is highly expressed on NK cells in mice and humans(348, 349, 356, 412-414). Tim-3 is a type I membrane glycoprotein that was first identified as a cell marker of terminally differentiated CD4+ T helper type 1 (Th1) cells18. Galectin-9 (Gal-9), a 40-kD S-type β-galactoside binding lectin, is a known ligand for Tim-3 and is highly expressed in tissues of the immune system, such as the bone marrow, lymph nodes, thymus and spleen(350, 369). The functional role of the Tim-3/Gal-9 pathway in T cells was first described to

negatively regulate the Th1 response(350). In contrast, stimulation of Tim-3 expressing dendritic cells (DCs) results in the secretion of pro-inflammatory cytokines(356, 386). Therefore, the Tim-3/Gal-9 interaction is considered to mediate both inhibitory and activating signaling pathways that have important roles in infection, autoimmunity, inflammation, peripheral tolerance and tumor immunity(354, 373, 399, 404, 415).

The potential of NK cells to control human hematological malignancies has been increasingly recognized in recent years(291, 294, 416). Initial studies were based on the KIR-ligand incompatibility model in which transplantation across the HLA class I barriers triggers alloreactive NK cell responses(291, 294, 416). More recently, specific donor NK cell receptor repertoires have been associated with less relapse and improved survival for patients receiving unrelated donor transplants for both HLA-matched and HLA-mismatched settings(304, 417). Therefore, to enhance the therapeutic effects of NK cells, a better understanding of the mechanisms that underlie target cell recognition is needed. While Tim-3 is highly expressed on NK cells compared to other lymphocyte populations(349, 414), the functional relevance of this is not completely understood. These experiments were designed to test the hypothesis that Tim-3 acts to mediate NK cell effector function.

## Materials and Methods

**Cell Isolation**. Adult peripheral blood (PB) was obtained from the Memorial Blood Center (Minneapolis, MN). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation using a Histopaque gradient (Sigma-Aldrich, St. Louis, MO) and NK cells were negatively selected using the magnetic-activated cell sorting (MACS) NK Cell Isolation Kit as per the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Additional samples from patients who received double unit umbilical cord blood transplants using cyclosporine and mycophenolate mofetil graft versus host disease (GvHD) prophylaxis were analyzed. All samples were obtained after informed consent in accordance with the Declaration of Helsinki, using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota.

**Cell Lines**. NK92 cells were cultured at 37°C with 5% $CO_2$ in NK92 medium (alpha medium containing 12.5% fetal bovine serum, 12.5% horse serum (HyClone Laboratories, Logan, UT), 0.2 mM inositol, 0.1 mM β-mercaptoethanol, 0.02 mM folic acid (Sigma-Aldrich), 100 U/mL penicillin, 100U/mL streptomycin (Invitrogen, Carlsbad, CA)) containing 500 U/mL recombinant human IL-2 (Chiron, Emeryville, CA). Raji cells were cultured at 37°C with 5% $CO_2$ in RPMI medium supplemented with 10% fetal bovine serum. K562 cells were cultured at 37°C with 5% $CO_2$ in Iscove's medium supplemented with 20% fetal bovine serum.

**Cell Transfection**. The full-length human Tim-3 cDNA or Gal-9 cDNA, generated by RT-PCR from RNA obtained from primary human NK cells and Jurkat cells respectively, was

cloned into the murine stem cell virus (MSCV) enhanced green fluorescent protein (eGFP) vector upstream of the internal ribosomal entry sequence using Gateway Cloning Technology (Life Technologies, Invitrogen). The Tim-3 MSCV or Gal-9 MSCV vector was transfected into 293T cells along with a pCL packaging vector to generate virus particles. In a 6-well plate, 2 $x10^6$ NK92 or Raji cells were suspended in 3mL of thawed retrovirus supernatant containing 10 μg/mL of protamine sulfate, centrifuged at 2500 RPM for 120 minutes at 33ºC, rested for 2 hours at 33ºC, 5%$CO_2$ and repeated once again the following day. Cells were then cultured for 24-48 hours. $eGFP^+$ cells were then selected using the fluorescence-activated cell sorter (FACS) Aria (BD Biosciences, San Jose, CA) and protein expression was evaluated via western blot and FACS analysis using monoclonal anti-human Tim-3 antibody and anti-human Gal-9 antibody (R&D Systems, Minneapolis, MN).

**Flow Cytometry**. Single cell suspensions were stained with the following monoclonal antibodies (mAbs): PE/Cy7-conjugated CD56 (HCD56; BioLegend, San Diego, CA), ECD-conjugated CD3 (UCHT1; Beckman Coulter, Indianapolis, IN), PE-conjugated Tim-3 (no. 344823; R&D Systems), PerCP/Cy5.5-conjugated anti-human CD107a (LAMP-1) (H4A3; BioLegend) and Pacific Blue-conjugated anti-human IFN-γ (4S.B3; BioLegend). Phenotypic acquisition of cells was performed on the LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

**Western Blot Analysis**. Western-blot analyses were carried out according to standard protocols with antibodies raised against human Gal-9 (no. BAF2045; R&D Systems; 1:200), IκBα (no. 9242; Cell Signaling, Danvers, MA; 1:1000), p44/42 MAPK (Erk1/2)

(no. 4348S; Cell Signaling; 1:1000) and β-actin (no. 4967; Cell Signaling; 1:1000) (β-actin was used as a loading control). Protein expression was detected using the SuperSignal West Pico Chemiluminescent Substrate kit (no. 34087, Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. For analysis of IκBα degradation and ERK phosphorylation, purified primary PB NK cells were stimulated with plate-bound anti-Tim-3 mAb (clone 2E2; Biolegend; 10 μg/mL) for the indicated time points, harvested and centrifuged at 4000 rpm for 5 minutes at 4ºC and then immediately lysed in NP-40 lysis buffer (no. FNN0021; Invitrogen) supplemented with protease and phosphatase inhibitors. Lysates were clarified by centrifugation at 13000 RPM for 10 minutes at 4ºC.

**Cytokine Production and CD107a Degranulation Assay**. NK92 cells, freshly isolated PBMC or purified PB NK cells were incubated for 16-hours with IL-12 (1 ng/mL) and IL-18 (10 ng/mL; both from R&D Systems). Cells were washed in 1X PBS, treated with 10 μg/mL of anti-Tim-3 mAb or immunoglobulin controls (rat IgG2a; R&D Systems) and incubated for 15 minutes at 37ºC. For HLA blocking experiments using primary AML and CML tumor cells, targets were pre-incubated for 15 minutes with 20 μg/mL of anti-human HLA mAb (kindly provided by Lopez Botet) or immunoglobulin control (human IgG1; R&D Systems). Anti-human CD107a mAb was added alone or with target cells (Raji eGFP, Raji Gal-9, primary AML or primary chronic myelogenous leukemia (CML) tumor cells; effector:target ratios 10:1 or 2:1) or recombinant human Gal-9 (rhGal-9; Gal Pharma, Japan) and incubated for 1-hour. BD GolgiStop (1:1500) and BD GolgiPlug (1:1000; both from BD Biosciences) were added and cells were further incubated for 5-hours. Cells were then harvested and stained with mAb CD56, CD3, and Tim-3 before

fixation and permeabilization. Permeabilized cells were then stained for intracellular IFN-γ using anti-human IFN-γ mAb. IFN-γ and CD107a expression was evaluated by FACS analysis. Select chemokines/cytokines were measured in supernatant. Purified PB NK cells were exposed to 20 nM rhGal-9 for 4 hours with or without priming for 16 hours with IL-12 (1 ng/mL) and IL-18 (10 ng/mL). Supernatant levels of IFN-γ, GM-CSF, IL-10, IL-8, MIP-1α, MIP-1β and RANTES were determined by multiplex assay using the Luminex system (Austin, TX) and human-specific bead sets (R&D systems; sensitivity 0.3-2.0 pg/mL).

## Results

### Tim-3 is expressed on human NK cells

Tim-3 has been described to have diverse innate and adaptive immunomodulatory roles(354, 373, 386, 399, 404, 415). While NK cells express Tim-3(349, 356), little is known about its function. We first examined Tim-3 expression on peripheral blood mononuclear cells (PBMC) from normal healthy volunteers. Among resting lymphocytes, the $CD56^{+}/CD3^{-}$ NK cell population had the highest percentage of Tim-3-expressing cells (49±2%) compared to $CD56^{+}/CD3^{+}$ NKT (6±1%, $P$<0.001, n=10) and $CD56^{-}/CD3^{+}$ T cell populations (1±0%, $P$<0.001, n=10; **Figure 1A**). The unique subset of IFN-γ producing $CD56^{bright}$ NK cells(288) exhibited significantly lower resting Tim-3 expression compared to $CD56^{dim}$ NK cells (53±6% versus 72±5% [$P$<0.001, n=20]). Tim-3 expression was upregulated in response to IL-2, IL-15, IL-12 and IL-18 with doses as low as 0.1 ng/mL and had a minimal response to IL-7 or IL-21 (**Figure 1B and 1C**). Together IL-12 and IL-18 potently increased Tim-3 expression in a 1:1 ratio beginning at 5 ng/mL and in a 1:10 ratio beginning at 0.5:5 ng/mL (**Figure 1C**). No significant differences were observed in Tim-3 expression between $CD56^{bright}$ and $CD56^{dim}$ NK cells with lower concentrations of IL-2 and IL-15 priming. However, with low concentrations of IL-12 and IL-18 priming Tim-3 expression in $CD56^{bright}$ NK cells was significantly higher compared to $CD56^{dim}$ NK cells (Tim-3 MFI 31±4 vs. 17±2 at IL-12 (0.1 ng/mL) and IL-18 (1 ng/mL)[$P$=0.005, n=8]). Tim-3 expression induced with IL-12 (1 ng/mL) and IL-18 (10 ng/mL) cytokine stimulation decreased 24 hours after cytokine-withdraw and was relatively absent after 48 hours. In T cells, it has been shown that the transcription factor T-bet binds to the Tim-3 promoter and is important for Tim-3 transcription(412). Real-time PCR analysis of IL-2, IL-15, IL-12, IL-18 and IL-12/IL-18 stimulated NK cells

revealed an increase in Tim-3 mRNA expression (except for IL-18) that also correlated with an increase in T-bet mRNA expression, suggesting a similar mechanism of Tim-3 induction for NK cells as seen in T cells (**Appendix Figure A1**).

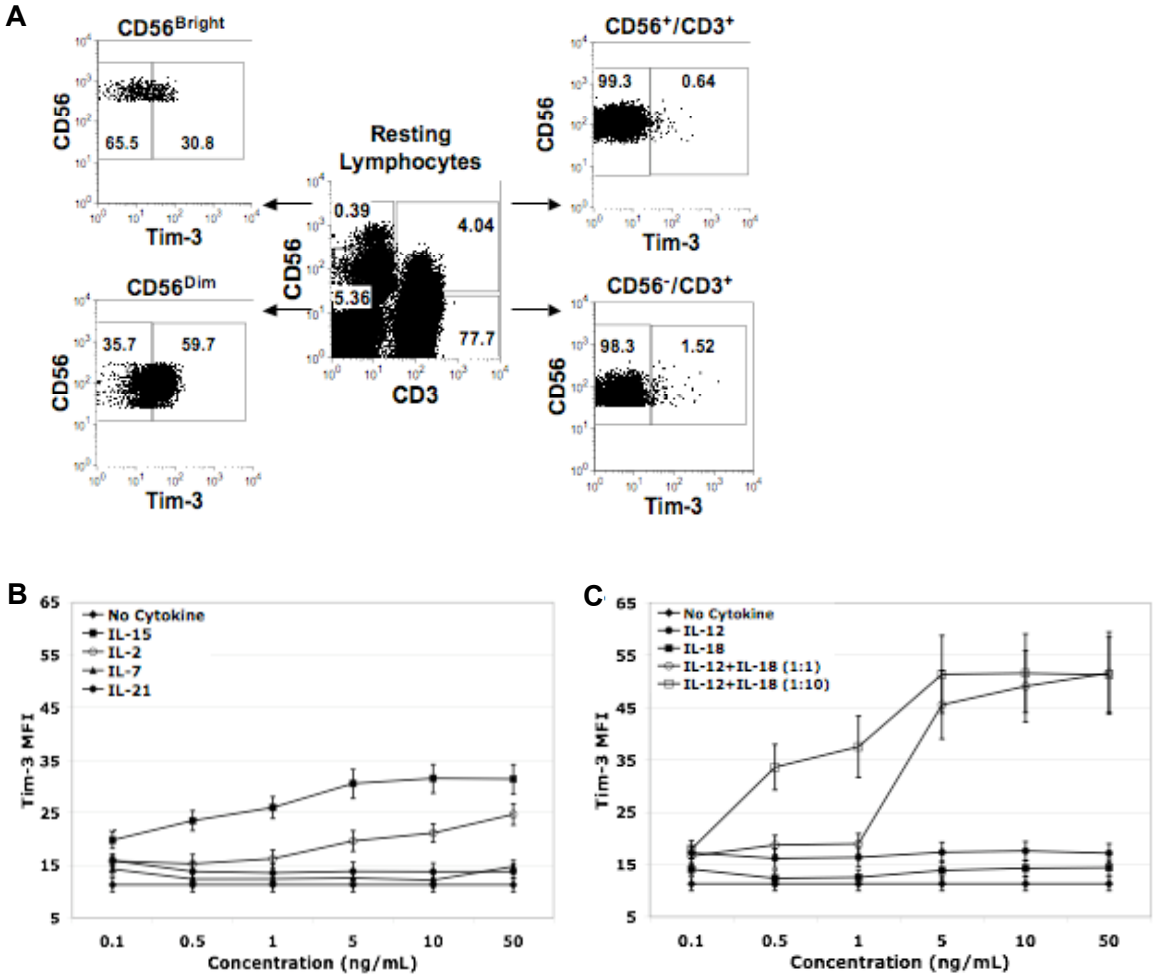



**Figure 1: Tim-3 is expressed on resting NK cells and is upregulated upon activation.** (A-C) PBMC and purified PB NK cells were stained with anti-CD56, anti-CD3 and anti-Tim-3 monoclonal antibodies (mAb). A) Tim-3 expression on resting lymphocyte populations was determined by FACS analysis and a representative donor is shown in flow plot A. B) PBMC were enriched for $CD56^{+}/CD3^{-}$ NK cells using a negative immunomagnetic bead depletion strategy. NK cells were incubated overnight in basal media or media containing IL-15, IL-2, IL-7, IL-21 at the indicated concentrations. Tim-3 Mean Fluorescence Intensity (MFI) was determined by FACS analysis (n=8). C) PBMC were enriched for $CD56^{+}/CD3^{-}$ NK cells using a negative immunomagnetic bead depletion strategy. NK cells were incubated overnight in basal media or media containing IL-12 alone, IL-18 alone, IL-12 and IL-18 (concentration ratio of 1:1) and IL-12 and IL-18 (concentration ratio of 1:10) at the indicated concentrations. Tim-3 MFI was determined by FACS analysis (n=8).

**Tim-3 is an activating co-receptor on NK cells and promotes IFN-γ production**

As Tim-3 is highly expressed on resting NK cells and upregulated with activation, we hypothesized that Tim-3 may be important in mediating NK cell function. To test this, we first overexpressed Tim-3 in the IL-2 dependent NK cell line, NK92, which has low endogenous Tim-3 expression (34±4% for NK92 native cell line versus 91±2% for NK92 Tim-3 cell line). The NK92 native, NK92 eGFP control and NK92 Tim-3 cell lines were then treated with soluble rhGal-9 in the presence of a Tim-3 blocking mAb or isotype control and intracellular IFN-γ levels were evaluated (**Figure 2A**). rhGal-9 concentrations of 10 nM to 100 nM induced significant increases in IFN-γ production in the NK92 Tim-3 cell line compared to the NK92 native and NK92 eGFP control cell lines. Gal-9 has been shown to have a dose dependent apoptotic effect(373). While induction of apoptosis was observed at high concentrations (>50 nM) of rhGal-9, there was no apoptosis at lower concentrations used to evaluate function. Blocking the Tim-3/Gal-9 interaction eliminated the induction of IFN-γ levels in the NK92 Tim-3 cell line at all rhGal-9 concentrations tested. These results show that engagement of Tim-3 by rhGal-9 is specific and identifies Tim-3 as an activating co-receptor that induces NK92 cells to produce IFN-γ.

We next evaluated the effect of Tim-3 on NK cell function when its ligand was presented in a cellular context. The NK-resistant target Burkitt's lymphoma-derived Raji cell line, which does not endogenously express Gal-9, was transduced with full-length Gal-9 (**Figure 2B**). The NK92 cell lines were then co-incubated with Raji eGFP control or Raji Gal-9 targets and intracellular IFN-γ levels were measured (**Figure 2C**). We observed a significant increase in IFN-γ production by the NK92 Tim-3 cell line (compared to the NK92 native and eGFP control cell lines) when exposed to the Raji Gal-9 targets

compared to the Raji eGFP control targets (31±1% for Raji eGFP control versus 53±4% for Raji Gal-9 [*P*<0.001]). Moreover, Tim-3 blockade of the NK92 Tim-3 cell line significantly decreased IFN-γ levels, which was greatest in the Raji Gal-9 targets, but also observed in the Raji eGFP control targets. This is consistent with the fact that Tim-3 is capable of engaging other moieties(365, 366) and suggests that Raji expresses other Tim-3 ligands.

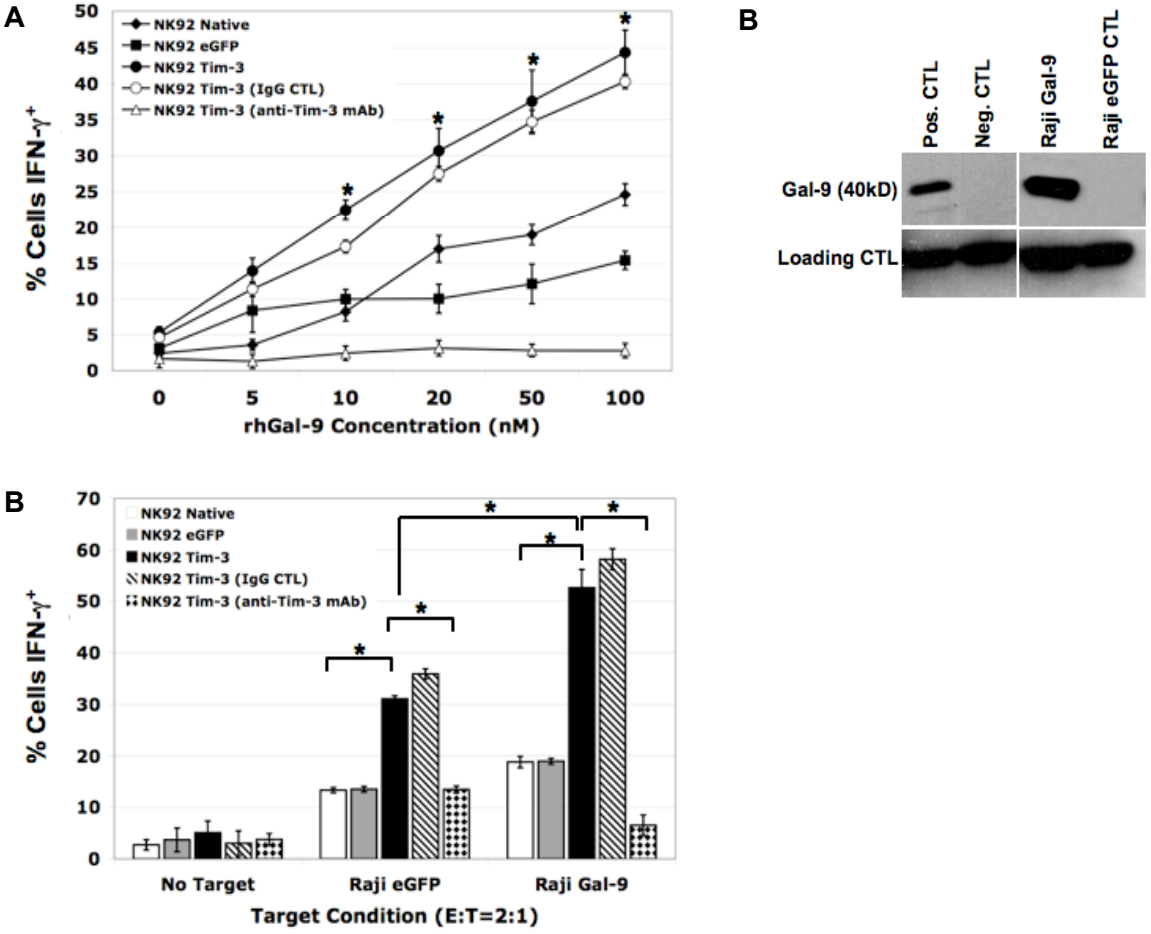
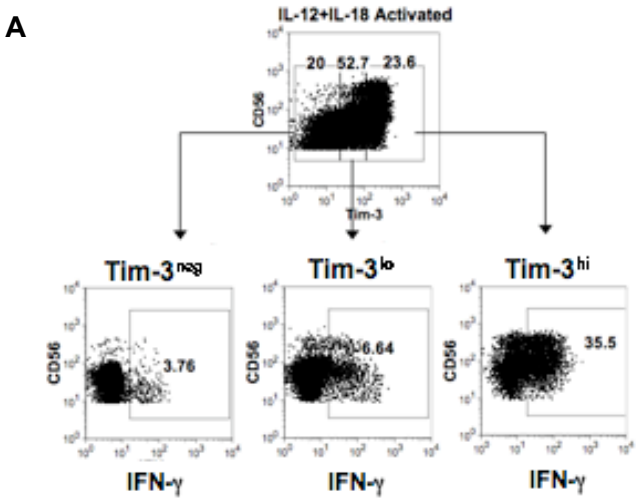


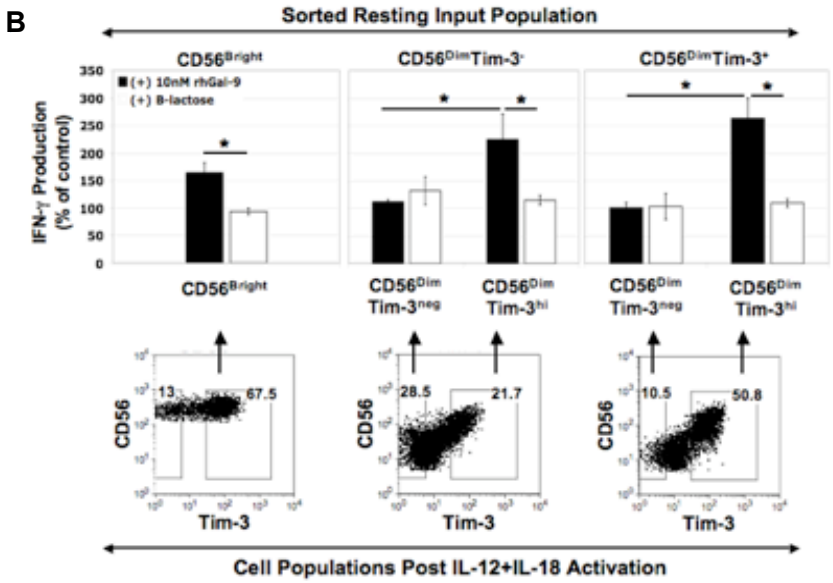

**Figure 2: The Tim-3 ligand Gal-9 increases IFN-γ production in the Tim-3-transduced NK92 cell line.** A) NK92 cell lines were stimulated with rhGal-9 for 4 hours in the presence of a blocking Tim-3 mAb or isotype control antibody and intracellular IFN-γ production was determined by FACS analysis (**P*<0.01, NK92 Tim-3 vs. NK92 Native (n=5) and NK92 Tim-3 vs. NK92 Tim-3 Blocked (anti-Tim-3 mAb, n=5). B) The full-length human Gal-9 gene was cloned into a MSCV vector and the Raji cell line was transduced as described. Gal-9 protein expression (40kD) was confirmed by Western blot analysis. The Jurkat cell line has endogenous Gal-9 expression and was used as the positive control. The murine stromal cell line EL08-1D2 was used as the negative control. C) NK92 cell lines were incubated in the presence of Raji eGFP and Raji Gal-9 target cell lines for 5 hours and intracellular IFN-γ production was determined via FACS analysis (**P*<0.001, n=5; error bars represent SEM).

Having established the function of Tim-3 in the NK92 cell line, we were interested in investigating how Tim-3 affects the function of primary NK cells. We first examined the IFN-γ response of primary peripheral blood NK cells to rhGal-9 without cytokine stimulation; modest increases in IFN-γ production were noted in resting NK cells with rhGal-9 treatment alone despite Tim-3 expression. These results suggest that Tim-3 acts as an activating co-receptor and that cytokine priming is a requirement for enhanced Tim-3 function. Therefore, NK cells were isolated from normal healthy volunteers and activated overnight with 1 ng/mL of IL-12 and 10 ng/mL of IL-18 based on Figure 1C and the finding that these concentrations were a weak IFN-γ stimulus. We also chose these cytokines to explore further as murine IL-12 knockout studies have shown that IL-12 has a vital role in the physiologic anti-tumor and anti-viral immune response, which is mediated in part through NK cell IFN-γ production(418, 419). A unique Tim-3 expression pattern was found on post IL-12 and IL-18-activated NK cells, which divided cells into four distinct populations: Tim-3 was homogeneously up-regulated on all $CD56^{bright}$ NK cells while $CD56^{dim}$ NK cells were stratified into 3 defined populations based on $Tim\text{-}3^{hi}$, $Tim\text{-}3^{lo}$ and $Tim\text{-}3^{neg}$ expression levels. Analysis of these populations revealed that the Tim-3 expressing NK cells were the predominant IFN-γ producing cells, with the $Tim\text{-}3^{hi}$ NK cells producing the highest IFN-γ levels (30±6% for $Tim\text{-}3^{hi}$, 17±5% for $Tim\text{-}3^{lo}$, and 11±4% for $Tim\text{-}3^{neg}$, (n=15); **Figure 3A**). We next sorted $CD56^{bright}$, $CD56^{dim}/Tim\text{-}3^{-}$ and the higher fraction of the $CD56^{dim}/Tim\text{-}3^{+}$ resting NK cell populations and stimulated these cells overnight with 1 ng/mL of IL-12 and 10 ng/mL of IL-18. Post activation, the majority of the $CD56^{bright}$ NK cells exhibited a $Tim\text{-}3^{hi}$ phenotype (72±%5), with 32±3% of the $CD56^{dim}/Tim\text{-}3^{-}$ population and 74±4% of the $CD56^{dim}/Tim\text{-}3^{+}$ population acquiring a $Tim\text{-}3^{hi}$ phenotype (**Figure 3B**). IFN-γ production within these activated NK cell

populations in response to stimulation with 10 nM rhGal-9 was next evaluated. There was a significant increase in IFN-γ production within the Tim-$3^{hi}$ expressing populations compared to the Tim-$3^{neg}$ populations when exposed rhGal-9. This increase in IFN-γ production within the Tim-3 expressing NK cell populations was abrogated by the addition of β-lactose, a β-galactoside that binds and blocks Gal-9 activity. In agreement with the intracellular data, Luminex analysis revealed an increase in IFN-γ production of resting bulk $CD56^+$ NK cells in response to a 4-hour exposure to 20 nM rhGal-9 (basline: 0±0 pg/mL vs. rhGal-9 stimulated: 30±10 pg/mL; *P*<0.05; n=6). To confirm the Tim-3 specificity of this response to rhGal-9, we subsequently sorted the Tim-$3^+$ and Tim-$3^-$ fractions of IL-12 and IL-18 activated NK cells and measured IFN-γ production in response to rhGal-9 in the presence of a Tim-3 blocking mAb (**Figure 3C**). There was a significant decrease in IFN-γ production within the Tim-$3^+$ NK cells with the application of the blocking mAb, confirming that the increase in IFN-γ levels in response to rhGal-9 was induced via interaction with Tim-3.

The production of other cytokines and chemokines induced by 4-hour exposure to 20 nM rhGal-9 was also examined in purified bulk $CD56^+$ NK cells primed with IL-12 (1 ng/mL) and IL-18 (10 ng/mL). An increase in MIP-1α (0 nM: 670±120 pg/mL vs. 20 nM: 2331±203 pg/mL; *P*<0.001, n=4), MIP-1β (535±92 pg/mL vs. 3262±1209 pg/mL; *P*=0.04, n=4) and RANTES (603±188 pg/mL vs. 1530±301 pg/mL; *P*=0.04, n=4) was observed. No differences were observed for GM-CSF, IL-10 and IL-8. While the addition of β-lactose potently blocked the effect of 20 nM rhGal-9 (MIP-1α: 727±102 pg/mL, *P*<0.001; MIP-1β: 514±86 pg/mL, *P*=0.06; RANTES: 574±220 pg/mL, *P*=0.04), addition of the Tim-3 blocking mAb had a minimal effect. As the activation thresholds and kinetics of NK cell


A
IL-12+IL-18 Activated
20
52.7
23.6
CD56
Tim-3
Tim-3neg
Tim-3lo
Tim-3hi
3.76
6.64
35.5
IFN-γ


B
Sorted Resting Input Population
CD56Bright
CD56DimTim-3-
CD56DimTim-3+
(+) 10nM rhGal-9
(+) B-lactose
IFN-γ Production (% of control)
CD56Bright
CD56Dim Tim-3neg
CD56Dim Tim-3hi
CD56Dim Tim-3neg
CD56Dim Tim-3hi
13
67.5
28.5
21.7
10.5
50.8
CD56
Tim-3
Cell Populations Post IL-12+IL-18 Activation

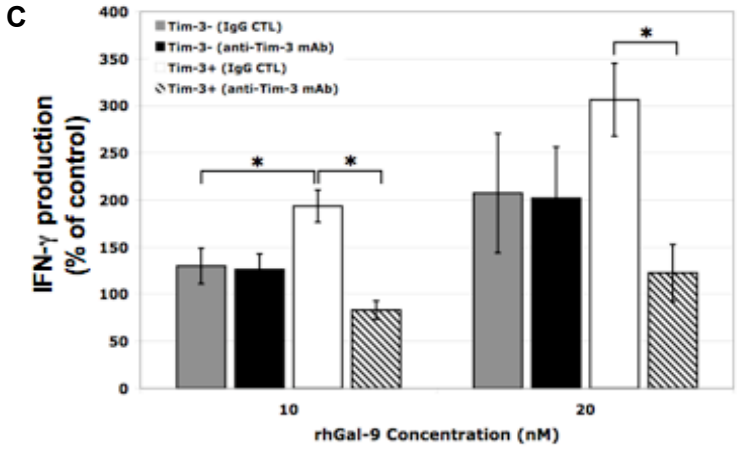

C
Tim-3- (IgG CTL)
Tim-3- (anti-Tim-3 mAb)
Tim-3+ (IgG CTL)
Tim-3+ (anti-Tim-3 mAb)
IFN-γ production (% of control)
rhGal-9 Concentration (nM)

**Figure 3: Primary Tim-3$^+$ NK cells specifically produce IFN-γ in response to rhGal-9.** A) Purified PB NK cells were incubated overnight in media containing IL-12 (1 ng/mL) and IL-18 (10 ng/mL) and intracellular IFN-γ production was determined by FACS analysis. One representative donor is shown in flow plots A. B) Resting NK cell populations CD56$^{bright}$, CD56$^{dim}$/Tim-3$^-$ and CD56$^{dim}$/Tim-3$^+$ were sorted and incubated overnight in media containing IL-12 (1 ng/mL) and IL-18 (10 ng/mL). Each sorted, activated population was exposed to 10 nM rhGal-9 with and without β-lactose (30 mM) blocking and the percent of control intracellular IFN-γ production (calculated as [(%Cells IFN-γ+ at 10 nM/%Cells IFN-γ+ at 0 nM)*100]) was determined within the Tim-3$^{neg}$ and Tim-3$^{hi}$ fractions via FACS analysis (*$P$<0.005, n=5). C) IL-12 (1 ng/mL) and IL-18 (10 ng/mL) activated NK cells were sorted into Tim-3$^+$ and Tim-3$^-$ cell populations and exposed to rhGal-9 (0 nM, 10 nM and 20 nM) with and without blocking using anti-Tim-3 mAb. The percent of control intracellular IFN-γ production (calculated as [%Cells IFN-γ$^+$ at 10 nM or 20 nM/%Cells IFN-γ$^+$ at 0 nM)*100]) determined via FACS analysis (*$P$<0.05, n=5; error bars represent SEM).

chemokine and cytokine responses are different(246), our data suggest Tim-3 may be less important for the early chemokine response and have a more prominent role in the IFN-γ response.

Initial experiments evaluating the mechanism by which rhGal-9 induces NK cell IFN-γ production revealed the involvement of MEK1/2 and NFκB signaling components (**Appendix Figure A2**). To further understand the direct mechanism by which Tim-3 enhances IFN-γ, purified NK cells were stimulated with an agonistic anti-Tim-3 mAb and intracellular $Ca^{2+}$ mobilization (**Appendix Figure A3**), activation of extracellular signal-regulated kinase (ERK) (**Figure 4A**) and degradation of the NFκB inhibitor IκBα (**Figure 4B**) were examined. Upon Tim-3 crosslinking, $Ca^{2+}$ flux was induced above isotype control baseline levels. Comparison of Tim-3-induced $Ca^{2+}$ flux levels to that of the potent activating receptor CD16 revealed weaker signaling capability of Tim-3, further indicating Tim-3 acts as a co-activating receptor. After 15 minutes of Tim-3 engagement an increase in ERK phosphorylation was also observed. Moreover, 30 minutes of Tim-3 engagement led to degradation of IκBα, indicating Tim-3 is involved in NFκB signaling. Together, these results establish Tim-3 as an activating co-receptor, which requires IL-

12 and IL-18 priming to enhance IFN-γ cytokine production.

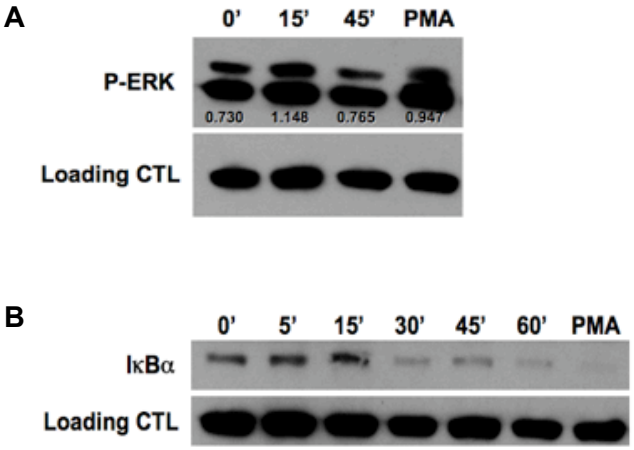



**Figure 4: Tim-3 engagement induces IκBα degradation and ERK activation.** (A-B) Purified PB NK cells were crosslinked with plate-bound anti-Tim-3 mAb for the indicated times. Cells were stimulated with PMA for 15 minutes as a positive control. A) Western blot analysis of ERK phosphorylation. Scanning densitometry was used to determine the relative expression levels of protein after normalizing to β-actin as a loading control. B) Western blot analysis of total IκBα degradation. Data in A and B are representative of four independent experiments.

To examine the effect of Tim-3 in primary human NK cells when Gal-9 is presented in a cellular context, we co-incubated IL-12 (1 ng/mL) and IL-18 (10 ng/mL) activated PBMC with Raji eGFP control or Raji Gal-9 target cells. We observed an increase in NK cell IFN-γ production when exposed to Raji Gal-9 target cells compared to the Raji eGFP control target cells and Tim-3 blockade significantly reduced this increase against the Raji Gal-9 targets (9±1% versus 5±1% [$P$<0.05]; **Figure 5A**). We also evaluated CD107a expression, a marker of degranulation that correlates with NK cell-mediated lysis of target cells. While there was a slight increase in CD107a expression after co-culture with the Raji Gal-9 target cells compared to the Raji eGFP control target cells and blocking Tim-3 resulted in a small decrease, these differences were not significant (**Figure 5B**). Collectively, these data demonstrate Tim-3 functions to activate NK cells significantly

enhancing their ability to produce IFN-γ and has a lesser effect on degranulation when primed with IL-12 and IL-18.

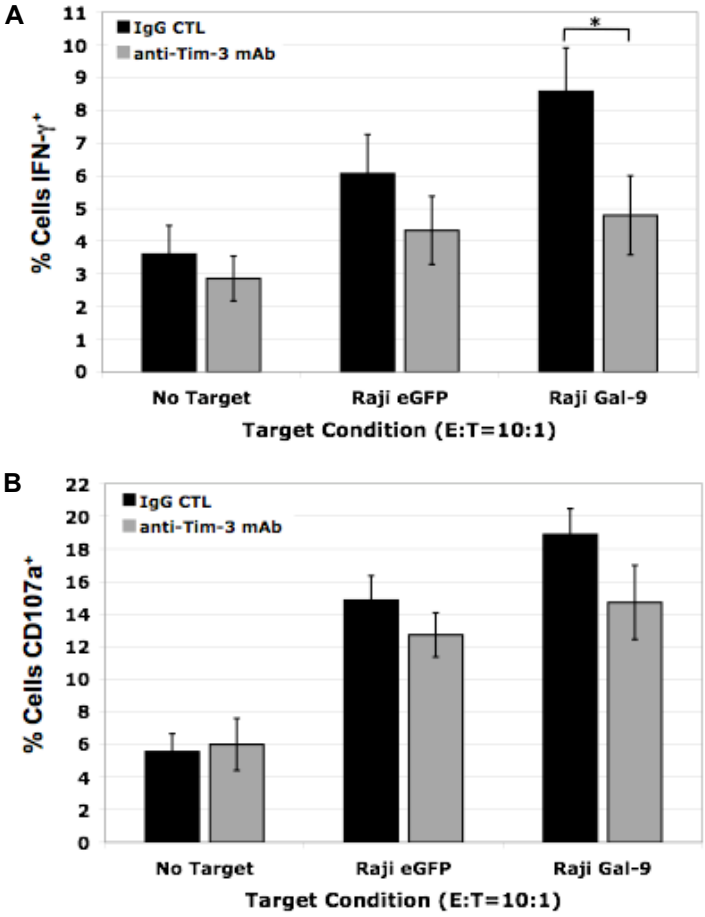



**Figure 5: Blocking Tim-3 decreases IFN-γ production in primary human NK cells and has a minimal effect on degranulation.** (A-B) IL-12 (1 ng/mL) and IL-18 (10 ng/mL) activated PBMC were co-incubated with Raji eGFP and Raji Gal-9 target cell lines in the presence of a blocking Tim-3 mAb or isotype control for 5 hours at an Effector:Target (E:T) ratio of 10:1. A) Intracellular IFN-γ production (*$P$<0.05, n=6) and B) CD107a expression (n=6) was determined by FACS analysis (error bars represent SEM).

### Diminished Tim-3 expression on NK cells correlates with impaired IFN-γ production *in vivo*

Studying patients undergoing allogeneic HCT for the treatment of leukemia provides a unique *in vivo* opportunity to investigate donor NK cell function in the context of human

disease(420). Therefore, Tim-3 expression on NK cells from normal healthy volunteers and HCT donor/recipient pair patient samples was next evaluated. In post-transplant recipients at both the 3 months and 6 months time points, Tim-3 NK cell expression was significantly lower than that of the paired donor (53±2% for paired donor versus 23±7% in the recipient at 3 months [*P*=0.005], and 32±5% in the recipient at 6 months [*P*=0.007]; **Figure 6A**). When these cells were co-incubated with the highly NK-sensitive target cell line K562 and intracellular IFN-γ levels were measured, we observed a significant impairment in the ability of post transplant recipient NK cells to produce IFN-γ when compared to their paired donors (**Figure 6B**).

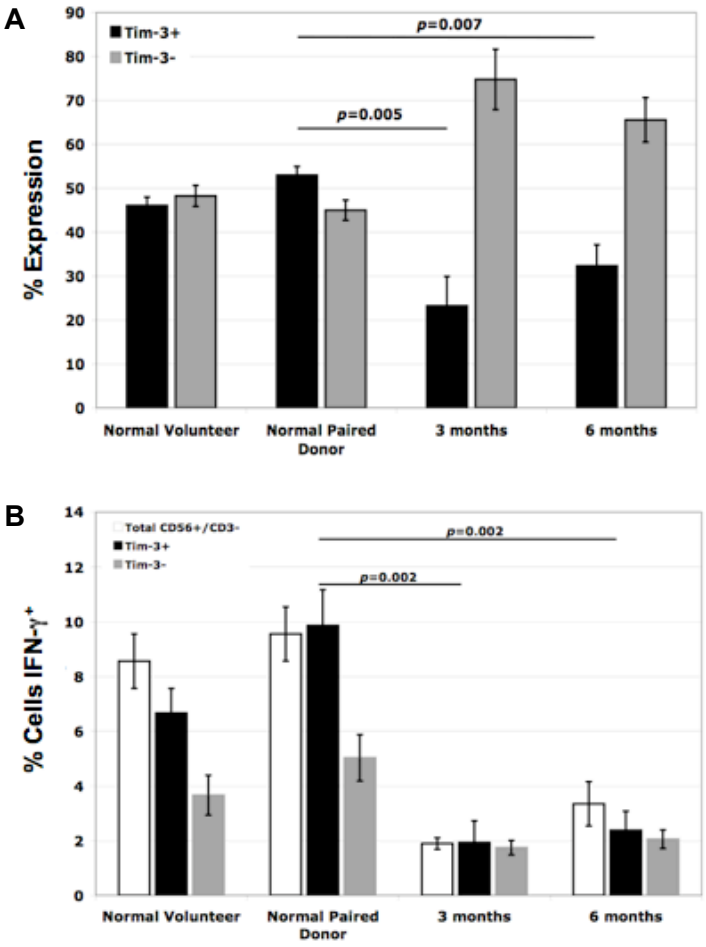


**Figure 6: NK cells from post transplant recipients have reduced Tim-3 expression compared to normal paired donors, which correlates with decreased IFN-γ production.** A) PBMC from normal healthy volunteers, normal pair donors and post transplant recipient patient samples (from time points of 3 and 6 months) were stained with anti-CD56, anti-CD3 and anti-Tim-3 mAb. Tim-3 expression was evaluated by FACS analysis within the gated $CD56^{+}/CD3^{-}$ NK cell population (n=4). B) PBMC from normal healthy volunteers, normal pair donors and post transplant recipient patient samples (from time points of 3 and 6 months) were co-incubated with K562 target cell line at an E:T ratio of 2:1 for 5 hours and intracellular IFN-γ levels were measured within the total $CD56^{+}/CD3^{-}$, $Tim\text{-}3^{+}$ and $Tim\text{-}3^{-}$ NK cell populations via FACS analysis (n=4; error bars represent SEM).

As our data above demonstrate, Tim-3 has a pivotal role in enhancing NK cell IFN-γ production. When we evaluated IFN-γ levels within the $Tim\text{-}3^{+}$ populations of the donor/recipient pairs we also found significant decreases in IFN-γ levels of the post-transplant recipient NK cells compared to the paired donors (10±1% for the donor versus 2±1% in the recipient at 3 months [$P$<0.002]), suggesting that the post-transplant defect may go beyond just reduced expression of Tim-3 alone.

**Tim-3 blockade decreases NK cell IFN-γ production against primary AML tumor targets**

To evaluate the functional contribution of Tim-3 and its impact in NK cell targeting of leukemia cells we examined the Gal-9 expression profile of human primary AML and CML samples via western blot and immunohistochemistry. Analysis revealed primary CML samples to be Gal-9 negative while primary AML samples had high levels of Gal-9 expression (**Figure 7A**; immuohistochemistry, **Appendix Figure A4**). PBMC were then isolated from normal healthy volunteers and co-incubated with primary AML tumor cells from patients and IFN-γ levels were evaluated (**Figure 7B**). Tim-3 blockade resulted in a significant reduction of NK cell IFN-γ levels after target cell exposure. To better evaluate the contribution of Tim-3 to NK cell activation, we applied a pan-HLA blocking antibody to the primary AML target cells to eliminate overriding inhibitory signals and then

measured IFN-γ production with and without Tim-3 blockade. As expected, IFN-γ levels

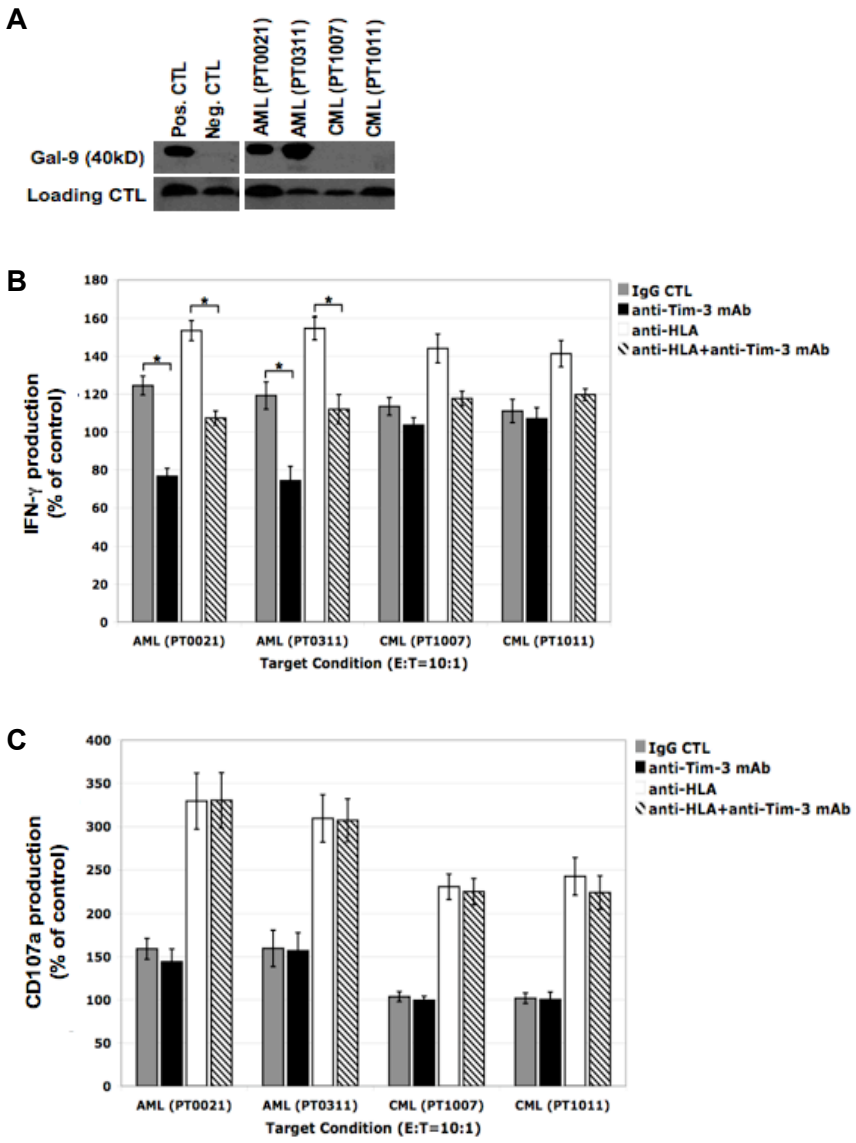



**Figure 7. Tim-3 blockade decreases IFN-γ production of activated human NK cells against primary AML tumor cells, but has no effect on degranulation.** A) Western blots were performed on cell lysates of AML (n=2) and CML (n=2) tumor cells to evaluate Gal-9 (40kD) expression. The Jurkat cell line has endogenous Gal-9 expression and was used as the positive control. The murine stromal cell line EL08-1D2 was used as the negative control. (B-C) PBMCs were incubated overnight in media containing IL-12 (1 ng/mL) and IL-18 (10 ng/mL) and a 5-hour CD107a/IFN-γ assay was performed as described using primary human AML and CML tumor cell targets. The percent of control intracellular IFN-γ (B) or CD107a (C) production of NK cells when exposed to targets with and without Tim-3 and HLA blocking alone and in combination (calculated for each blocking condition as [(%Cells IFN-$\gamma^+$ or CD107a$^+$ in presence of target/% Cells IFN-$\gamma^+$ or CD107a$^+$ without targets)*100] was determined by FACS analysis (*$P$<0.005, n=6; error bars represent the SEM).

increased with the application of the HLA blocking antibody alone. When both the anti-

HLA and anti-Tim-3 blocking antibodies were applied we observed a significant decrease in NK cell IFN-γ production, thus confirming the functional contribution of Tim-3 in NK cell targeting of primary AML cells. This effect was specific as chronic myelogenous leukemia (CML) patient samples, which lack Gal-9 expression did not reveal significant functional differences with Tim-3 blockade (**Figure 7B**).

As data in Figure 5B showed a minimal role of Tim-3 in NK cell degranulation against the Raji target cell lines when primed with IL-12 and IL-18, we next evaluated if this was also true for primary leukemia cells. PBMC isolated from normal healthy volunteers were co-incubated with primary AML and CML tumor cells from patients in the presence of a blocking Tim-3 mAb or isotype control and CD107a expression levels were measured (**Figure 7C**). In agreement with the Raji cell line data, there were no differences in CD107a degranulation with Tim-3 blockade for either of the primary AML or CML targets.

## Discussion

In this study we have examined the expression and function of a novel immune receptor Tim-3 in primary human NK cells. Among resting lymphocyte populations, NK cells have the highest percentage of cells expressing Tim-3. In response to cytokine stimulation Tim-3 expression increased and engagement of Tim-3 with the Gal-9 ligand induced significant increases in IFN-γ production that were abrogated by Tim-3 blockade. Tim-3 directly signals as engagement of Tim-3 led to intracellular $Ca^{2+}$ mobilization, ERK activation and degradation of the NFκB inhibitor IκBα. In contrast to its effect on cytokine production when primed with IL-12 and IL-18, Tim-3 blockade minimally affected NK cell degranulation. Reconstituted post-transplant NK cells from HCT recipients have reduced levels of Tim-3 expression that correlated with impaired IFN-γ production and IFN-γ production by NK cells targeting Gal-9 positive primary AML tumors was significantly reduced with Tim-3 blockade. Our data collectively show that Tim-3 functions as an activating co-receptor in human NK cells to enhance IFN-γ production when engaged by its ligand Gal-9 supporting a model whereby Tim-3 is upregulated by inflammatory cytokines and NK cell effector function is enhanced by Tim-3/Gal-9 interactions.

The functional role of the Tim-3/Gal-9 pathway was first described as a mechanism to negatively regulate the Th1 response, inhibiting IFN-γ production and inducing cell death(350, 386). Subsequently, this interaction has been described to have important roles in infection, autoimmunity, inflammation and tumor immunity serving to downregulate T cell responses(354, 386, 399, 404, 415). In contrast, stimulation of Tim-3 expressing DCs or macrophages results in the secretion of pro-inflammatory cytokines, thus exposing a dual function for Tim-3 in immunoregulation(356). A recent

study examining the role of Tim-3 in NK cells in the context of Hepatitis B Virus (HBV) infection showed increased cytotoxicity of NK cells isolated from chronic Hepatitis B patients against HepG2.215 targets with the blockade of Tim-3, while IFN-γ levels were not significantly affected(405). Our results demonstrate an immunopotentiating role for Tim-3, enhancing IL-12 and IL-18 primed NK cell IFN-γ production with little effect on NK cell degranulation in the context of tumor immunity. This raises the question of how triggering one receptor can lead to two distinct functional outcomes. One possibility is that Tim-3 function is influenced by the surrounding microenvironment. This concept is supported by findings describing Tim-3 function in $CD8^+$ cytotoxic T cells. In a mouse model of fibrosarcoma, increased anti-tumor activity, as indicated by enhancement of IFN-γ, perforin and granzyme B function, was reported in $CD8^+$/Tim-$3^+$ T cells in the presence of Gal-9 expressing DCs(400). In contrast, shRNA knockdown of Tim-3 in a mouse model of HBV infection resulted in significant increases in IFN-γ production from hepatic $CD8^+$ T cells(421). All of this supports the notion of a functional threshold that governs the activating or inhibitory potential of Tim-3, causing the receptor to act as rheostat that fine-tunes the cell response.

Although the molecular basis for Tim-3 signaling is still evolving, it is proposed that Tim-3 may facilitate both positive and negative signaling cascades through differential tyrosine phosphorylation of the cytoplasmic tail. The intracellular tail of Tim-3 contains six conserved tyrosine residues and a Src homology 2 (SH2) binding motif that, upon Tim-3 crosslinking, displayed differential tyrosine phosphorylation patterns in DCs and T cells(356). These results indicate that Tim-3 is able to initiate distinct signaling events that lead to different functional outcomes in a cell specific manner. Furthermore, a recent

study examining the role of the cytoplasmic tail tyrosine residues of Tim-3 in T cells shows that the dominant activity of the three most C-terminal tyrosine residues is inhibitory with two of the more N-terminal tyrosine residues displaying an activating function(368). Differential function through a single receptor in the same cell, however, is not without precedent in NK cell biology. One such receptor is 2B4 (CD244), which has been shown to initiate both positive and negative signaling events that are mediated, in part, by differential tyrosine phosphorylation of the cytoplasmic tail and the availability of two distinct adaptor proteins, SAP and EAT-2(205, 206, 422). Therefore, it is plausible that differential tyrosine phosphorylation within the cytoplasmic tail of Tim-3 may serve to mediate the recruitment of alternative adaptor proteins that ultimately lead to NK cell activation or inhibition.

While Gal-9 is a known ligand of Tim-3 and interacts with the N-linked carbohydrates attached to the IgV region via its two carbohydrate recognizing domains (CRDs), Tim-3 has been described to contain another conserved binding domain that has Gal-9 independent binding activities(350, 365, 366). Our data showing an increase in NK92 Tim-3 IFN-$\gamma$ production in the presence of Gal-9 negative Raji eGFP control targets suggest there are additional ligands for Tim-3 that have a functional role. Therefore, regulation of positive or negative signaling pathways through Tim-3 may be ligand-dependent and rely upon a balance between the interplay of the multiple Tim-3 binding activities. This is the case for the related protein Tim-1, where two different antibodies with distinct binding affinities for the IgV domain have been shown to induce opposing functions(423). Consequently, the Tim-3-interacting ligand may influence the effect of Tim-3 on cell function, ultimately contributing to the dual roles of this receptor exhibited within the same cell.

Stimulation with both soluble rhGal-9 and membrane bound Gal-9 demonstrated a preferential effect of the Tim-3/Gal-9 pathway on NK cell IFN-γ production compared to degranulation when primed with IL-12 and IL-18. Emerging evidence has revealed a clear dichotomy in signals that regulate NK cell cytokine production versus cytotoxicity upon stimulation of a single receptor(288-290). These divergent pathways involve the use of different kinases and adaptor proteins to facilitate each function. We found that rhGal-9 was not a strong enough Tim-3 stimulus for resting NK cells to elicit either cytokine production or degranulation. However, when we primed with IL-12 and IL-18 Tim-3 enhanced this stimulus and increased IFN-γ production. Taken together, this suggests that Tim-3 is dependent upon the recruitment of key adaptor molecules through prior activation before signaling can occur. Furthermore, the function of Tim-3 as a co-receptor may primarily act to fine-tune a cellular response from cytokine exposure and/or signals delivered from other NK cell receptors.

As NK cells do not require pre-sensitization for anti-tumor activity and do not induce GvHD in an allogeneic transplant setting, they are considered to be an attractive option for immunotherapy(291, 292, 294, 416). To enhance the therapeutic effects of donor NK cells, it is critical to identify functional receptors essential for the interaction of malignant tumor cells and effector NK cells. We have demonstrated that engagement of a novel NK cell receptor Tim-3 positively regulates IFN-γ–producing NK cells, which have a central role in tumor immunosurvelliance. The anti-tumor mechanisms of IFN-γ include both direct and indirect targeting, functioning to inhibit cellular proliferation(424), promote apoptosis through the up-regulation of caspases, FAS and TRAIL(425), inhibit

angiogenesis(426) and prime the transition of the immune response from innate to adaptive immunity. As a result, enhancement of IFN-γ production by Tim-3 has important implications for NK cell effector function against cancer and infected cells.

Donor-derived NK cells are recognized to mediate beneficial GvL reactions in HCT and ultimately provide protection against relapse for patients with AML(291, 292, 427). We have shown that Tim-3$^{+}$ NK cell IFN-γ function is enhanced against Gal-9 positive AML primary tumors compared to Gal-9 negative CML primary tumors. These results are consistent with the finding that NK cell effects are strongest in patients with AML compared to other myeloid leukemias. Our results demonstrating reduced Tim-3 expression in post-transplant reconstituted NK cells from HCT recipients, which correlated with an impairment to produce IFN-γ, indicate that Tim-3 expression on NK cells is developmentally regulated and further supports our hypothesis that Tim-3 function in NK cells is important in the context of HCT. Enhancing Tim-3 expression early post-transplant may, therefore, have an essential role in augmenting the NK cell immune response against residual tumor cells. Collectively, our data show that Gal-9 positive tumors are more susceptible to NK cell recognition and targeting via interaction with Tim-3. Furthermore, the Tim-3/Gal-9 pathway may have a critical role in clearing residual disease. Understanding this mechanism can lead to strategies to overcome early post-transplant limitations, providing protection against relapse and infection, and ultimately improving survival outcomes.

## Authorship Contributions

MKG designed the research plan, performed experiments, data analysis and interpretation, and wrote the manuscript; TRL assisted with cloning experiments and data analysis; VM performed experiments, data analysis and manuscript preparation; MF performed data analysis and manuscript preparation; MSO assisted with cloning, cell culture and performed experiments; SAC performed data analysis and manuscript preparation; MRV performed data analysis and manuscript preparation; FC performed data analysis and manuscript preparation; CJH performed immunohistochemistry experiments and manuscript preparation; AP-M performed chemokine/cytokine assays and manuscript preparation; TN provided rhGal-9 reagent and manuscript preparation; MH provided rhGal-9 reagent and manuscript preparation; BRB designed the research plan, performed data analysis, interpretation and manuscript preparation; JSM designed the research plan, performed data analysis, interpretation and manuscript preparation, and was responsible for all aspects of the work.

# CHAPTER 2

## BISPECIFIC AND TRISPECIFIC KILLER CELL ENGAGERS DIRECTLY ACTIVATE HUMAN NK CELLS THROUGH CD16 SIGNALING AND INDUCE CYTOTOXICITY AND CYTOKINE PRODUCTION

Manuscript is under review with revisions by the American Association for Cancer Research in the journal *Molecular Cancer Therapeutics*.

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This study evaluates the mechanism by which bispecific and trispecific killer cell engagers (BiKEs and TriKEs) act to trigger human NK cell effector function and investigates their ability to induce NK cell cytokine and chemokine production against human B-cell leukemia. We examined the ability of BiKEs and TriKEs to trigger NK cell activation through direct CD16 signaling, measuring intracellular $Ca^{2+}$ mobilization, secretion of lytic granules, induction of target cell apoptosis and production of cytokine and chemokines in response to the Raji cell line and primary leukemia targets. Resting NK cells triggered by the recombinant reagents led to intracellular $Ca^{2+}$ mobilization through direct CD16 signaling. Co-culture of reagent-treated resting NK cells with Raji targets resulted in significant increases in NK cell degranulation and target cell death. BiKEs and TriKEs effectively mediated NK cytotoxicity of Raji targets at high and low effector-to-target (E:T) ratios and maintained functional stability after 24 and 48 hours of culture in human serum. NK cell production of IFN-$\gamma$, TNF-$\alpha$, GM-CSF, IL-8, MIP-1$\alpha$ and RANTES was differentially induced in the presence of recombinant reagents and Raji targets. Moreover, significant increases in NK cell degranulation and enhancement of IFN-$\gamma$ production against primary ALL and CLL targets were induced with reagent treatment of resting NK cells. In conclusion, BiKEs and TriKEs directly trigger NK cell activation through CD16, significantly increasing NK cell cytolytic activity and cytokine production against tumor targets, demonstrating their therapeutic potential for enhancing NK cell immunotherapies for leukemias and lymphomas.

## Introduction

With over 70,000 cases anticipated in the US for 2012, non-Hodgkin's lymphoma (NHL) is the most common adult hematologic malignancy, 85% of which are of B-cell origin(428, 429). Monoclonal antibodies (mAbs), such as rituximab, have shown to be therapeutic for the treatment of NHL(430). Despite this success, there are limitations that decrease the overall efficiency of mAb therapies(431). With the development of CD16-directed bispecific and trispecific single chain fragment variable (bscFv and tscFv) recombinant reagents, most of these undesired limitations are avoided while eliciting high effector function as they lack the Fc-portion of whole antibodies and have a targeted specificity for CD16(345, 346, 432). As a result, recombinant reagents are attractive for clinical use enhancing natural killer (NK) cell immunotherapies.

The ability of NK cells to recognize and kill targets is regulated by a sophisticated repertoire of inhibitory and activating cell surface receptors. NK cell cytotoxicity can occur by natural cytotoxicity, mediated via the natural cytotoxicity receptors (NCRs), or by antibodies, such as rituximab, to trigger antibody dependent cell-mediated cytotoxicity (ADCC) through CD16, the activating low affinity Fc$\gamma$ receptor for IgG highly expressed by the $CD56^{dim}$ subset of NK cells(47, 155, 181, 433). Natural cytotoxicity is triggered via NCRs by *de novo* expression of NK cell activating receptor ligands on target cells. In the absence of cytokine stimulation, these receptors inefficiently elicit a cytotoxic or cytokine response independently, but together they are able to function synergistically to activate a resting NK cell and promote effector function(247, 433). In contrast, ADCC is mediated when CD16 binds to opsonized targets through Fc engagement and signals through immunoreceptor tyrosine-based activation motifs (ITAMs) of the associated

FcεRIγ chain and CD3ζ chain subunits(176). The signal delivered via CD16 is potent and induces both a cytotoxic and cytokine response in the absence of cytokine stimulation that can further be enhanced by co-engagement of other activating receptors(247).

In this study, we demonstrate the ability of a CD16/CD19 BiKE and a CD16/CD19/CD22 TriKE to trigger NK cell activation through direct signaling of CD16 and induce directed secretion of lytic granules and target cell death. Furthermore, we show for the first time the ability of these reagents to induce NK cell activation that leads to cytokine and chemokine production.

## Materials and Methods

**Cell isolation and purification**. Peripheral blood mononuclear cells (PBMC) were isolated from adult blood (Memorial Blood Center, Minneapolis, MN) by centrifugation using a Histopaque gradient (Sigma-Aldrich) and NK cells were purified by removing T-cells, B-cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells via magnetic beads per the manufacturer's protocol (Miltenyi Biotec). Primary acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL) and chronic lymphoblasitc leukemia (CLL) samples were obtained from the Leukemia MDS Tissue Bank (LMTB) at the University of Minnesota. All samples were obtained after informed consent and in accordance with the Declaration of Helsinki, using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota.

**Flow Cytometry.** Cell suspensions were stained with the following monoclonal antibodies (mAbs): PE/Cy7-conjugated CD56 (HCD56; BioLegend), ECD-conjugated CD3 (UCHT1; Beckman Coulter), FITC-conjugated CD16 (3G8; BD Biosciences), PE-conjugated CD19 (SJ25C1; BD Biosciences), APC-conjugated CD20 (2H7; BioLegend), FITC-conjugated CD22 (H1B22; BioLegend), PerCP/Cy5.5-conjugated anti-human CD107a (LAMP-1) (H4A3; BioLegend), Pacific Blue-conjugated anti-human IFN-$\gamma$ (4S.B3; BioLegend) and AF488-conjugated cleaved caspase-3 (9669; Cell Signaling Technology). Phenotypic acquisition of cells was performed on the LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.).

**Construction, expression and purification of bscFv CD16/CD19 and tscFv CD16/CD19/CD22 reagents**. Synthesis of hybrid genes encoding the bscFv and tscFv reagents were accomplished using DNA shuffling and DNA ligation techniques as previously described(434). The fully assembled gene (from 5' end to 3' end) consisted of an NcoI restriction site, an ATG initiation codon, the $V_H$ and $V_L$ regions of anti-human CD16 (NM3E2)(345), a 20-amino acid segment of human muscle aldolase, the $V_H$ and $V_L$ regions of humanized anti-CD22(434), humanized anti-CD19(434) and a XhoI restriction site. The resultant gene fragment was spliced into the pET21d expression-vector and DNA sequencing analysis (Biomedical Genomics Center, University of Minnesota) was used to verify that the gene was correct in sequence and cloned in frame. Genes encoding the BiKEs and monospecific scFV controls were created in the same manner. For bacterial protein expression and purification by ion exchange and size exclusion chromatography, methods were used as previously described(434).

**Proliferation Assay**. The Burkitt's lymphoma Raji cell line (ATCC) was cultured at 37°C with 5% $CO_2$ in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). $2x10^4$ Raji cells were treated with varying concentrations of CD16/CD19/CD22 or non-specific control CD16/EpCAM and 1 nM of a targeted diphtheria toxin-CD19/CD22 (DT-CD19/CD22) was added and a $^3$H-thymidine proliferation was performed as previously described(434).

**Cytokine/Chemokine Production, CD107a and Caspase-3 Assay**. PBMC or purified NK cells were incubated overnight at 37ºC, 5% $CO_2$ in basal medium (RPMI supplemented with 10% fetal calf serum). Cells were washed, treated with bscFv CD16/CD19, tscFv CD16/CD19/CD22, rituximab (Genentech), CD22 parental antibody

(derived from the RFB4 hybridoma), CD19 parental antibody (derived from the HD37 hybridoma), scFv anti-CD16 or scFv CD19/CD22 (negative controls) and CD107a and intracellular IFN-γ assays were performed as previously described(435). For evaluation of caspase-3 activation, Raji target cell cleaved caspase 3 expression was evaluated by FACS analysis using a live forward/side scatter gate and a $CD56^-/CD20^+$ gate to determine cleaved caspase 3 positive Raji populations relative to an isotype control (rabbit IgG, Cell Signaling Technology). For Luminex analysis of cytokines/chemokines, NK cells, Raji cells and NK cells co-cultured with Raji cells were treated with the BiKE, TriKE, rituximab, negative controls or no reagent for 6 hours at 37ºC, 5%$CO_2$. Supernatant levels of IFN-γ, TNF-α, GM-CSF, IL-8, MIP-1α, and RANTES were determined by multiplex assay using the Luminex system (Luminex Co.) and human-specific bead sets (R&D systems; sensitivity 0.5-3.0 pg/mL) and final pg/mL values adjusted for background for each condition were determined using the following equation: [NK cells + Raji cells pg/mL] – [(NK cells alone pg/mL) + (Raji cells alone pg/mL)].

**51-Chromium Release Cytotoxicity Assay.** Direct cytotoxicity assays were performed by standard 4-hour $^{51}Cr$-release assays using effector cells pre-treated with reagents and Raji cells as targets. For examination of reagent stability, reagents were pre-incubated for 24 and 48 hours at 37ºC, 5% $CO_2$ in 100% human serum. $^{51}Cr$ released by specific target cell lysis was measured by a gamma scintillation counter and the percent specific cell lysis was calculated.

**$Ca^{2+}$ Flux Assay.** $Ca^{2+}$ flux was measured using the Fluo-4 NW Calcium Assay Kit (Invitrogen) within effector NK cells as per manufacturer's protocol. $Ca^{2+}$ mobilization was evaluated by FACS analysis. After 30 seconds of acquisition, 10μg/mL of purified anti-CD16 mAb (3G8; BD Biosciences), biotinylated scFv anti-CD16, purified mIgG (negative control; R&D Systems), or 1μg/mL of ionomycin (positive control; Sigma) was added and events were acquired for 1 minute. After 1 minute, 10μg/mL of purified goat anti-mouse (GAM; BioLegend) or purified streptavidin (SA; Pierce Thermo Scientific) was added to cross-link receptors and events were acquired for an additional 4-5 minutes. The median Fluo-4 relative fluorescence was analyzed as a function of time in seconds.

## Results

### Generation of bscFv CD16/CD19 BiKE and tscFv CD16/CD19/CD22 TriKE

Recombinant bscFv and tscFv reagents were generated targeting the NK cell receptor CD16 and B-cell antigens CD19 and CD22, antigens that have been shown to be therapeutic for the treatment of diffuse large B-cell lymphoma and ALL(436, 437) (**Figure 1A**). BiKEs and TriKEs were purified by ion exchange and size exclusion chromatography. The fractions collected during the final step of purification of the CD16/CD19/CD22 TriKE are shown (**Figure 1B**). High (95%) purity was obtained as demonstrated by Coomassie Blue staining (**Figure 1C**). Similar results were obtained for the CD16/CD19 BiKE (data not shown).

### BiKE and TriKE antigen binding is cell specific and directly signals through CD16 to induce NK cell activation

Raji cells were coated with varying concentrations of the TriKE, treated with a CD19/CD22-targeted diphtheria toxin (DT-CD19/CD22) and a $^{3}$H-thymidine proliferation assay was performed (**Figure 1D**). All concentrations of the TriKE blocked the effects of the inhibitory toxin while the non-specific control reagent (CD16/EpCAM) had no effect, demonstrating effective targeting of the B-cell antigens by the CD19 and CD22 end of the reagents.

We next evaluated the ability of the BiKE and TriKE to directly target and trigger NK cell activation. $Ca^{2+}$ mobilization, a primary indicator of cell activation, was measured after specifically cross-linking CD16 on the surface of NK cells. Resting NK cells were loaded with a $Ca^{2+}$ binding dye solution and a time course analysis of changes in intracellular

$Ca^{2+}$ concentration was performed by flow cytometry (**Figure 1E**). Without cross-linking, both the CD16 mAb and the biotinylated scFv CD16 elevated baseline $Ca^{2+}$ levels, which were further enhanced upon cross-linking with the secondary antibody. The $Ca^{2+}$ flux kinetics induced by the biotinylated scFv CD16 were faster than the response elicited by the CD16 mAb, which may be explained by the high affinity interaction of streptavidin and biotin molecules. These results demonstrate the CD16 end of the BiKE and TriKE is capable of engaging the NK cell and inducing a potent signal through CD16, which results in $Ca^{2+}$ mobilization.

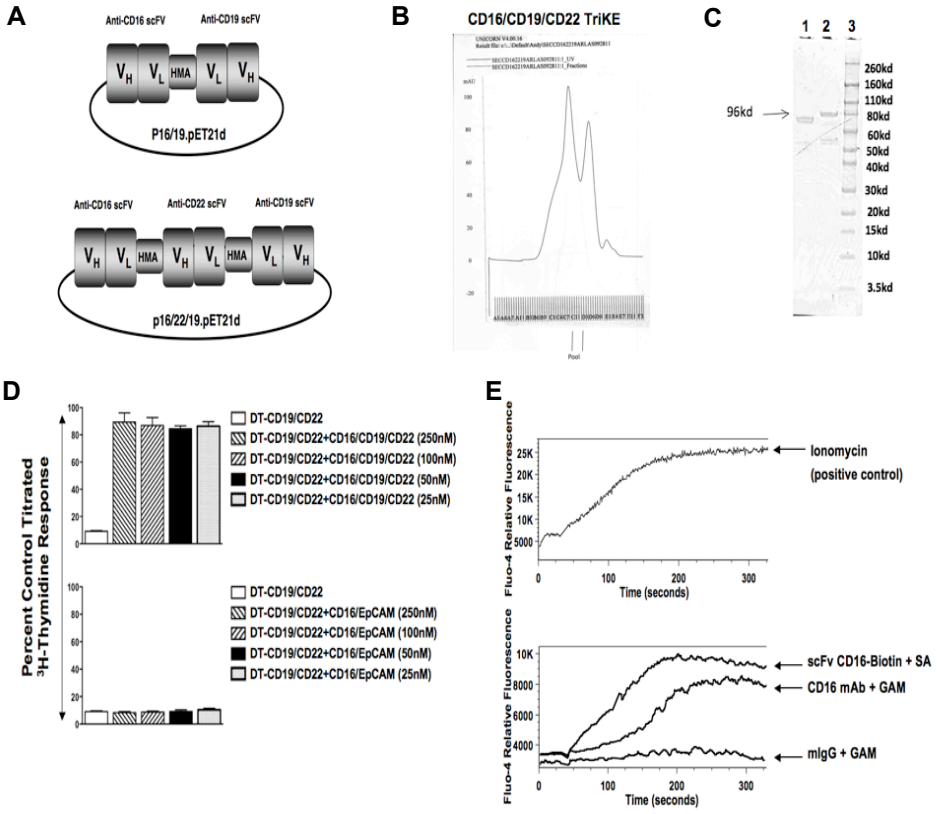


**Figure 1. BiKEs and TriKEs specifically target both effector and target cells**. A) Construct-diagrams of bscFv CD16/CD19 and tscFv CD16/CD19/CD22 reagents. B) Ion exchange and size exclusion chromatography purification trace of the CD16/CD19/CD22 TriKE. C) Coomassie Blue staining of TriKE protein isolates (molecular weight of ~96 kD). Lane 1 = non-reduced recombinant protein, Lane 2 = reduced recombinant protein, Lane 3 = molecular weight ladder. D) Proliferation assay of $^{3}H$-pulsed Raji cells treated with CD16/CD19/CD22 (top graph) or a non-specific control (bottom graph). E) Intracellular $Ca^{2+}$ flux assay in resting NK cells after CD16 receptor cross-linking. Plots display a representative donor (of 4 experiments) showing the median Fluo-4 relative fluorescence plotted as a function of time in seconds.

**BiKEs and TriKEs induce directed secretion of NK cell lytic granules and lysis of B-cell targets**

As $Ca^{2+}$ flux is a prerequisite for function(438), we evaluated the ability of these recombinant reagents to mediate killing of Raji targets, a lymphoma cell line with high expression of CD19, CD20 and CD22 (**Figure 2A**). Resting NK cells were co-cultured with Raji targets with or without reagent treatment and CD107a expression, a marker of NK cell cytotoxicity(266, 439), was measured via FACS analysis (**Figure 2B**). NK cell CD107a expression significantly increased in the presence of the BiKE and TriKE compared to untreated NK cells or parental antibodies. This response was target cell restricted as no functional response was induced with treated NK cells in the absence of targets (data not shown). The level of induced degranulation was equal to the function induced by the mAb rituximab. To analyze the correlation of NK cell CD107a expression induced by the recombinant reagents with direct target cell lysis(266, 439), $^{51}$Cr-labeled Raji targets were co-cultured with resting NK cells treated with or without reagents and target cell killing was determined (**Figure 2C**). Both the BiKE (for E:T=2.2:1, 70.6%±4.5%; $P<0.01$) and TriKE (59.8%±3.9%; $P<0.01$) mediated significant lysis of the target cells at all E:T ratios compared to the untreated (18.7%±3.0%) and control treated NK cells. Furthermore, analysis of caspase activation in Raji target cells after two or four hours of co-culture with NK cells led to increases in cleaved caspase-3 expression in BiKE and TriKE coated-Raji targest remaining in the live cell gate compared to the uncoated-Raji targets (**Figure 2D**). These results demonstrate BiKE and TriKE reagents specifically engage both targets and effectors to mediate target cell killing, activating NK cells to secrete lytic granules and induce target cell death via a caspase 3 apoptosis pathway.

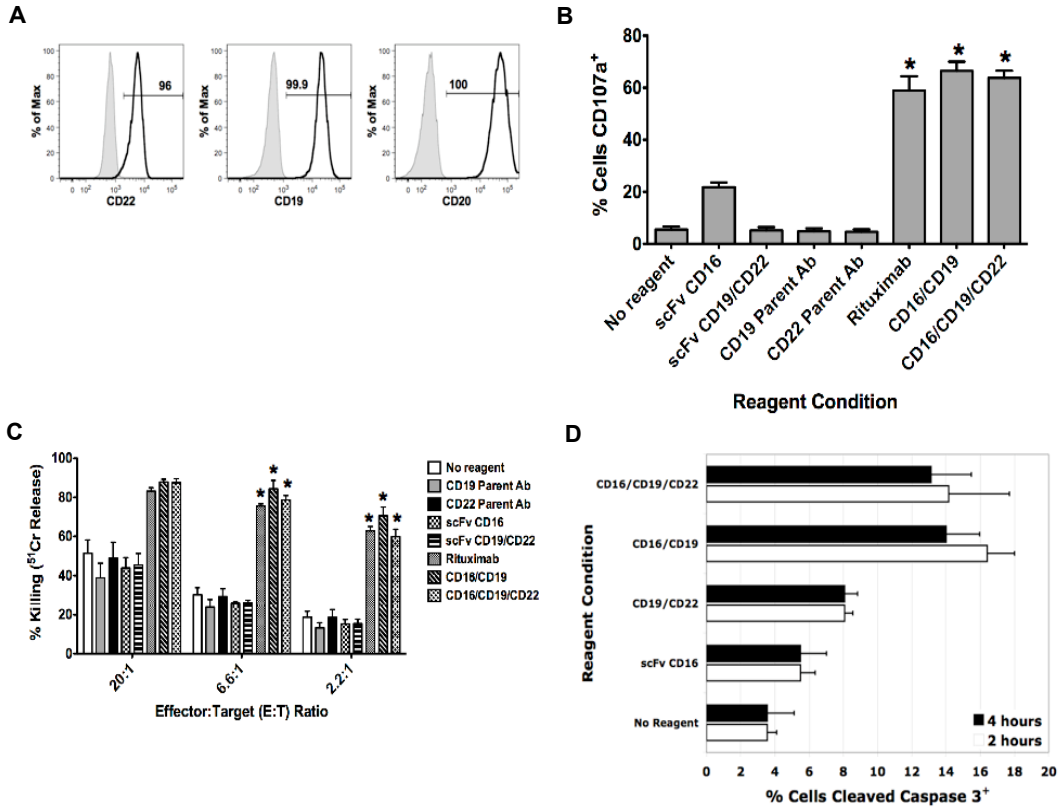


**Figure 2. BiKEs and TriKEs enhance NK cell killing of Raji tumor target cells**. A) FACS plots of CD19, CD20 and CD22 (open histogram; isotype control shaded histogram) receptor expression on Raji targets. B) CD107a expression of resting NK cells co-cultured with Raji target cells (E:T=2:1) with or without (10μg/mL) reagent treatment (*$P$<0.01 for test condition compared to no reagent, n=3; error bars represent SEM). C) $^{51}$Cr labeled Raji target cell lysis by resting treated (10μg/mL) or untreated NK cells treated. Each experiment was carried out in triplicate and repeated with three donors, with aggregate results shown (*$P$<0.01 for test condition compared to no reagent; error bars represent SEM). D) Cleaved caspase 3-expression in Raji targets induced by treated or untreated resting NK cells (E:T=2:1; experiment was performed twice with two NK donors; error bars represent SEM).

## BiKEs and TriKEs are stable and mediate target cell killing at high and low E:T ratios in a dose-dependent manner

To determine the potency of the BiKE and TriKE reagents, resting PBMC were pre-treated with or without reagents at varying concentrations, exposed to Raji target cells and NK cell function was measured by FACS analysis (**Figure 3A**). Both NK cell degranulation and IFN-γ production increased in a dose dependent manner. While rituximab induced NK cell function equally well at low and high concentrations, it peaked

without reaching the functional levels seen for the higher concentrations of the BiKE and TriKE reagents. The human FcγR family consists of six known members which are expressed by various effector immune cells(431). As PBMC contain multiple FcγR receptor expressing populations in addition to NK cells(330), these cells may compete with NK cells for engagement of the Fc portion of rituximab diluting the NK cell effect. As the effector-targeting end of BiKEs and TriKEs is specific for the low affinity FcγRIII (CD16)(345), this further supports the notion of increased NK cell specificity of the bscFv and tscFv recombinant reagents.

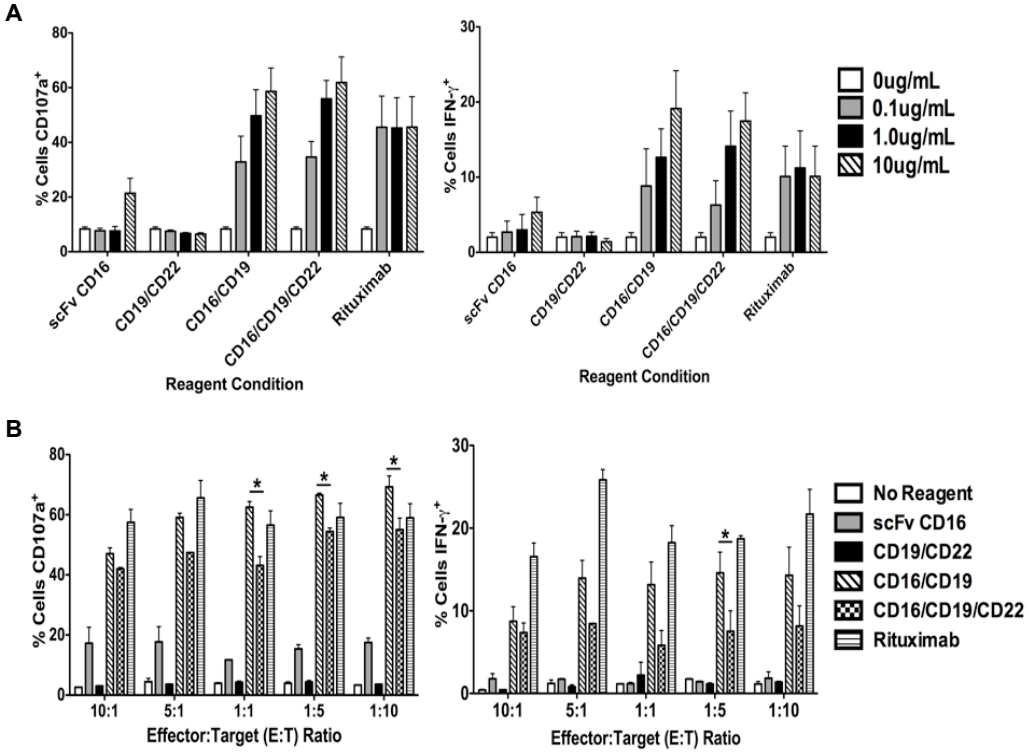


**Figure 3. BiKE and TriKE reagents display a dose-dependent functional effect.** A) PBMC treated with varying doses of reagents were co-cultured with Raji target cells (E:T=10:1) and percent NK cells CD107a (left graph) or IFN-γ (right graph) positive were evaluated (n=3; error bars represent SEM). B) Purified NK cells treated with or without 10μg/mL of reagent were co-cultured with Raji target cells at varying E:T ratios and NK cell CD107a expression (left graph) and IFN-γ production (right graph) were evaluated (*P<0.05, n=2; error bars represent SEM).

NK cells were next tested at varying E:T ratios (**Figure 3B**). NK cells efficiently degranulated at both high and low E:T ratios. The BiKE induced greater CD107a

expression compared to the TriKE at the 1:1, 1:5 and 1:10 E:T ratios suggesting the BiKE may be more efficient at recruiting effector NK cells for target cell lysis. Furthermore, production of IFN-γ by NK cells against Raji targets in the presence of the BiKE and TriKE was also maintained at both high and low E:T ratios (**Figure 3C**). As the activation threshold for NK cell cytokine production differs from that of NK cell cytotoxicity(247, 288, 440), these results indicate the recombinant reagents are capable of inducing both activation requirements and function consistently at both low and high E:T ratios.

The stability of antibody-derived proteins is a vital property that greatly affects the therapeutic efficacy. To evaluate the functional stability, BiKEs and TriKEs were incubated in 100% human serum for 24- or 48-hours, after which resting NK cells were treated with or without reagents, co-cultured with $^{51}$Cr-labeled Raji target cells and target cell lysis was measured (**Figure 4A**). As the results demonstrate, the reagents remain stable and are capable of mediating target cell lysis. In addition, after human serum incubation the TriKE induced greater degranulation (**Figure 4B**), target cell lysis and IFN-γ production (**Figure 4C**) compared to the BiKE, implying structural variations in the recombinant reagents may impact their biological properties, which has been suggested(441). These data also support the premise that degranulation and cytotoxicity data correlate well as readouts for CD16 triggering.

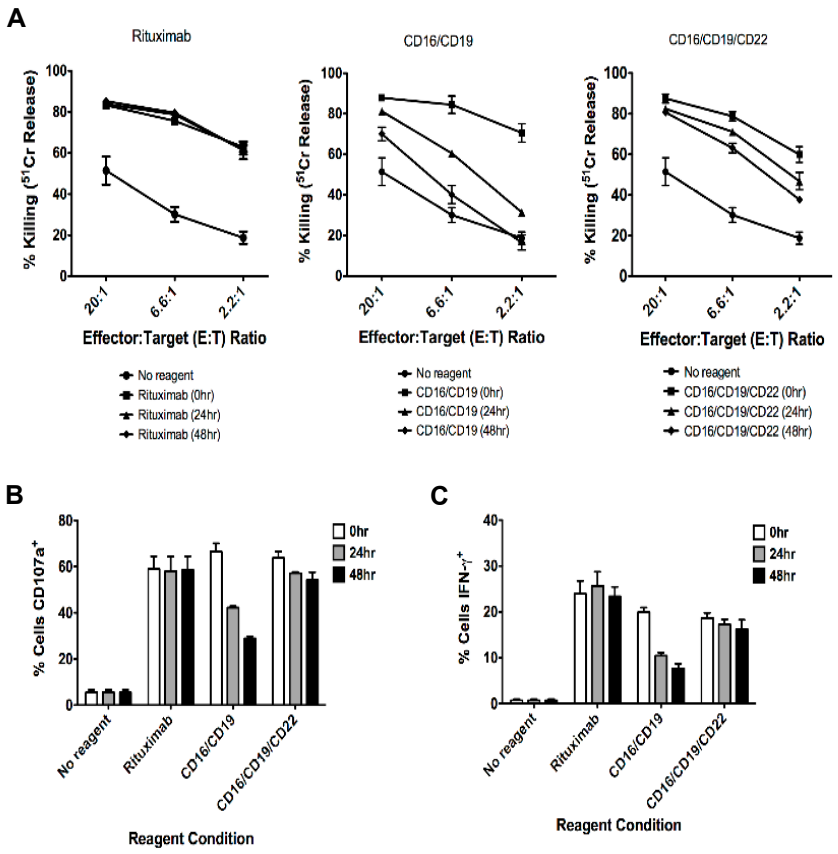


**Figure 4. BiKE and TriKE reagents are functionally stable after culture in human serum**. Reagents were cultured in 100% human serum at 37ºC for 24- or 48-hours. Resting NK cells were treated with or without 10μg/mL of reagent and co-cultured with $^{51}$Cr-labeled Raji target cells (A) or unlabeled Raji target cells (B-C). Percent target cell killing (A), CD107a expression (B) and intracellular IFN-γ production (C) were evaluated. (For the $^{51}$Cr-release assay, each experiment was carried out in triplicate and repeated with three donors with aggregate results shown. For the degranulation and IFN-γ assay, n=3, error bars represent SEM).

## BiKEs and TriKEs induce NK cell chemokine and cytokine production against B-cell targets

Analysis of intracellular IFN-γ production of resting NK cells with or without reagent treatment against Raji target cells showed significant increases in cytokine production for both BiKE and TriKE treated NK cells compared to untreated NK cells (**Figure 5A**). Further analysis of cytokine and chemokine production was performed via Luminex on supernatants harvested from resting NK cells co-cultured with Raji targets. The BiKE

induced significant increases in NK cell production of IFN-γ, GM-CSF, TNF-α, RANTES, MIP-1α and IL-8 and treatment with the TriKE resulted in a different profile with significant increases in GM-CSF, TNF-α, RANTES and MIP-1α compared to untreated NK cells (**Figure 5B**).

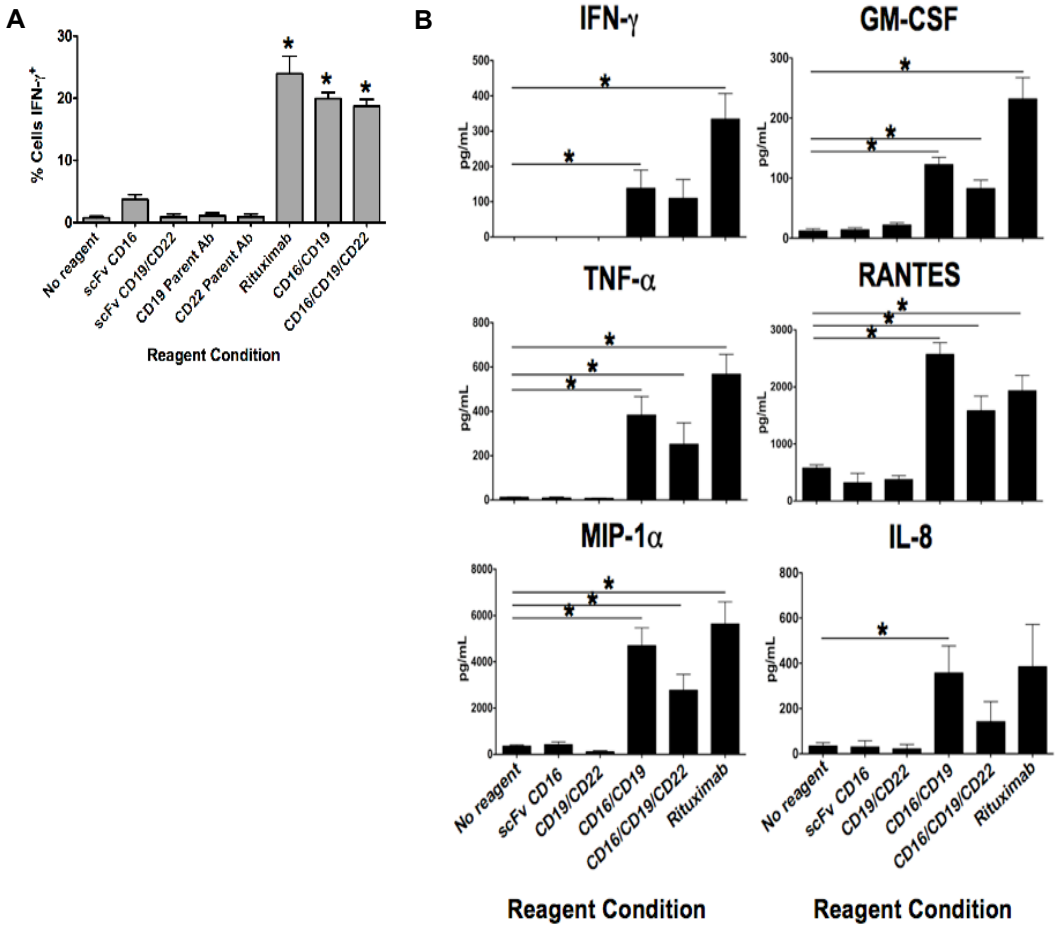


**Figure 5. NK cell chemokine and cytokine production induced by Raji target cells is enhanced in the presence of BiKEs and TriKEs.** A) FACS analysis of treated (10μg/mL) or untreated NK cell IFN-γ production against Raji targets. (*$P$<0.01 for test condition compared to no reagent, n=3; error bars represent SEM). B) Luminex analysis of cytokine/chemokine levels on supernatants harvested from resting treated (10μg/mL) or untreated NK cells co-cultured with Raji targets. Final pg/mL values were determined for each condition using the following equation: [NK cell + Raji cell pg/mL] – [(NK cell alone pg/mL) + (Raji cell alone pg/mL)] (*$P$<0.05, n=6; error bars represent SEM).

Interestingly, the potency with which rituximab induced NK cell IFN-γ (rituximab vs. bscFv $P$=0.003; vs. tscFv $P$=0.001), GM-CSF ($P$=0.026; $P$=0.003), TNF-α ($P$=0.108;

*P*=0.0002) and MIP-1$\alpha$ (*P*=0.207; *P*=0.03) production was greater than that of the recombinant reagents. Furthermore, there were significant differences observed between the BiKE and TriKE reagents for NK cell production of GM-CSF (P<0.05) and RANTES (*P*<0.01), showing CD16 triggering by each reagent generates a unique profile of significantly increased chemokine and cytokine production. As differences in signaling thresholds for the induction of NK cell chemokine and cytokine responses have been demonstrated(246), the differential effects induced by BiKEs, TriKEs and the mAb rituximab suggest other receptor-ligand interactions may be differentially engaged to ultimately determine effector function.

**NK cell function against primary ALL and CLL tumors is enhanced in the presence of BiKEs and TriKEs**

Primary leukemia cells, pre-B ALL and B-CLL cells, were next tested with allogeneic normal NK cells. NK cell degranulation was significantly enhanced against both ALL and CLL primary targets in the presence of the BiKE and TriKE compared to untreated NK cells with no increases observed for the primary AML targets further demonstrating B-cell target specificity (**Figure 6A**). Moreover, CD107a expression induced by the TriKE was significantly greater than the levels induced by rituximab. To evaluate whether this increased function was due to the surface expression levels of CD19, CD20 and CD22 on the primary ALL, CLL and AML leukemia targets, expression of these B-cell antigens was measured via FACS analysis (**Figures 6B and 6C**). Variations in surface expression of these receptors between patient samples were observed within each type of primary leukemia. The overall CD19, CD20 and CD22 expression profile of the primary leukemia targets suggests there may be an advantage to targeting two tumor-

antigens rather than one. Lastly, evaluation of NK cell IFN-γ production against primary leukemia targets was also performed. NK cells treated with BiKE and TriKE reagents displayed significant increases in IFN-γ production against primary CLL targets and enhancement (not statistically significant) against primary ALL targets compared to untreated NK cells (**Figure 6D**).

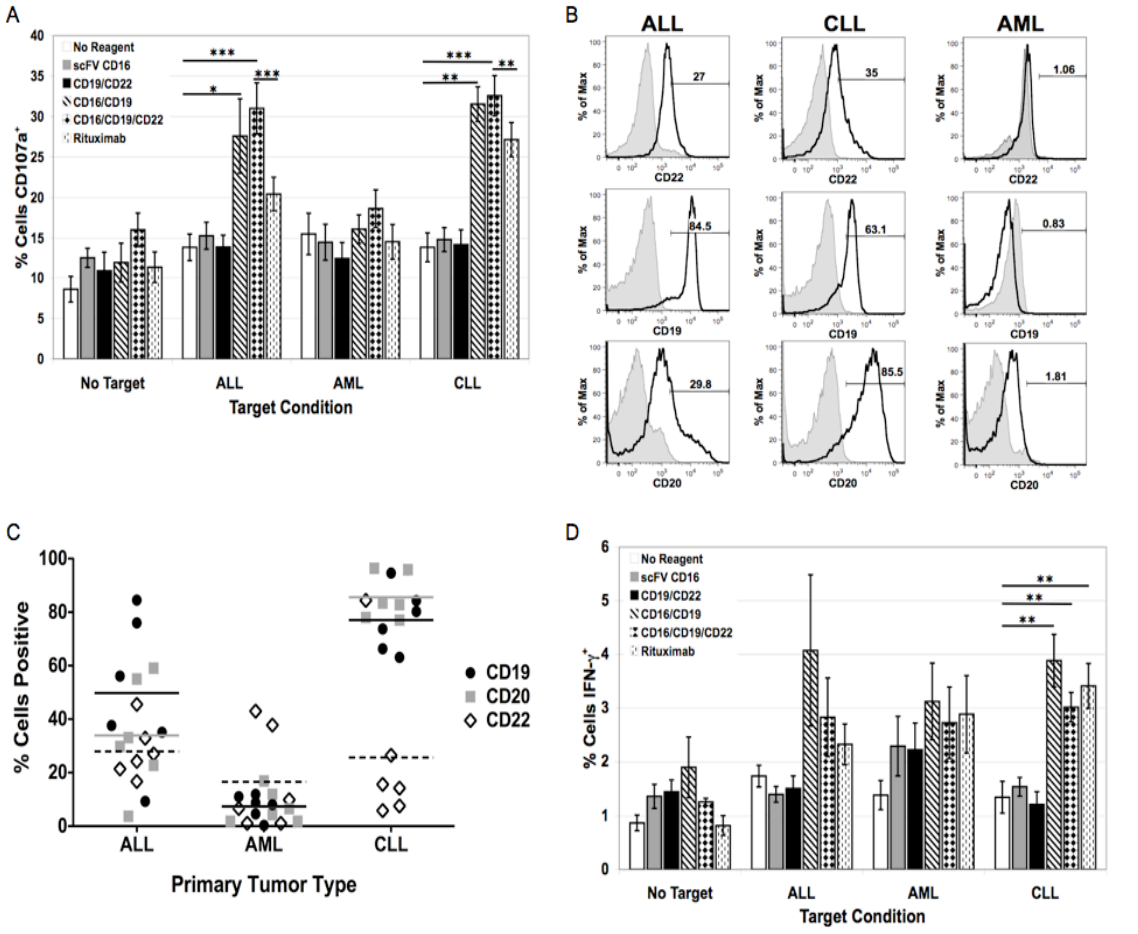


**Figure 6. NK cell function against primary leukemia targets is enhanced in the presence of BiKEs and TriKEs**. A-B, FACS analysis of CD19, CD20 and CD22 (open histograms; isotype control shaded histogram) surface expression on ALL, CLL and AML primary tumor cells. For each primary tumor the histogram plots represent one of six donors (A) with the scatter plot displaying aggregate data (B). C-D) Resting NK cells were co-cultured with primary ALL, CLL or AML tumor cells with or without 10μg/mL of reagent and CD107a (C) or IFN-γ (D) expression was evaluated (**P*<0.01, ***P*<0.001, ****P*<0.0001, six primary leukemia samples of each tumor type were tested against two allogeneic normal NK donors; error bars represent SEM).

## Discussion

In this study we examined the ability of CD16/CD19 BiKE and CD16/CD19/CD22 TriKE recombinant reagents to enhance NK cell function against B-cell tumor targets. While *in vitro* NK cell targeting of B-cell malignancies with bispecific recombinant reagents has been described(346, 432, 441), the mechanism by which these reagents directly function to enhance NK cell activity has not been addressed. Our results demonstrate engagement of CD16 by the CD16 end of the recombinant reagents actively induces $Ca^{2+}$ mobilization in the effector NK cell. As triggering of CD16 leads to phospholipase C-$\gamma$ activation and $Ca^{2+}$ mobilization via signaling through the associated ITAM-bearing Fc$\varepsilon$RI$\gamma$ chain and CD3$\zeta$ chain(176), our data definitively demonstrate BiKE and TriKE reagents directly signal through CD16 to activate NK cells. Furthermore, it has been demonstrated that NK cell cytotoxicity results from a combination of distinct signals, which includes CD16(244, 442). As NK cell engagement by BiKEs and TriKEs led to significant increases in NK cell degranulation and directed target cell lysis, this further demonstrates the ability of these reagents to actively propagate signals for NK cell activation.

Cytokine and chemokine production is another critical component of NK cell function. We definitely show the ability of BiKE and TriKE reagents to induce a pro-inflammatory profile of cytokines and chemokines. When comparing the effects of the BiKE to the TriKE there is a general increase in overall cytokine and chemokine production induced by the BiKE. Furthermore, NK cell cytokine and chemokine production induced by the mAb rituximab was significantly enhanced compared to the recombinant reagents. It has been demonstrated that CD16 engagement is sufficient to induce significant TNF-$\alpha$

secretion, but requires the addition of co-activating receptor engagement to induce an equivalent IFN-γ response(246). Therefore, the functional potency of the recombinant reagents is likely influenced by the presence of co-activating receptor ligands present on the engaged target cell, whose expression may be altered by signaling through the target antigen, which has been shown for CD20 engagement by rituximab(443). CD19 is a B-cell co-receptor involved in the positive regulation of B-cell function and up-regulation of B-cell co-stimulatory ligands(444-448). As our data show enhanced NK cell effector function, both in cytotoxicity and cytokine and chemokine production, after engagement of target cells via the recombinant reagents targeting CD19, it is likely a CD19 triggering event in the target cell occurs upon cross-linking that up-regulates B-cell co-stimulatory molecules that serve to further drive NK cell activation through interactions with activating co-receptors, such as 2B4, ICOS, OX40, 4-1BB and CD28, all of which are expressed on activated NK cells(449-453). In contrast to CD19, CD22 is an ITIM-containing B-cell receptor involved in the negative regulation of B-cell function(446, 454). Our data demonstrate the BiKE induced greater NK cell degranulation and cytokine production compared to the TriKE. As CD22 and CD19 deliver opposing signals that regulate B-cell activation(454) and as antigen expression on Raji target cells is virtually uniform for both (shown in Figure 2A), this could suggest engagement of the inhibitory CD22 receptor by the TriKE modulates the CD19 triggering event, which may lead to a lower surface density of co-stimulatory ligand expression and thus lower reagent-induced effector function as observed for the TriKE.

The ability of rituximab to induce significantly greater amounts of pro-inflammatory cytokines suggests it either mediates stronger cross-linking of CD16 or CD20 ligation leads to target cell modifications capable of enhancing effector function. As surface

densities of CD19, CD20 and CD22 were essentially the same on the Raji target cells and the binding affinity of the scFv CD16 is higher ($K_d$=$10^{-8}$ M)(345) than that of IgG ($K_d$=$10^{-6}$ M)(175), this points to the later. Activated B-cells express CD40 and the CD40-CD40L ligand interaction has been shown to induce B-cell IL-12 production(455, 456). As activated NK cells express CD40L, effector-target cell interaction could promote B-cell production of IL-12, a known stimulus for IFN-γ production and a cytokine that enhances the effectiveness of mAb therapy(457). NK cell IFN-γ production has a greater threshold of activation (i.e. requires the addition of co-activating signals)(246) and as our results show rituximab induces a stronger cytokine and chemokine NK cell response compared to the BiKE despite the lower affinity interaction with CD16, this suggests an important role for target cell co-stimulatory ligand expression for which CD19 engagement may be a weaker stimulus.

Donor-derived NK cells are among the first cells to reconstitute the recipient's immune cell repertoire after HCT and are key mediators of graft versus leukemia (GvL) reactions against minimal residual disease (MRD) providing protection against relapse(292). Consequently, the use of BiKEs and TriKEs to enhance NK cell target recognition and function holds great therapeutic promise for the treatment of cancer. We have demonstrated BiKEs and TriKEs successfully enhance NK cell function against primary ALL and CLL tumors. Notably, the TriKE was significantly superior to rituximab in targeting of ALL and CLL. Lower expression of CD20 compared to CD19 on the ALL tumors could explain these differences. However, expression of CD20 on CLL was greater than that of CD19, which suggests targeting CD22 in addition to CD19 provided an advantage. This has been demonstrated with the combinatorial use of the

epratuzumab, a humanized anti-CD22 mAb, and rituximab, which produced enhanced anti-tumor activity compared with either mAb alone(436). In contrast, as B-cell ALL has been shown to express the inhibitory receptor CD32 (Fc$\gamma$RIIA)(458), it is likely this receptor competes with CD16 for Fc binding of rituximab resulting in decreased effector function. This notion may further be supported by the fact that B-cell CLL has been shown to be CD32 negative(459) and our results demonstrate a greater ability of rituximab to induce NK cell effector function against CLL compared to ALL.

Altogether, our data demonstrate BiKEs and TriKEs directly activate NK cells through CD16, overcoming inhibition by MHC class I molecules and inducing target-specific cytotoxicity, cytokine and chemokine production. Moreover, there are implications that the tumor-antigens targeted by the recombinant reagents influence effector function. Therefore, careful evaluation of effector-target cell interactions induced by BiKEs and TriKEs will further serve to optimize their clinical efficacy.

## Author Contributions

MKG designed the research plan, performed experiments, data analysis and interpretation, and wrote the manuscript; MRV performed data analysis, interpretation and manuscript preparation; DAT generated recombinant reagents and performed experiments; BZ and VM performed experiments, data analysis and manuscript preparation; SZ performed experiments; AP-M performed chemokine/cytokine assays, data analysis, interpretation and manuscript preparation; DAV designed the research plan, performed data analysis, interpretation and manuscript preparation; JSM designed the research plan, performed data analysis, interpretation and manuscript preparation, and was responsible for all aspects of the work.

# CONCLUSION

Originally described as large granular lymphocytes with natural cytotoxicity against tumor cells, NK cells have subsequently been recognized as a separate lymphocyte lineage, with both cytotoxicity and cytokine-producing effector functions that are tightly regulated by a sophisticated repertoire of activating and inhibitory cell surface receptors. Much progress has been made over the last twenty years in the dissection of the molecular mechanisms that allow NK cells to distinguish normal healthy cells (self) from abnormal cells due to infection or transformation (non-self). These data have been essential in defining recognition strategies utilized by NK cells to identify targets and in describing the “dynamic equilibrium concept” of NK cell effector function regulation, namely the process by which the integration of antagonist signals upon receptor-ligand interactions mediate the dynamic equilibrium that governs NK cell function and determines whether or not NK cells will eliminate the encountered targets.

As discussed in Chapter 1, a newly described NK cell receptor, Tim-3, plays an important role in the co-activation of NK cell cytokine production. A recent report has also demonstrated an inhibitory role for Tim-3 in regulating NK cell cytotoxicity. Lanier and colleagues showed that when an NK cell line or resting primary NK cells were co-triggered by an activating receptor, such as NKG2D or CD16, and Tim-3, NK cell cytotoxicity was suppressed(460). However, when NK cells activated overnight with IL-2 were co-triggered, Tim-3 did not reduce cytolytic activity. Moreover, triggering of Tim-3 alone did not induce any significant changes in NK cell function, which agrees with our data that suggests Tim-3 is a weak signaling co-receptor that requires cell priming by cytokines or engagement of other surface receptors to mediate function. Intriguingly, our

data shows an immunopotentiating role for Tim-3 in regulating cytokine production, while the Lanier group demonstrates a negative immunoregulatory role for Tim-3 in regulating cytotoxicity. As noted in the introduction, divergent signaling pathways for cytokine production and cytotoxicity have been shown as well as differential signaling thresholds that are required to trigger each function. As demonstrated by Long and colleagues, activation of resting NK cells is stringently regulated and a hierarchy exists among activating receptors for their ability to activate and induce function in resting NK cells, with CD16>NKp46>2B4>NKG2D>DNAM-1>CD2(247). Furthermore, it was determined that signaling by a combination of synergistic receptors, which are not activating on their own with the exception of CD16, is required to override an activation threshold. It was subsequently shown that the E3 ubiquitin ligase c-Cbl is responsible for this basal level of inhibition and ultimately imposes the requirement for receptor co-stimulation and synergistic activation of resting NK cells(270). c-Cbl was found to bind to phosphorylated Vav-1 and inhibit Vav-1 dependent signals required for activation. As a result, it was shown that strong Vav-1 signaling mediated by synergistic activation is required to overcome c-Cbl inhibition. In a reverse-ADCC (R-ADCC) assay using the mouse mastocytoma cell line P815, Lanier and colleagues demonstrated suppression of cytotoxicity was greater when NKG2D and Tim-3 were co-triggered compared to co-triggering of CD16 and Tim-3(460). As c-Cbl contains an SH2 domain, which mediates binding to phosphotyrosine motifs, it is possible that Tim-3 recruits c-Cbl via its SH2 binding domain to the signaling complex facilitating the downregulation of Vav-1 dependent signals and dampening NK cell activation induced by NKG2D, a weaker activating receptor. Upon stronger stimulation, such as CD16, activating molecular components may be more readily available and compete with c-Cbl for binding to the cytoplasmic domain of Tim-3 and reduce the inhibitory effect of Tim-3 on NK cell

activation. In fact, Kane and colleagues have demonstrated the direct association of the Src family tyrosine kinase Fyn and the p85 subunit of PI3K to the cytoplasmic tail of Tim-3(368). Notably, Ndhlovu et al. showed that NK cell cytotoxicity of IL-2 activated NK cells stimulated with CD16 was not increased with Tim-3 co-stimulation(460), whereas we demonstrated Tim-3-mediated enhancement of IFN-γ production by IL-12 and IL-18 activated NK cells. A likely explanation may be that stimulation with IL-2 and CD16 saturated the cytolytic response, which has a lower threshold of activation, and as Tim-3 functions as a weak co-receptor, its effects may have been masked. We found this to be the case when evaluating the effect of Tim-3 on IFN-γ production; its function was only discernable in the presence of lower doses of IL-12 and IL-18 cytokine priming as well as against NK-resistant targets, such as Raji cells. Therefore, moderate stimulation of resting NK cells, signal strength of which is enough to induce degranulation, may generate intracellular microenvironment conditions that promote a negative regulatory role for Tim-3, whereas stronger stimulation, such as CD16 or IL-12 and IL-18 activation, may generate an intracellular microenvironment that favors an activating role for Tim-3. We have generated preliminary data further investigating this by performing R-ADCC assays and found the following: (i) Resting NK cells co-stimulated with Tim-3 and CD16 or NKp46 (the top two activating receptors in the hierarchy) did not display any overt changes in degranulation while IFN-γ production was enhanced in both cases. Conversely, co-triggering of Tim-3 with activating receptors NKG2D or 2B4 (both of which are lower in the hierarchy) resulted in suppression of degranulation with variable results for IFN-γ production (**Appendix Figure A5**). (ii) NK cells stimulated overnight with IL-12 (1ng/mL) and IL-18 (10ng/mL) and co-triggered with Tim-3 and CD16 or NKp46 again did not lead to any substantial changes in CD107a expression, whereas IFN-γ

production appeared to be enhanced noting variation between donors. Interestingly, co-stimulation of IL-12 and IL-18 primed NK cells with either NKG2D or 2B4 with Tim-3 resulted in suppression of degranulation while IFN-γ production was enhanced in Donor 120 with Donor 121 displaying no substantial changes (**Appendix Figure A6**), which may suggest ITAM-containing receptors influence the effect of Tim-3 signaling on cytotoxicity differently than non-ITAM bearing receptors as recruitment of proximal signaling molecules varies between the two. For example, the DAP10 signaling protein associated with NKG2D directly recruits PI3K to its phosphotyrosine motif as has been shown for Tim-3. Therefore, it is possible there is competition for PI3K binding creating more opportunity for negative regulatory proteins, such as c-Cbl, to associate with Tim-3 causing suppression of NKG2D-induced cytotoxicity. By contrast, activating ITAM-bearing receptors, such as CD16 and NKp46, do not directly bind PI3K and thus would allow greater access for Tim-3 to recruit and interact with PI3K, displacing negative regulatory molecules and amplifying activation signals.

To further investigate the role of the priming stimulus on Tim-3 function, NK cells were stimulated with IL-15 (1ng/mL), IL-2 (100U/mL) or IL-12 (1ng/mL) and IL-18 (10ng/mL) overnight and degranulation and IFN-γ production was measured in a R-ADCC assay after co-stimulation with Tim-3 and the activating receptor CD16 or the inhibitory receptor NKG2A. Priming with IL-15, IL-2 or IL-12 and IL-18 resulted in enhanced degranulation and IFN-γ production with Tim-3 triggering alone (noting variability among priming conditions), while stimulation with both CD16 and Tim-3 resulted in slight increases in effector function (**Appendix Figures A7-A9**). As the combination of cytokine priming and CD16 stimulation potently activates NK cells, the effector

responses again may be saturated making it difficult to discern the functional contribution of Tim-3. As expected, engagement of NKG2A suppressed both degranulation and IFN-γ production. However, co-triggering with Tim-3 functioned to offset this inhibition. Taken together, these results suggest that under the influence of cytokine priming Tim-3 may function to amplify this activation state and promote both NK cell cytotoxicity and cytokine production. Notably, while slight, there were hints that Tim-3 could amplify NKG2A inhibition in resting NK cells when co-engaged (**Appendix Figure A5**). However, if this were the case, one would anticipate function to be more suppressed than what was observed. While the P815 model is useful in targeting receptors of interest, cross-reactivity of human LFA-1 with mouse ICAM-1 makes it difficult to dissect the function of inhibitory signals, as well as individual activating events(461). Therefore, further investigation using the *Drosophila* S2 cell model described by the Long group(251) may be warranted as these cells do not express ligands for adhesion and allow for the examination of individual receptor contribution to NK cell effector function.

While Ndhlovu et al. demonstrated suppression of NK cell cytotoxicity by two different antibodies that crosslink Tim-3, there were differences in their effectiveness with the 344801 clone (Tim-3 801) displaying more potent suppression than the 344823 clone (Tim-3 823) when crosslinked in combination with NKG2D. This suggests that engagement of different Tim-3 epitopes may result in distinct conformational changes that influence the interactions of the Tim-3 cytoplasmic tail with signaling molecules. It may be likely that exposure of the membrane proximal tyrosines, which have been found to facilitate cell activation, is greater when engaged with the Tim-3 823 mAb allowing for phosphorylation and subsequent interactions with activating signaling components,

whereas the Tim-3 801 mAb may induce a different conformation that limits their exposure favoring the phosphorylation and interaction of the three membrane distal tyrosines with negative regulatory signaling molecules. Furthermore, the avidity and affinity with which each antibody interacts with Tim-3 may be different and ultimately influence the functional outcome. In fact, this has been shown for Tim-1 in T-cells where engagement by two different antibodies induces opposite functions(423). Kuchroo and colleagues demonstrated that the 3B3 clone (Tim-1 3B3) potently induced T-cell activation while the RMT1-10 clone (Tim-1 RMT1-10) suppressed T-cell function. It was found that Tim-1 3B3 and Tim-1 RMT1-10 bound closely related epitopes in the IgV domain, but with significantly different avidity and affinity with Tim-1 3B3 displaying both greater avidity and affinity compared to Tim-1 RMT1-10. It is thought that higher-avidity receptor-ligand interactions facilitate the formation of a stable signaling complex, such as large supramolecular activation clusters, which have been shown to mediate full T-cell activation(462). As the 2E2 clone for Tim-3 exhibits agonistic effects on Tim-3 function(351), further characterization of the physical properties of interaction between agonistic and antagonistic antibodies (such as Tim-3 801 and Tim-3 823) and Tim-3 may help explain the mechanisms by which this receptor mediates both inhibition and activation of effector function.

As Tim-3 has the potential to simultaneously engage more than one ligand, distinct combinations of interacting ligands may also influence Tim-3 function; for example, engagement of one ligand by the FG-CC' binding cleft, such as PS, and a second ligand, such as Gal-9, via N-linked carbohydrates present in the IgV domain. Furthermore, Tim-3/Gal-9 interactions likely contribute to the formation of the galectin-glycoprotein lattice, the content of which may also influence Tim-3 function. It has been

demonstrated that galectin-1 (Gal-1) increases TCR signaling through galectin lattice stabilization of the agonist–antigen/TCR complex(463). Moreover, galectin-3 (gal-3) has been shown to regulate receptor residency time at the cell surface influencing the magnitude and duration of receptor signaling(464). Therefore, it is possible that in a state of activation Tim-3 is crosslinked and stabilized in an activating signaling complex by Gal-9 and is capable of amplifying the activating signals induced by the surrounding activating receptors. During the formation of an inhibitory synapse, Tim-3/Gal-9 interactions may stabilize inhibitory signaling complexes and facilitate the association of negative regulatory signaling molecules with Tim-3 resulting in the amplification of inhibitory signals. For T-cells, the formation of a Gal-9 lattice mediated by interaction with Tim-3 may facilitate the exhausted phenotype associated with Tim-$3^+$ cells. Along these lines, we have preliminary data showing a concentration-dependent apoptotic effect of Gal-9 on NK cells, which may be impart due to activation-induced cell death (AICD) facilitated by Tim-3/Gal-9 interactions. Treatment of resting NK cells with high dose rhGal-9 led to the induction of apoptosis, which was significantly blocked by β-lactose and partially blocked in the presence of a recombinant human Tim-3/Fc chimeric protein (Tim-3 Fc chimera) or a Tim-3 blocking mAb (**Appendix Figure A10**). Interestingly, induction of apoptosis in IL-15 activated NK cells by rhGal-9 was only blocked in the presence of β-lactose while both the Tim-3 Fc chimera and Tim-3 mAb had no effect, revealing Tim-3 is less influential in the Gal-9-induced apoptotic process in a cytokine-primed activated state and suggests other surface molecules may contribute to this effect (**Appendix Figure A11**). Indeed, studies have demonstrated activating receptors such as CD16 and CD2 are capable of inducing NK cell AICD in cytokine-primed cells(465, 466). Together, these data imply the cell surface receptor repertoire,

which is altered by cytokine priming, influences the effect of Tim-3 in this process. Analysis of the Gal-9-induced apoptotic effect in $CD56^{bright}$ and $CD56^{dim}$ NK subpopulations, which have differences in their receptor repertoire, supports this hypothesis. While treatment with rhGal-9 induced apoptosis in both $CD56^{dim}$ and $CD56^{bright}$ NK cells, the effect of Tim-3 blockade in resting NK cells was greater in the $CD56^{bright}$ subpopulation ($CD56^{bright}$ vs. $CD56^{dim}$: Tim-3 Fc, $P$=0.009; Tim-3 mAb, $P$=0.006; **Appendix Figure A12**). In a resting state, $CD56^{dim}$ NK cells have relatively high expression of both Tim-3 and CD16 whereas $CD56^{bright}$ NK cells have lower expression of Tim-3, virtually lack expression of CD16 and express the co-activating receptor NKp46. Therefore, in the $CD56^{dim}$ subpopulation formation of the Gal-9 lattice may crosslink the potent activating receptor CD16 inducing activating signals that may be amplified by Tim-3. However, as CD16 delivers such a strong signal the effect of blocking Tim-3 co-signals does not provide sufficient protection from Gal-9-induced apoptosis. By contrast, receptors expressed on resting $CD56^{bright}$ NK cells, such as NKp46, are activating co-receptors, as described by the Long group(247), and do not induce potent signals on their own. Consequently, Tim-3 amplification of co-receptor activation signals may play a more prominent role in eliciting Gal-9-induced apoptosis within this subpopulation as our data demonstrate. Furthermore, as $CD56^{bright}$ NK cells are potent producers of IFN-γ, which we showed Tim-3 functions to enhance, it is possible activation mediated by formation of a Gal-9 lattice stimulates IFN-γ production that ultimately feeds back to the $CD56^{bright}$ NK cells and contributes to AICD. As Gal-9 expression is induced by IFN-γ(467, 468), a possible Gal-9 functional threshold may exist to regulate the NK cell immune response, namely during initiation low to moderate levels of secreted or membrane bound Gal-9 functions to induce NK cell activation.

However, as the immune response progresses the generation of pro-inflammatory cytokines leads to increased levels of Gal-9 expression that may then function to limit the NK cell immune response. Notably, cytokine-priming with IL-15 abolished the differences of the Gal-9 effect between $CD56^{bright}$ and $CD56^{dim}$ NK cells, which may be due to global activation by IL-15 and the upregulation of activating receptor expression in both subpopulations (**Appendix Figure A13**). Identification of the receptors that most prominently contribute to NK cell AICD, the specific role Tim-3 plays and the mechanism by which Gal-9 interaction mediates this process requires further investigation.

The ability of Tim-3 to serve as an NK cell co-receptor, which we have shown can enhance NK cell effector function, may be useful to investigate for clinical therapeutic purposes. Our group and others have demonstrated the effectiveness of NK cell-directed BiKEs and TriKEs in targeting human cancers. New reagents currently in development aim to further optimize the induction of NK cell effector responses and tumor targeting by developing trispecific reagents with bivalent specificity on the effector cell end engaging both CD16 and a co-activating receptor. Initial work using this format has been done in T cells. Huang and colleagues demonstrated a single chain trispecific reagent targeting T cell co-receptors CD3 and CD28 and CEA (Carcinoma Embryonic Antigen) tumor antigen effectively activated T cells without any additional co-stimulation resulting in CEA-positive tumor cell targeting and elimination(469). Zhang et al. subsequently showed the co-stimulatory molecule 41BB (CD137) to be superior to CD28 in the expansion of $CD8^+$ memory T cells and generation of antigen-specific $CD8^+$ CTLs with enhanced cytolytic functions(470). This led to recent work by Xiong and colleagues that revealed combination-therapy with a recombinant human 4-1BB ligand (4-1BBL)/anti-CD20 fusion protein and an anti-CD3/anti-CD20 bispecific diabody significantly

increases T-cell cytotoxicity against human B-cell lymphoma targets *in vitro* and enhances *in vivo* tumor clearance in an xenograft B-cell lymphoma mouse model(471). These results, therefore, indicate the generation of trispecific reagents co-targeting activating and co-activating receptors may provide additional therapeutic benefits in the anti-tumor response. As data suggest Tim-3 is capable of amplifying activating signals, development of a recombinant reagent that co-targets CD16 and Tim-3 against a tumor-associated antigen may prove to be therapeutically advantageous. However, recognizing the multi-functional nature of this receptor, further characterization of the driving mechanisms and circumstances under which the various responses are elicited by Tim-3 is likely needed before the full development and application of this potential reagent can be realized. Notably, pair-wise combinations of activating co-receptors that function synergistically have been well defined by the Long group(247), and may also prove to be beneficial in the design and development of NK-specific recombinant reagents aimed at inducing tumor-specific effector function. They demonstrated CD16-induced activation was enhanced by co-ligation of NKG2D, 2B4, CD2 and DNAM. Interestingly, co-stimulation with NKp46 did not result in increased function. As NKG2D, 2B4, CD2 and DNAM-1 are non-ITAM bearing receptors and both CD16 and NKp46 signal via ITAM motifs, these results suggests ITAM-based signals do not enhance one another. Furthermore, ligation of NKp46 alone induced lower levels of $Ca2^{+}$ mobilization compared to CD16 ligation alone despite the association of both receptors with ITAM-bearing signaling chains. It is possible differences in receptor-signaling chain associations facilitate these divergent results as CD16 complexes with ITAM-signaling chains via an aspartate-aspartate interaction and NKp46 is joined by an arginine-aspartate interaction. Further studies are needed to understand the basis for these interactions and the impact it may have on functional outcomes. Nonetheless, it appears

that synergy is achieved through the use of different signaling modules by each receptor to induce activation as Long and colleagues demonstrated 2B4, which recruits SAP and Fyn, synergizes with NKp46 (ITAM-bearing), NKG2D (recruits and activates PI3K, PLC-γ and Vav-1) and DNAM-1 (phosphorylated by PKC)(472), NKG2D and DNAM-1 each synergize with NKp46 and 2B4, and CD2 (recruits the adaptor protein CD2AP)(473) exclusively synergizes with ITAM-associated receptors, NKp46 and CD16. Further work on the identification of the signaling components employed by each receptor is necessary and will help reveal the mechanistic basis for these collaborative interactions. Ultimately, these results suggest the development of NK cell-targeted activation reagents should be carefully designed to manipulate and take full advantage of synergistic activating receptor/co-receptor combinations. Moreover, as discussed in Chapter 2, it is also important to understand how engagement of the tumor-associated antigen influences the expression of co-activating receptor ligands on the target cell, as this too may play a critical role in determining the success with which these therapeutic reagents activate the effector response to eradicate the targeted tumor.

In conclusion, NK cells are critical mediators of the immune response against infected and transformed cells. Their function is driven by dynamic engagement of the different cell surface receptors that convey distinct signals, which ultimately lead to NK cell activation or inhibition. Here we show a novel NK cell immune receptor, Tim-3, is important in enhancing NK cell IFN-γ production when engaged by its ligand Gal-9, which may facilitate the targeting of Gal-$9^{+}$ tumors. Furthermore, we demonstrate targeting of the potent activating receptor CD16 by recombinant reagents directly triggers NK cell cytotoxicity and chemokine/cytokine production in a tumor-specific

manner, further revealing the overall potential of exploiting the NK cell receptor repertoire for immunotherapeutic purposes. Understanding the specific receptor-ligand interactions that influence NK cell effector function and the distinct pathways induced by receptor triggering will provide important insights on how to take full advantage of the biological properties of NK cells and optimize their use for the treatment of human disease.

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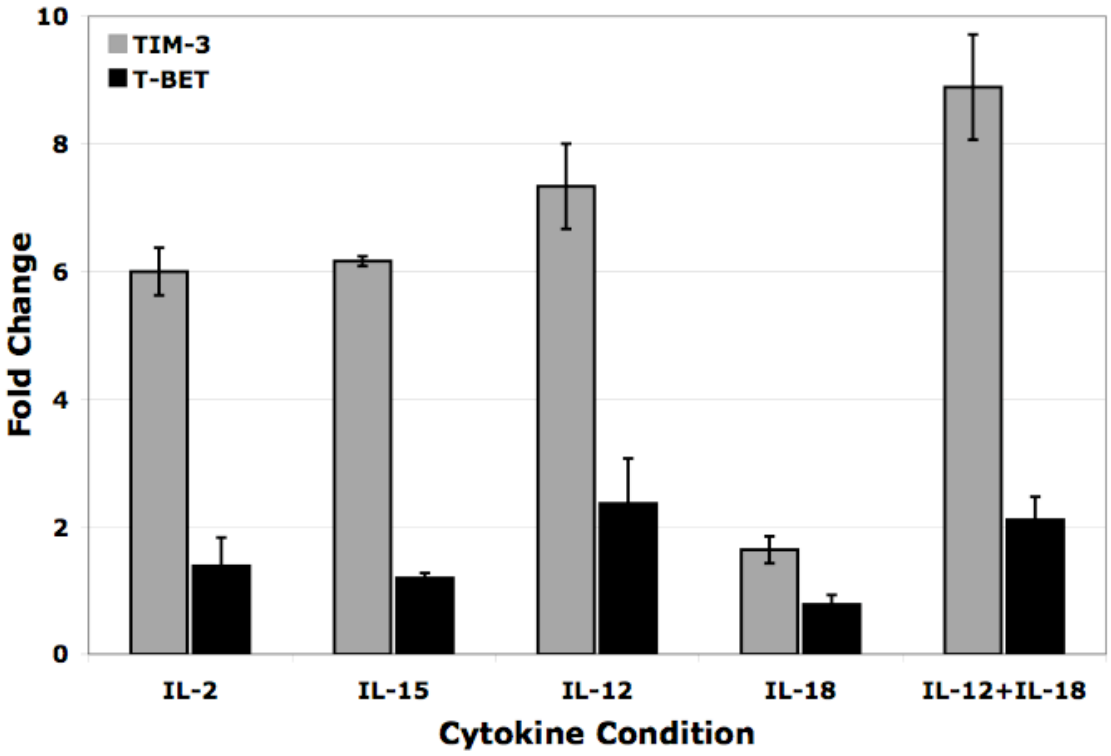
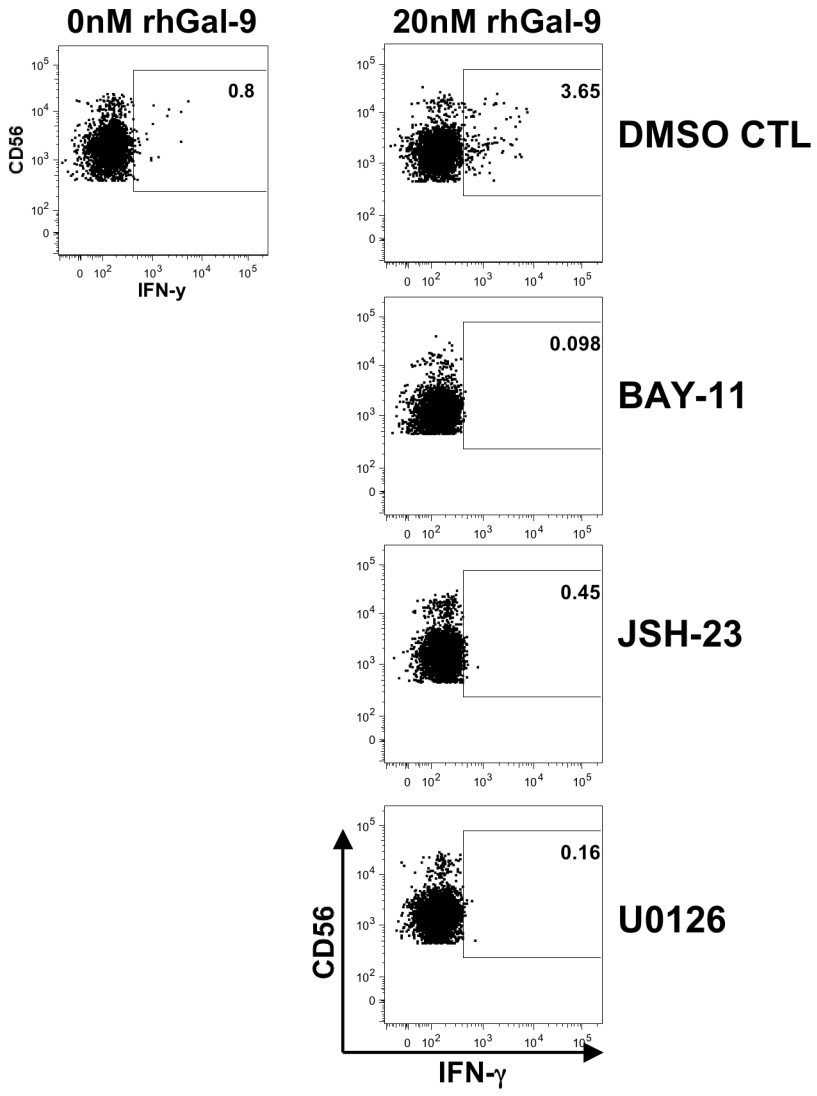
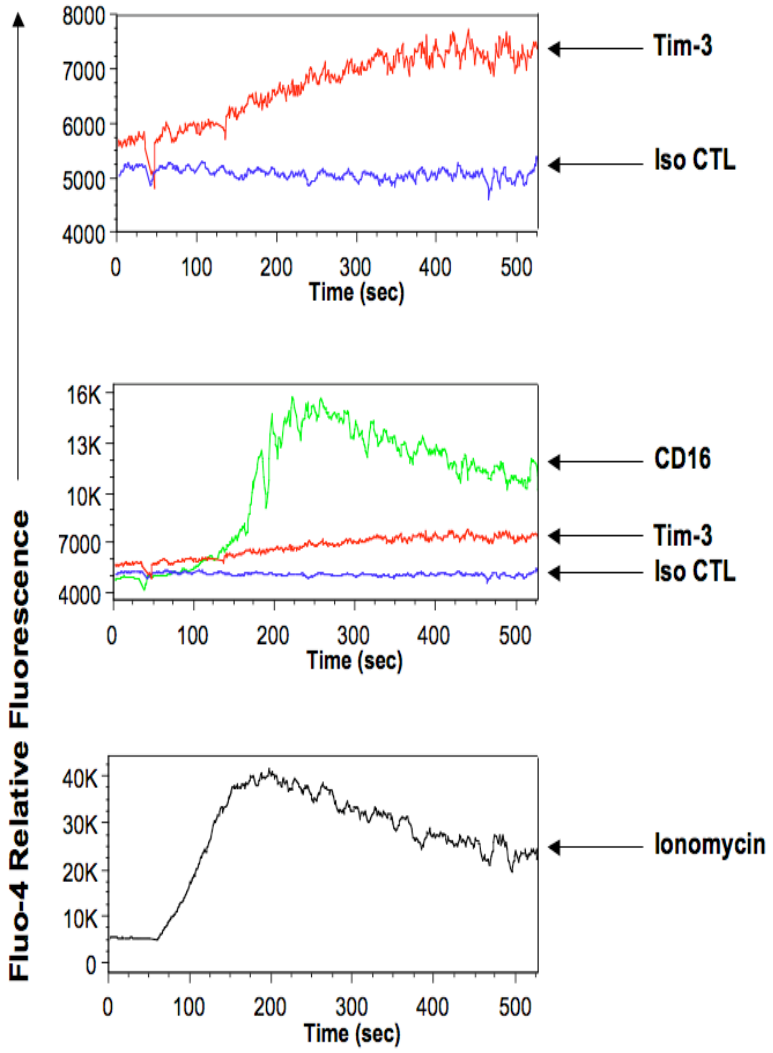
# APPENDIX



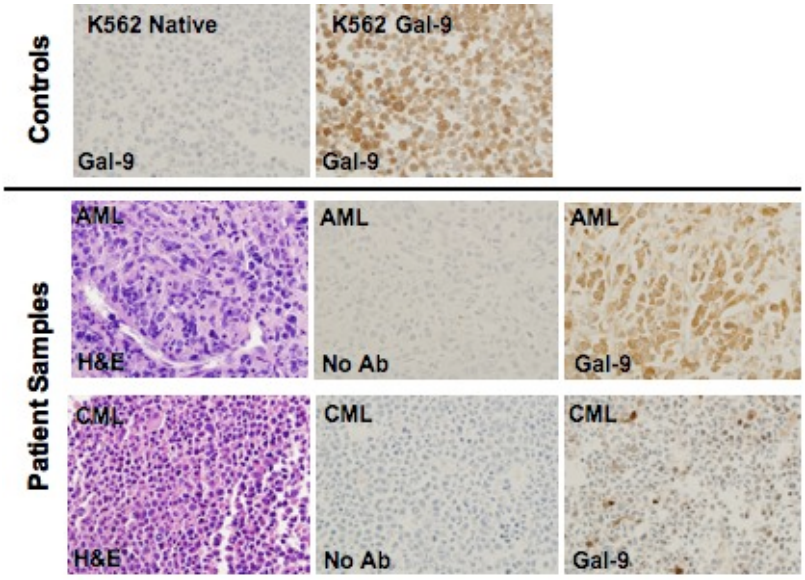

**Appendix Figure A1. Increased levels of the transcription factor T-bet correlate with increased levels of Tim-3.** Resting NK cells (n=3) were stimulated with IL-2 (5 ng/mL), IL-15 (5 ng/mL), IL-12 (5 ng/mL), IL-18 (5 ng/mL) and IL-12 (1 ng/mL)+IL-18 (10ng/mL) for 4 hours and levels of T-bet and Tim-3 were evaluated by qRT- PCR. The change in relative expression levels between resting and cytokine stimulated conditions is represented on the y-axis and was calculated using the following formula: Fold Change ($\Delta\Delta$CT) = (2^[$\Delta CT_{sample}$ (resting control – cytokine stimulated)]/2^[$\Delta CT_{reference}$ (resting control – cytokine stimulated)].



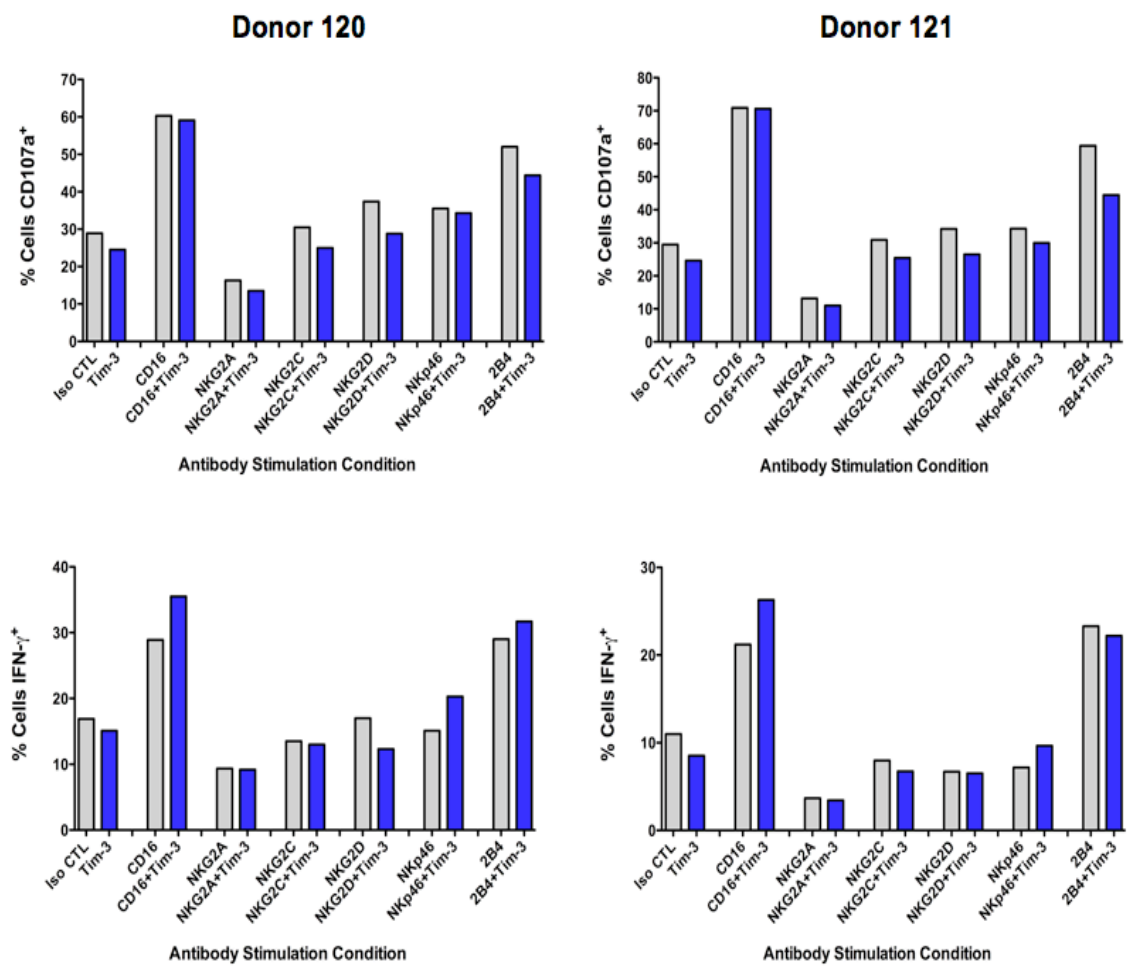

**Appendix Figure A2**. **IFN-γ production induced by rhGal-9 in resting NK cells involves MEK1/2 and NFκB signaling pathways**. To evaluate signaling pathways involved in rhGal-9 induced IFN-γ production, resting NK cells were stimulated with 20 nM rhGal-9 for 4 hours in the presence of 0.1% DMSO control, 10 μM NFκB inhibitors (BAY-11 [Santa Cruz Biotechnology, sc-202490] and JSH-23 [Santa Cruz Biotechnology, sc-222061]) or 10 μM MEK1/2 inhibitor (U0126 [Promega, V112A]) and IFN-γ production was measured by FACS. (Flow plots are representative of two donors.)



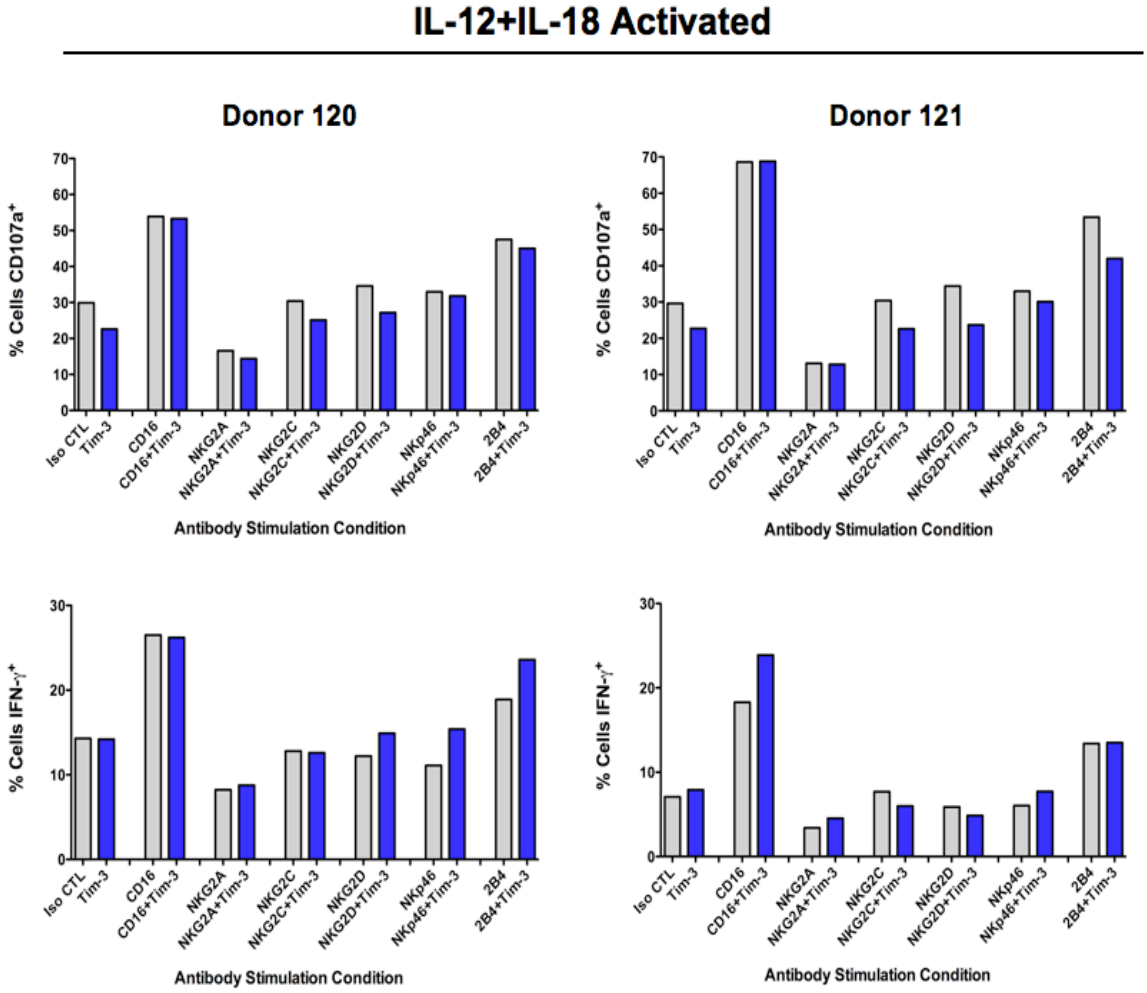

**Appendix Figure A3: NK cell $Ca^{2+}$ mobilization induced by Tim-3 crosslinking.** Resting NK cells were stained with a 2X Fluo-4 calcium-binding dye solution and the change in intracellular $Ca^{2+}$ concentration was measured over time via flow cytometry. Baseline fluorescence was acquired for 1 minute after the addition of the Tim-3 or CD16 primary mAb, after which the goat anti-mouse (GAM) secondary was added to crosslink the receptors and $Ca^{2+}$ flux was recorded for an additional 5-6 minutes. Positive (ionomycin) or negative (mIgG) controls were also recorded. Plots display a representative donor (of 2 experiments) showing the median Fluo-4 relative fluorescence plotted as a function of time in seconds.



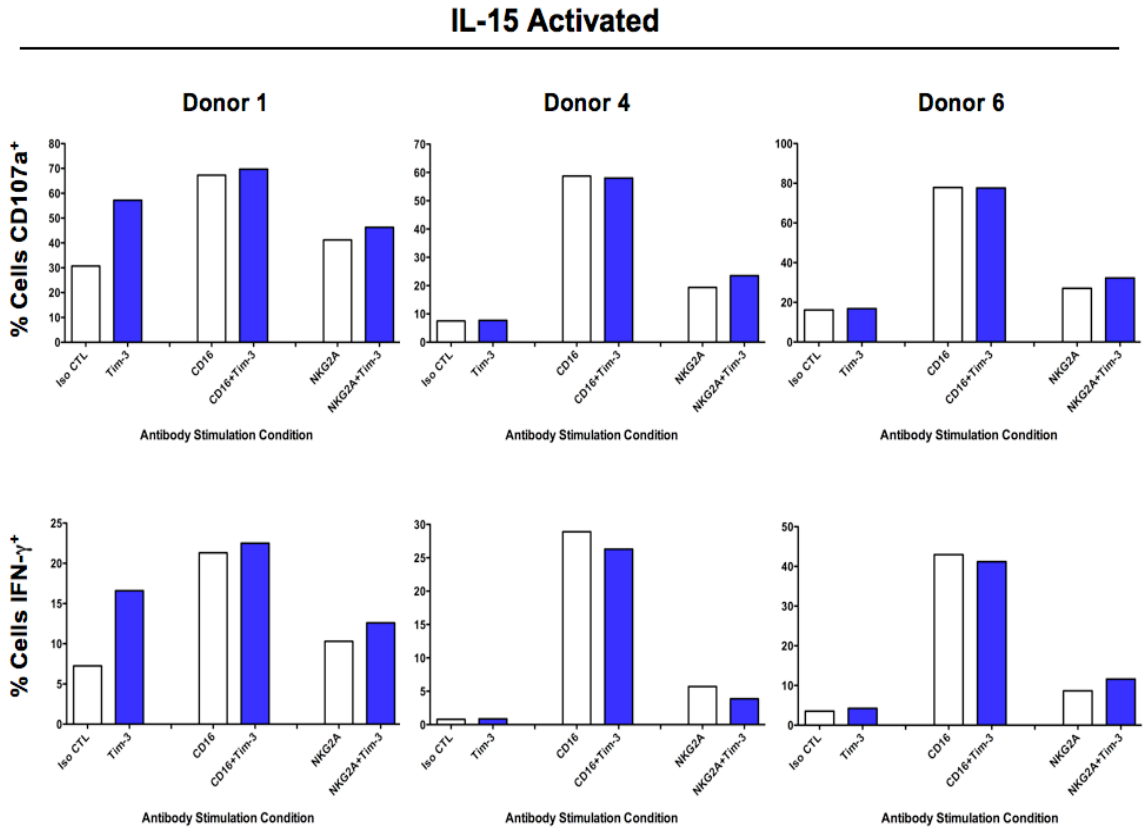

**Appendix Figure A4: Immunohistochemistry analysis of Gal-9 expression in primary tumor samples.** AML and CML patient samples were stained with hematoxylin and eosin (H&E) or an antibody against Gal-9 (anti-human Gal-9, R&D systems BAF2045). Native K562 or K562 transduced with full-length Gal-9 were used as controls. Figure represents one of 5 patient samples for each tumor type.




**Appendix Figure A5: The effect of Tim-3 as a co-receptor on purifed, resting NK cell effector function.** Purified NK cells were incubated overnight in basal medium. Resting NK cells were coated with 10 μg/mL mAbs for 15 minutes at 37ºC against Tim-3, CD16, NKG2A, NKG2C, NKG2D, NKp46 or 2B4 or each in combination with Tim-3, co-cultured with P815 cells (E:T = 2:1) and a degranulation and IFN-γ assay was performed. Induction of CD107a and IFN-γ production was measured via flow cytometry.




**Appendix Figure A6: The effect of Tim-3 as a co-receptor on purified, IL-12 and IL-18 primed NK cell effector function.** Purified NK cells were incubated overnight in basal medium containing IL-12 (1ng/mL) and IL-18 (10ng/mL). Activated NK cells were coated with 10 μg/mL mAbs for 15 minutes at 37ºC against Tim-3, CD16, NKG2A, NKG2C, NKG2D, NKp46 or 2B4 or each in combination with Tim-3, co-cultured with P815 cells (E:T = 2:1) and a degranulation and IFN-γ assay was performed. Induction of CD107a and IFN-γ production was measured via flow cytometry.




**Appendix Figure A7: The effect of Tim-3 as a co-receptor on IL-15 primed NK cell effector function.** PBMC from cryopreserved donors were incubated overnight in basal medium containing IL-15 (1 ng/mL). IL-15 activated PBMC were coated with 10 μg/mL of mAbs for 15 minutes at 37ºC against Tim-3, CD16 or NKG2A or each in combination with Tim-3, co-cultured with P815 cells (E:T = 10:1) and a degranulation and IFN-γ assay was performed. Induction of NK cell CD107a and IFN-γ production was measured via flow cytometry by gating on the $CD56^+/CD3^-$ population.

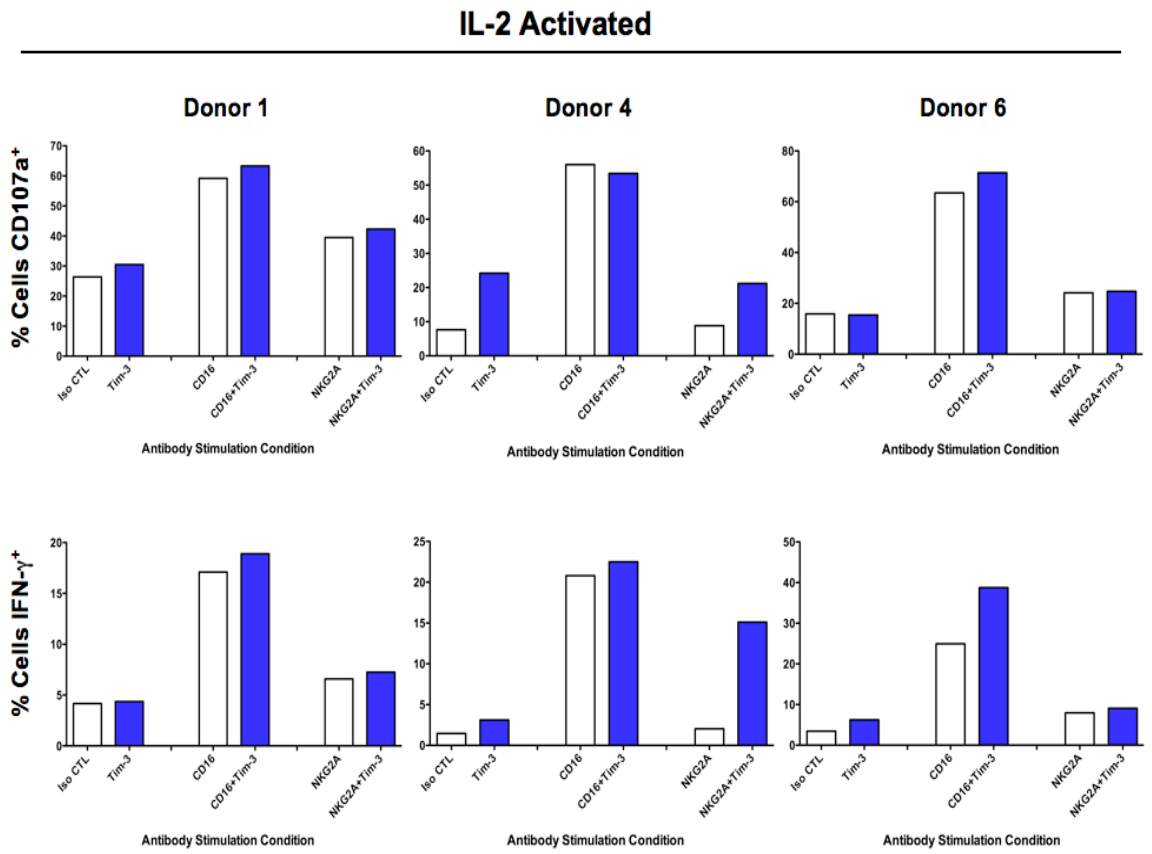
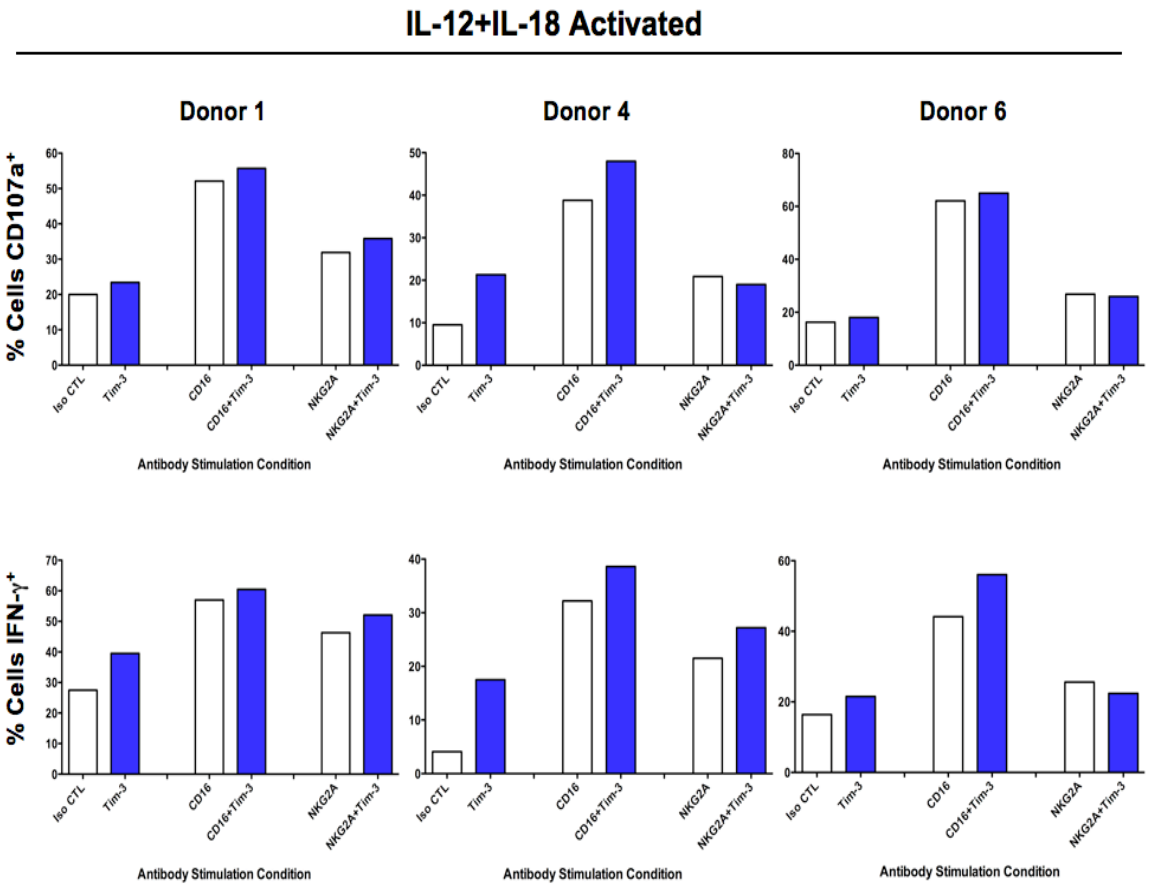
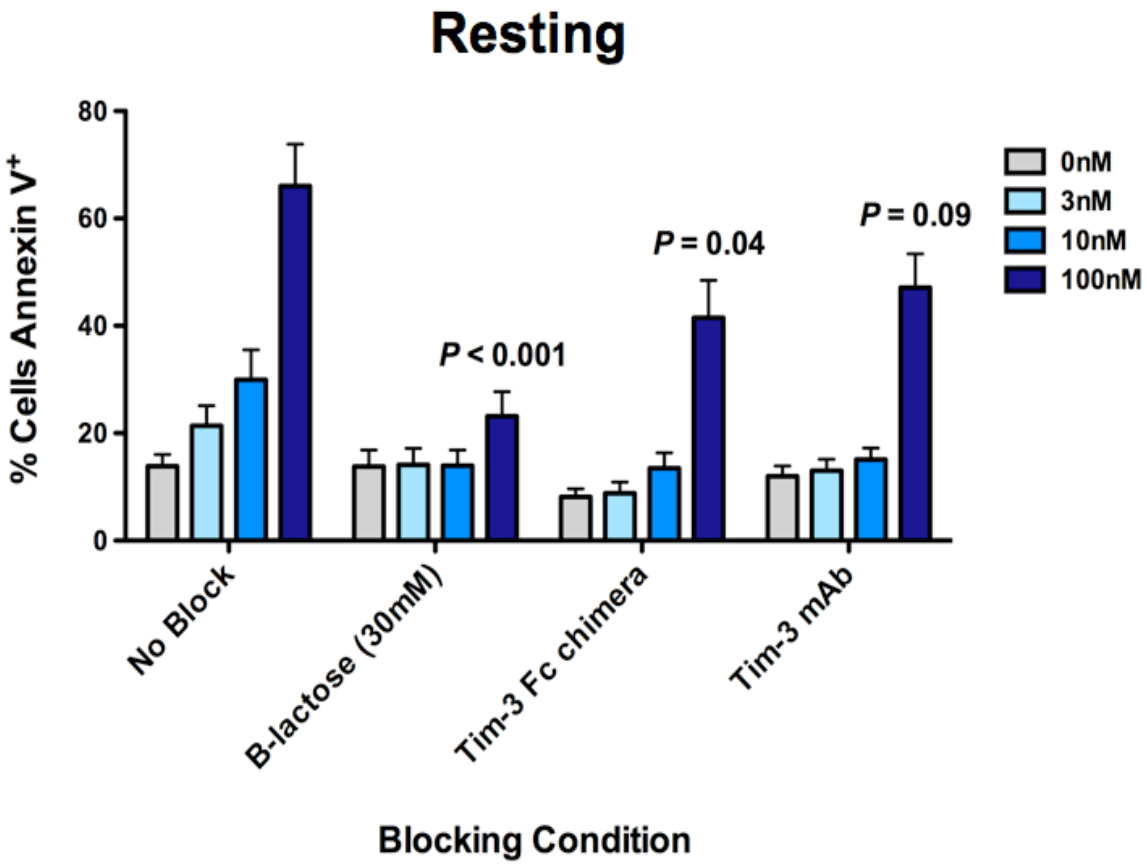


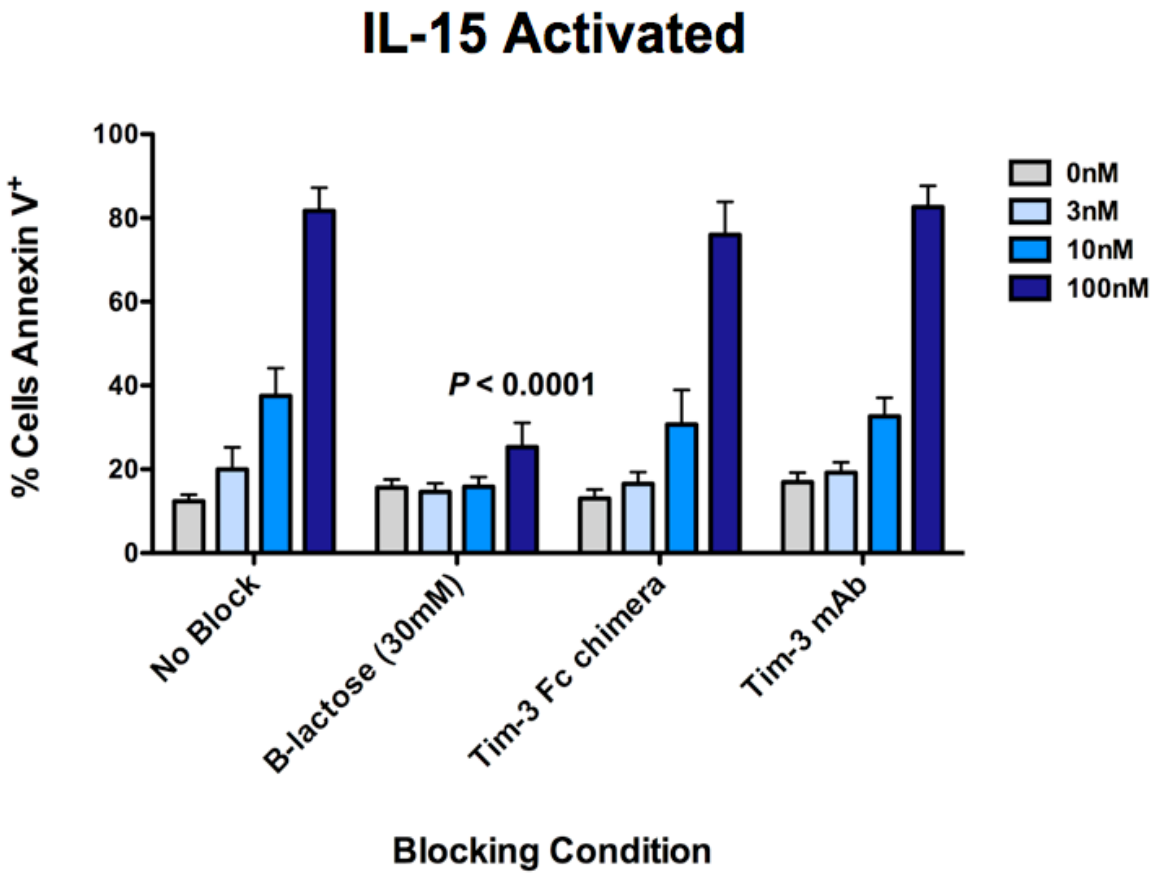

**Figure Appendix A8: The effect of Tim-3 as a co-receptor on IL-2 primed NK cell effector function.** PBMC from cryopreserved donors were incubated overnight in basal medium containing IL-2 (100 U/mL). IL-2 activated PBMC were coated with 10 μg/mL of mAbs for 15 minutes at 37ºC against Tim-3, CD16 or NKG2A or each in combination with Tim-3, co-cultured with P815 cells (E:T = 10:1) and a degranulation and IFN-γ assay was performed. Induction of NK cell CD107a and IFN-γ production was measured via flow cytometry by gating on the $CD56^{+}/CD3^{-}$ population.



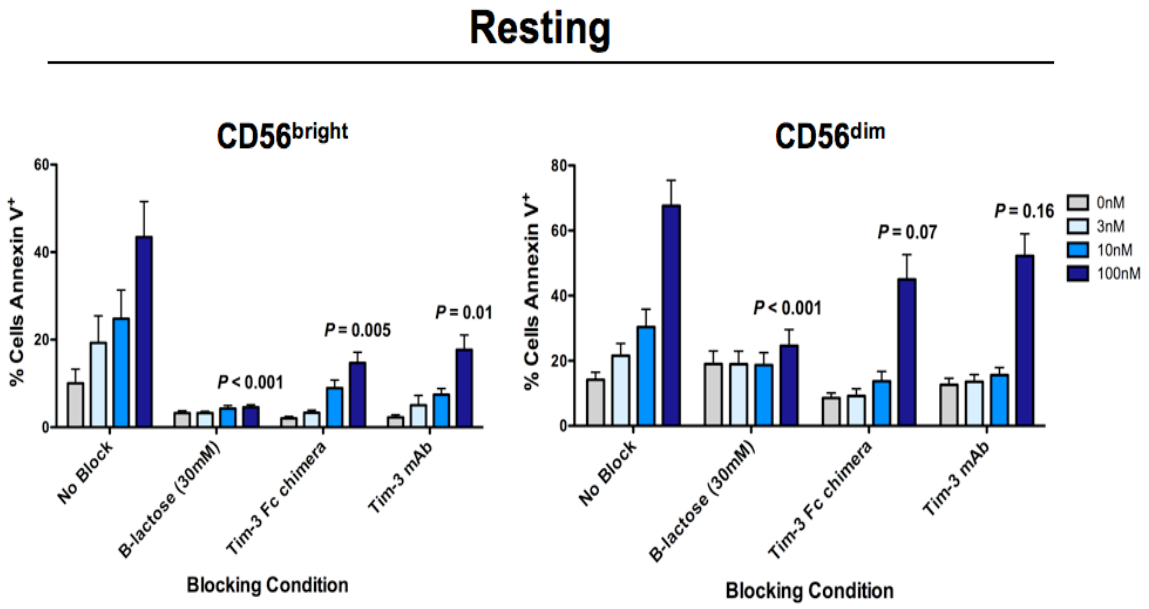

**Appendix Figure 9: The effect of Tim-3 as a co-receptor on IL-12 and IL-18 primed NK cell effector function.** PBMC from cryopreserved donors were incubated overnight in basal medium containing IL-12 (1 ng/mL) and IL-18 (10 ng/mL). IL-12 and IL-18 activated PBMC were coated with 10 μg/mL of mAbs for 15 minutes at 37ºC against Tim-3, CD16 or NKG2A or each in combination with Tim-3, co-cultured with P815 cells (E:T = 10:1) and a degranulation and IFN-γ assay was performed. Induction of NK cell CD107a and IFN-γ production was measured via flow cytometry by gating on the $CD56^{+}/CD3^{-}$ population.



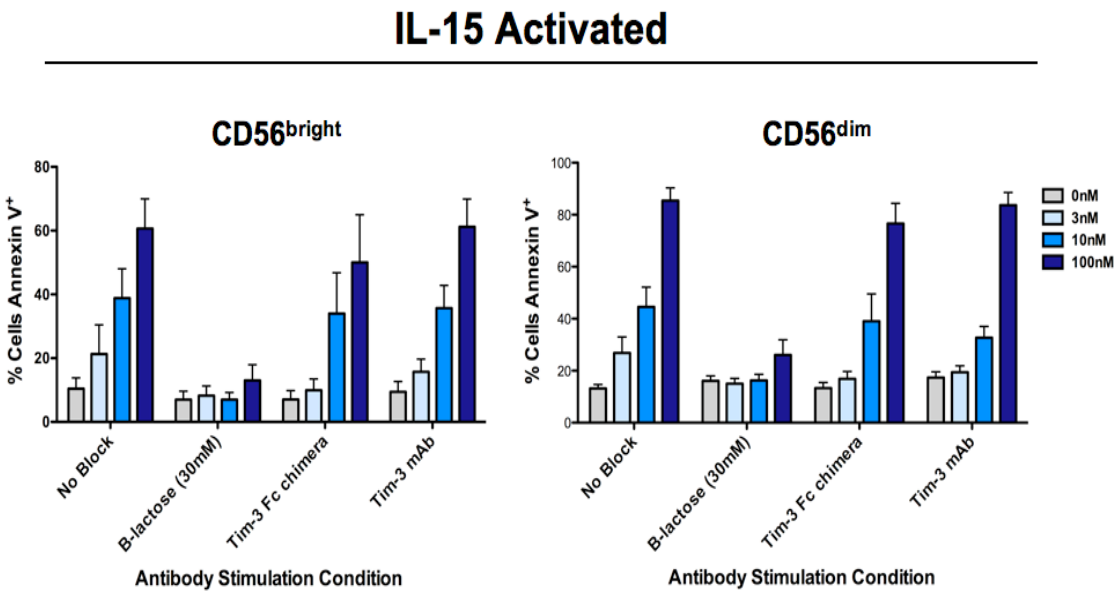

**Appendix Figure 10: Gal-9 induced apoptosis of resting NK cells.** Purified, resting NK cells were incubated in basal medium containing various concentrations of soluble rhGal-9 as indicated in the presence or absence of β-lactose (30mM), Tim-3 Fc chimera (10 μg/mL) or Tim-3 mAb (10 μg/mL) for 4 hours and the expression of Annexin V was evaluated via flow cytometry P-values represent statistical significance between each blocking condition and the no block condition at the 100nM rhGal-9 concentration (n=10); error bars represent SEM.




**Appendix Figure 11: Gal-9 induced apoptosis of IL-15 activated NK cells.** Purified, IL-15 (1 ng/mL) activated NK cells were incubated in basal medium containing various concentrations of soluble rhGal-9 as indicated in the presence or absence of β-lactose (30mM), Tim-3 Fc chimera (10 μg/mL) or Tim-3 mAb (10 μg/mL) for 4 hours and the expression of Annexin V was evaluated via flow cytometry P-values represent statistical significance between each blocking condition and the no block condition at the 100nM rhGal-9 concentration (n=10); error bars represent SEM.




**Appendix Figure 12: Differential effects of Gal-9 induced apoptosis in CD56$^{bright}$ and CD56$^{dim}$ resting NK cell subpopulations.** Purified, resting NK cells were incubated in basal medium containing various concentrations of soluble rhGal-9 as indicated in the presence or absence of β-lactose (30mM), Tim-3 Fc chimera (10 μg/mL) or Tim-3 mAb (10 μg/mL) for 4 hours and the expression of Annexin V was evaluated via flow cytometry gating on the CD56$^{bright}$ and CD56$^{dim}$ populations. P-values represent statistical significance between each blocking condition and the no block condition at the 100nM rhGal-9 concentration (n=10); error bars represent SEM.




**Appendix Figure A13: Gal-9 induced apoptosis in CD56$^{bright}$ and CD56$^{dim}$ IL-15 activated NK cell subpopulations.** Purified, IL-15 activated (1 ng/mL) NK cells were incubated in basal medium containing various concentrations of soluble rhGal-9 as indicated in the presence or absence of β-lactose (30mM), Tim-3 Fc chimera (10 μg/mL) or Tim-3 mAb (10 μg/mL) for 4 hours and the expression of Annexin V was evaluated via flow cytometry gating on the CD56$^{bright}$ and CD56$^{dim}$ populations. P-values represent statistical significance between each blocking condition and the no block condition at the 100nM rhGal-9 concentration (n=10); error bars represent SEM.