University of Tennessee Health Science Center
UTHSC Digital Commons

Theses and Dissertations (ETD)

College of Graduate Health Sciences

12-2013

NKG2D Ligands in Cancer

Neha Das Gupta

University of Tennessee Health Science Center

Follow this and additional works at: <https://dc.uthsc.edu/dissertations>

Part of the [Medical Cell Biology Commons](#), [Medical Immunology Commons](#), and the [Neoplasms Commons](#)

Recommended Citation

Gupta, Neha Das , "NKG2D Ligands in Cancer" (2013). *Theses and Dissertations (ETD)*. Paper 113.
<http://dx.doi.org/10.21007/etd.cghs.2013.0120>.

This Dissertation is brought to you for free and open access by the College of Graduate Health Sciences at UTHSC Digital Commons. It has been accepted for inclusion in Theses and Dissertations (ETD) by an authorized administrator of UTHSC Digital Commons. For more information, please contact jwelch30@uthsc.edu.

NKG2D Ligands in Cancer

Abstract

NK cell transplantation has been increasingly used to treat cancers that are resistant to chemotherapy. However, not all cancers are susceptible to NK cell killing. The prevalence and mechanisms of NK cell resistance have not been well elucidated. Because NKG2D is a major activating receptor on NK cells, we sought to test the hypothesis that NKG2D is the primary pathway in tumor cell recognition. Herein, we comprehensively assessed 20 cancer cell lines representing a broad array of cancer types. In line with our primary hypothesis, no cancer cell lines that expressed low levels of NKG2D ligands were susceptible to NK cell lysis. Furthermore, we found that only 30% of the cancer cell lines were susceptible to NK cell killing and all of them expressed high level of NKG2D ligands. The dependency on NKG2D pathway in these NK susceptible cells was confirmed by anti-NKG2D antibody blocking experiments. For the 70% of cell lines that were resistant to NK cells, 65% were due to silencing of NKG2D ligand expression, whereas 35% were due to insufficient NKG2D activation. For the former cell lines, NKG2D ligand expression was found to be downregulated at the mRNA or protein level. By histone acetylation or proteasome modulation, these regulatory checkpoints could be circumvented to obtain sufficient surface expression of NKG2D ligands to overcome NK resistance. For the latter cancer cell lines that were NK resistant despite high NKG2D ligand expression, KIR inhibition was found to override NKG2D activation in 80% of these cancers. MHC blockade successfully increased NK susceptibility of these cell lines. In the remaining 20%, downregulation of ICAM-1 was responsible for NK resistance. In conclusion, we successfully obtained sufficient evidence to support our hypothesis that NKG2D is the primary pathway for tumor cell recognition. Evasion of the NKG2D pathway is common through NKG2D ligand silencing, KIR inhibition, or inadequate expression of adhesion molecules to mediate immune-synapse formation. Because these resistance mechanisms are readily amenable to therapeutic alteration, NK cell transplantation holds promise for future cancer therapy.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Biomedical Sciences

Research Advisor

Wing Leung, M.D., Ph.D.

Keywords

Cancer, NK cells, NKG2D, Resistance, Therapy

Subject Categories

Diseases | Medical Cell Biology | Medical Immunology | Medical Sciences | Medicine and Health Sciences | Neoplasms

NKG2D LIGANDS IN CANCER

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Neha Das Gupta
December 2013

Copyright © 2013 by Neha Das Gupta.
All rights reserved.

DEDICATION

To my mom and dad who told me ‘*Go conquer the world*’ and to my husband Abhishek without whom this dream wouldn’t see the face of reality.

ACKNOWLEDGEMENTS

Just like all good things must come to an end, so must this long and fulfilling journey of 6 years in graduate school. As I look forward to new beginnings, I reflect upon the past 6 years and realize that I cannot move ahead without acknowledging all the people that have made this Ph.D. journey remarkably wonderful. First and foremost, this journey and my future as a scientist would be impossible without my advisor Dr. Wing Leung, to whom I shall be indebted forever. Dr. Leung has instilled in me values I will treasure throughout my future career as a scientist. He has always been my source of encouragement and taught me the value of focus, persistence and perseverance. I thank him for having immense faith in me and giving me the opportunity to pursue my project in his lab as his first graduate student. His selfless time, care and backing were sometimes all that kept me going. As a researcher, I owe what I am today to him and his teachings.

I am highly obligated to my incredible Ph.D. committee including Dr. Terrence Geiger, Dr. Elizabeth Fitzpatrick, Dr. John Cox and Dr. Roderick Hori. Their invaluable suggestions and ideas have greatly helped in the fruition of my research project. I thank all of them from the bottom of my heart.

I would also like to thank the faculty and staff at St. Jude and UTHSC who have had an instrumental role in my graduate career development. In particular, I would like to express my appreciation for Dr. Pat Ryan whose support as Director of the IPBS program was instrumental to my choices in graduate school. I would also like to thank Dr. Yang and Dr. Wong in the biostatistics department at St. Jude for their help with the statistical analyses. I would also like to call out Mrs. Tricia Page in the volunteer services for giving me the opportunity to serve patients and patient families at St. Jude. It is their smiles and inspiration that made every single day worthwhile.

I would like to acknowledge the role of the funding support I received from NIH and ALSAC through Dr. Leung, for making my research possible.

No words can fully describe what my lab family means to me. Their support and guidance has been key in my scientific development. Since the inception of this lab, the one person who has constantly supported me and helped me grow as a scientist is Dr. Wing Chan. His teachings and scientific brain storming discussions have shaped my scientific mind and for this I will be eternally grateful to him. I extend special thanks to Dr. Ying Li, Dr. Piya Rujkijyanont, Dr. Justin Chang and Dr. Duangchan Suwannasaen for their support, inspiration and willingness to lend a listening ear. I also express my deepest gratefulness to Mrs. Macal Tuggle-Brown, whose friendship and undying support provided the much needed sunshine during bleak times. I also thank Dr. Erin Sullivan, Dr. Susanne Wendt, Dr. Rafijul Bari, Mrs. Diane Woods, Mrs. Sarah Schell, Mrs. Barbara Rooney, Mr. Jim Houston and Mrs. Marti Holladay for their technical help and support. I deeply acknowledge the efforts of Mrs. Amy Doville, Mrs. Amy Prior and Mrs. Debi Rankin for all the help in their capacity as administrative staff.

I am very grateful to all my dear friends in Memphis who made this place a home away from home. It is their rock solid support and love that has resulted in my metamorphosis into a scientist. Without their kindness and care this feat would be unimaginable.

Last but not the least, I thank my parents for their blessings and unconditional love. What I am today is because of them. I would have never been able to pursue my dreams, if not for their sacrifices. I am extremely grateful to my husband Abhishek Gadi for being my support system and pushing me to go the extra mile to achieve my dreams. His confidence and belief in me has made this dream a reality.

In the end, I extend my respect and humble gratitude to The Almighty for having bestowed upon me countless blessings. Without his grace and divine intervention, this Ph.D. journey would have never been possible.

ABSTRACT

NK cell transplantation has been increasingly used to treat cancers that are resistant to chemotherapy. However, not all cancers are susceptible to NK cell killing. The prevalence and mechanisms of NK cell resistance have not been well elucidated. Because NKG2D is a major activating receptor on NK cells, we sought to test the hypothesis that NKG2D is the primary pathway in tumor cell recognition. Herein, we comprehensively assessed 20 cancer cell lines representing a broad array of cancer types. In line with our primary hypothesis, no cancer cell lines that expressed low levels of NKG2D ligands were susceptible to NK cell lysis. Furthermore, we found that only 30% of the cancer cell lines were susceptible to NK cell killing and all of them expressed high level of NKG2D ligands. The dependency on NKG2D pathway in these NK susceptible cells was confirmed by anti-NKG2D antibody blocking experiments. For the 70% of cell lines that were resistant to NK cells, 65% were due to silencing of NKG2D ligand expression, whereas 35% were due to insufficient NKG2D activation. For the former cell lines, NKG2D ligand expression was found to be downregulated at the mRNA or protein level. By histone acetylation or proteasome modulation, these regulatory checkpoints could be circumvented to obtain sufficient surface expression of NKG2D ligands to overcome NK resistance. For the latter cancer cell lines that were NK resistant despite high NKG2D ligand expression, KIR inhibition was found to override NKG2D activation in 80% of these cancers. MHC blockade successfully increased NK susceptibility of these cell lines. In the remaining 20%, downregulation of ICAM-1 was responsible for NK resistance. In conclusion, we successfully obtained sufficient evidence to support our hypothesis that NKG2D is the primary pathway for tumor cell recognition. Evasion of the NKG2D pathway is common through NKG2D ligand silencing, KIR inhibition, or inadequate expression of adhesion molecules to mediate immune-synapse formation. Because these resistance mechanisms are readily amenable to therapeutic alteration, NK cell transplantation holds promise for future cancer therapy.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION	1
Immunobiology of NK Cells	1
Effector functions of NK cells	1
Cytotoxicity.....	1
Cytokine production.....	2
Regulation of effector functions	2
The NKG2D Pathway	9
Clinical Significance of NK Cells	13
CHAPTER 2. HYPOTHESES AND AIMS.....	16
Problem at Hand	16
Central Hypothesis.....	16
Specific Aims.....	16
CHAPTER 3. MATERIALS AND METHODS.....	17
Cancer Cell Lines.....	17
Cell Purification.....	17
Phenotypic Analysis by Flow Cytometry	17
Retroviral Constructs and Transduction of Cells.....	18
NK Cytotoxicity Assay by BATDA	18
Drug Treatments	18
Quantification of MICA Transcripts by Real-Time PCR.....	19
CD107a Degranulation Assay	19
ELISA	19
Immunoprecipitation and Western Blotting	20
Immunofluorescence Confocal Microscopy	20
Statistical Analysis.....	21
Mean ligand expression score	21
Mean lysis	21
CHAPTER 4. RESULTS.....	23
Heterogeneity in NK Susceptibility and NKG2D Ligand Expression in Cancer Cell Lines.....	23
Level of NKG2D Ligand Expression Usually (but Not Always) Correlates with NK Susceptibility	23
NK Sensitivity of Group 1 Cells Involves the NKG2D Pathway.....	26
Pediatric Leukemia Patients Show a Group 2 rather than Group 1 Phenotype	26
Ectopic Expression of MICA Overcomes NK Resistance.....	26
Low Levels of MICA Surface Expression in Group 2 Cancer Cells Were in Part due to Transcription Silencing.....	30
Histone Deacetylase Inhibitors Can Upregulate MICA mRNA and Surface Expression in Neuroblastoma Cell Lines.....	30

Histone Deacetylase Inhibitor Treatment Only Marginally Upregulated MICA mRNA and Surface Protein in the Leukemia and Lymphoma Group 2 Cancer Cells ..	30
Group 2 Cells Express Low Levels of Intracellular MICA	35
Shedding of MICA Is Not Responsible for the Absence of Surface MICA in Group 2 Cancer Cell Lines.....	35
The Small Amount of MICA in Group 2 Cells Is Retained in the Endoplasmic Reticulum.....	35
Treatment with Proteasome Inhibitor Increases Surface Expression of MICA.....	35
NK Resistance of Group 3 Cell Lines Was Not due to PI-9 Overexpression	41
KIR Inhibition Overrides NKG2D Activation in Group 3 Cells	41
RD Cells Cannot Degranulate NK Cells.....	45
NK Resistant RD Cells Expressed Low Levels of ICAM-1	45
NKG2D Pathway Can Be Modulated in Patient Samples	45
CHAPTER 5. DISCUSSION	52
NK Resistance Is Predominant in Cancer	52
Substantial Heterogeneity in NKG2D Ligand Expression and NK Susceptibility	53
Lack of NKG2D Activation Is Commonly Responsible for NK Resistance.....	54
NKG2D Ligand Expression Does Not Necessarily Lead to NK Activation	58
Translational Significance of Our Findings.....	60
CHAPTER 6. CONCLUSION.....	64
LIST OF REFERENCES	66
APPENDIX. SUPPLEMENTARY FIGURES FOR CHAPTER 5	87
VITA.....	95

LIST OF TABLES

Table 3-1.	Weight for each NKG2D ligand	22
Table 3-2.	Four possible phenotypes in our cancer cell spectrum	22

LIST OF FIGURES

Figure 1-1. Regulation of NK cell function according to the 'missing-self' hypothesis.....	3
Figure 1-2. Regulation of NK cell function as explained by the dynamic equilibrium concept.	4
Figure 1-3. Outcome of NK cell response is dependent on the balance between activating and inhibitory signals.	6
Figure 1-4. Sequential steps involved in the NK lysis of target cells.	7
Figure 1-5. NKG2D receptor and ligands in humans.	10
Figure 4-1. Heterogeneity in NK susceptibility and NKG2D ligand expression of cancer cell lines.	24
Figure 4-2. Level of NKG2D ligand expression usually (but not always) correlates with NK susceptibility.	25
Figure 4-3. NK cytotoxicity of group 1 cells involves the NKG2D pathway.	27
Figure 4-4. NKG2D ligand expression and NK susceptibility of leukemia patients.....	28
Figure 4-5. Ectopic expression of MICA overcomes NK resistance in group 2 cancer cells.	29
Figure 4-6. Low level of MICA transcripts in group 2 cancer cells.	31
Figure 4-7. HDAC inhibitor treatment upregulates MICA mRNA and protein levels on neuroblastoma cell lines and increases their susceptibility to NK cells.	32
Figure 4-8. Effect of HDAC inhibitor treatment on MICA mRNA and protein levels in the remaining group 2 cancer cells.	33
Figure 4-9. Presence of intracellular MICA protein in group 2 cells.	36
Figure 4-10. MICA is not shed by group 2 cancer cell lines.	37
Figure 4-11. MICA is retained in the endoplasmic reticulum.	38
Figure 4-12. Treatment with proteasome inhibitor increases surface expression of MICA.	42
Figure 4-13. No significant difference in PI-9 levels between group 1 and group 3 cell lines.	43

Figure 4-14. KIR inhibition overrides NKG2D activation in group 3 cells.	44
Figure 4-15. RD cells cannot degranulate NK cells.	46
Figure 4-16. Low ICAM-1 expression on RD cells.	47
Figure 4-17. Modulation of NKG2D pathway in pediatric leukemia samples.....	48
Figure 5-1. Model for determining strategies for NK therapy.....	62
Figure A-1. Histone deacetylase inhibitor sodium valproate upregulates mRNA and protein levels of other NKG2D ligands in neuroblastoma.....	87
Figure A-2. Histone deacetylase inhibitor sodium valproate significantly upregulated ULBP2 mRNA and protein expression on group 2 leukemia and lymphoma cell lines.	88
Figure A-3. Bortezomib does not increase MICA mRNA.	89
Figure A-4. Bortezomib does not downregulate HLA-ABC in patient cells.	90
Figure A-5. Histone deacetylase inhibitor treatment marginally upregulated MICB mRNA and surface protein in the group 2 leukemia and lymphoma cell lines.	91
Figure A-6. Histone deacetylase inhibitor treatment marginally upregulated ULBP1 mRNA and surface protein in the group 2 leukemia and lymphoma cell lines.	92
Figure A-7. HLA inhibition does not further increase NK susceptibility of group 1 cells.	93
Figure A-8. HLA inhibition does not increase NK susceptibility of group 2 cancer cells.	94

LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell cytotoxicity
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
CAR	chimeric antigen receptor
DAP10	DNAX activating protein of 10KDa
DC	dendritic cell
EEA-1	early endosome antigen 1
Endo H	endoglycosidase H
ER	endoplasmic reticulum
FASL	FAS ligand
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM130	golgi matrix protein 130
GVH	graft versus host disease
GVL	graft versus leukemia
HCMV	human cytomegalovirus
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IL	interleukin
KIR	killer cell immunoglobulin-like receptor
LAMP-1	lysosomal associated membrane protein 1
mRNA	messenger ribonucleic acid
MIC	MHC class I related chain
NCR	natural cytotoxicity receptors
NK	natural killer
NKG2D	natural killer group 2 member D
PBMC	peripheral blood mononuclear cells
PI-9	protease inhibitor 9
TNF- α	tumor necrosis factor- α
TRAIL	tumor-necrosis-factor-related apoptosis-inducing ligand
TRAILR	TRAIL receptor
ULBP	UL16 binding protein

CHAPTER 1. INTRODUCTION

Immunobiology of NK Cells

The first documented description of natural killer (NK) cells dates back to 1975 when Rolf Kiessling described a unique subset of large granular lymphocytes that can directly lyse tumor cells without prior sensitization. Kiessling isolated and characterized these cells and named them natural killer due to their 'natural' occurrence and ability to kill tumor cells [1, 2]. In humans, NK cells are the third largest lymphocyte population comprising of 5-10% of peripheral blood lymphocytes. They are derived from CD34⁺ hematopoietic progenitor cells [3] in the bone marrow and are relatively short lived (approximately 2 weeks) [4]. NK cells are phenotypically characterized by the surface expression CD56 and the absence of CD3 (CD56⁺ CD3⁻) [5]. Furthermore, human NK cells can be divided into two functional subsets based on their surface expression of CD56: CD56^{bright} immunoregulatory cells and CD56^{dim} cytotoxic cells [6]. Around 90% of peripheral blood and spleen NK cells are CD56^{dim} and express perforin. These cells are cytotoxic through granzyme degranulation. In contrast, CD56^{bright} cells lack perforin and are predominant in the lymph nodes and tonsils. These cells readily produce IFN- γ in response to stimulation with interleukin (IL)-12, 15 and 18 [7, 8].

Effector functions of NK cells

Cytotoxicity

Natural cytotoxicity of NK cells is mediated via perforin/granzyme pathway or the death receptor pathway. Perforin/granzyme-mediated apoptosis is the principal pathway used by NK cells to eliminate virus-infected or tumor transformed target cells [9]. Studies in perforin-deficient mice have revealed that this protein is required for most NK cell cytotoxicity [10]. After effector-tumor-cell interaction (conjugation), cytotoxic granules become re-oriented towards the tumor cell, and their contents are secreted into the intercellular cleft between effector and tumor cells in a calcium-dependent manner. These granules contain perforin, which disturbs the tumor-cell membrane and allows the entry of serine proteases known as granzymes [9]. The two most-abundant granzymes, A and B, have been implicated in mediating apoptosis of target cells through caspase-dependent and -independent pathways [11]. Furthermore, NK cells can also induce apoptosis of their targets via death receptor pathways [12]. Some NK cells express FAS ligand (FASL) or tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL). These bind to and activate their receptors, FAS and TRAIL receptor (TRAILR), respectively, which are expressed by some tumor cells inducing tumor cell apoptosis [13, 14].

Cytokine production

NK cells are major producers of IFN- γ in physiological and pathological conditions [15]. They also produce both proinflammatory and immunosuppressive cytokines such as tumor necrosis factor- α (TNF- α) and IL-10 respectively. By production of cytokines, NK cells can act as regulatory cells to influence various cell types such as T cells, B cells and dendritic cells (DCs). NK cell derived IFN- γ and TNF- α helps NK cells cross talk with DCs in many ways including NK cell killing of immature DC and the promotion of DC maturation which leads to enhanced antigen presentation to T cells [16]. Production of IFN- γ by NK cells helps shape T cell responses in lymph nodes, possibly by a direct interaction between naive T cells and NK cells migrating to secondary lymphoid compartments from inflamed peripheral tissues [17, 18]. Furthermore, NK cells also secrete many chemokines that is key to their colocalisation with other hematopoietic cells such as DCs in areas of inflammation [19].

Regulation of effector functions

NK cells can distinguish between healthy cells and abnormal cells by using a sophisticated repertoire of cell surface receptors that control their activation, proliferation and effector functions. The activating receptors stimulate NK cell activity while the inhibitory receptors dampen NK cell responses. Regulation of NK cell function was initially explained by the “missing-self” hypothesis proposed by Karre *et al.* Their seminal study in 1986 noted that NK cells appear to be actively inhibited from responding when they encounter certain tumor cells that express MHC class I [20]. Because MHC class I molecules are expressed by all nucleated cells, they are an ideal universal marker of healthy self. The ability of NK cells to lyse cells ‘missing-self’ predicted the existence of inhibitory receptors and revealed a mechanism whereby NK cells provided immune surveillance for cells that had downregulated MHC class I, a frequent event in cellular transformation or virus infection to avoid recognition by CD8⁺ T cells [21, 22] (**Figure 1-1**). However, missing from the ‘missing-self’ hypothesis was any molecular basis of how NK cells are activated when they encounter tumor or virus infected cells or how NK cells efficiently lyse target cells that have an intact MHC class I surface expression.

Discovery of activating receptors on NK cells, led to the ‘dynamic equilibrium’ or ‘rheostat’ concept- a contemporary modification of the ‘missing-self’ hypothesis [23] (**Figure 1-2**). According to this concept, NK cells sense the density of various surface molecules expressed on the target cells [24]. The integration of these distinct signals dictates the quality and the intensity of the NK cell response. In essence, inhibitory receptors serve as a rheostat, regulating and dampening activating signals, rather than completely terminating them. When NK cells and target cells interact, the information is interpreted by an analog and not a binary process. Thus, when an NK cell encounters a target cell with no inhibitory or activating ligand, there is no target cell lysis by the NK cell (**Figure 1-2A**). If the target cell expresses the inhibitory MHC class I but no activating ligands, NK cells do not lyse the target cells due to the existence of

Figure 1-1. Regulation of NK cell function according to the 'missing-self' hypothesis.

(A) Normal cells expressing self MHC class I molecules engage inhibitory receptors on NK cells. Recognition of self MHC class I spares normal cells from NK lysis. (B) If the target cell loses expression of MHC class I molecules, owing to viral infection or transformation, then the MHC-binding inhibitory receptor at the surface of the NK cell is not engaged. Thus, the NK cell does not receive inhibitory signals and therefore lyses the target cell. In this case, the target cell is perceived by the NK cell to be 'missing self'.

Figure 1-2. Regulation of NK cell function as explained by the dynamic equilibrium concept.
 Depiction of possible outcomes of encounters between NK cells and target cells.

inhibitory signals (**Figure 1-2B**). When the target cells express activating but no inhibitory ligands, NK cells lyse the target cells (**Figure 1-2C**). However, in the presence of both activating and inhibitory signals, the outcome is determined by summation of strength of the signals (**Figure 1-2D**). The amount of activating and inhibitory receptors on the NK cells, the amount of ligands on the target cells as well as the qualitative differences in the signals transduced determine the extent of NK response. The failure of NK cells to lyse target cells can be either due to active inhibition or alternatively due to the absence of sufficient activation. In experimental models, when multiple activating receptors are engaged simultaneously [25] or when a sufficiently potent NK activating receptor is stimulated [26, 27], NK cells are capable of lysing target cells even in the presence of inhibitory signaling through ligation of inhibitory receptors and MHC class I molecules. In summary, the dynamic regulation of NK cell effector functions proposes that the balance of signals from inhibitory and activating receptors together dictate the fate of the target (**Figure 1-3**).

Lysis of target cells by NK cells involves 3 sequential steps, each of which are under tight regulation. First, NK cells adhere to target cells resulting in the formation of immune-synapse. This is followed by NK cell activation by integration of signals from the activating and inhibitory receptors and finally granule exocytosis resulting in target cell death (**Figure 1-4**). The NK cell detection system includes a variety of cell surface adhesion receptors, activating receptors and inhibitory receptors, the engagement of which regulates NK cell activities. Conjugate formation between NK effectors and target cells is a prerequisite for cytotoxicity, and is primarily mediated by binding of the NK surface adhesion molecules LFA-1 (CD11a/CD18) and CD2 to their respective target cell ligands, intracellular adhesion molecule-1 (CD54) and LFA-3 (CD58) [28]. In humans, the MHC class I specific inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) and the lectin-like CD94-NKG2A heterodimers [29]. These inhibitory receptors carry in their cytoplasmic tails immunoreceptor tyrosine based inhibition motifs (ITIMs), which become phosphorylated upon receptor engagement and recruit tyrosine phosphatases SHP-1 and SHP-2. These tyrosine phosphatases suppress NK cell responses by dephosphorylating protein substrates of the tyrosine kinases linked to the activating NK cell receptors. Activating receptors on human NK cells include the FcγRIIIA (CD16) receptor involved in antibody-dependent cell cytotoxicity (ADCC) [30], the natural cytotoxicity receptors (NCRs) including NKp46 [31], NKp44 [32], NKp30 [33] and the C type lectin-like superfamily member NKG2D [34]. Some of these activating receptors contain immunoreceptor tyrosine based activation motifs (ITAMs) in their cytoplasmic domains and some mediate signals through adaptor proteins like DNAX activating protein of 10kDa (DAP10). In addition, NK cells also express co-receptors that cannot directly mediate lysis of target cells but play an important role in reinforcing the strength of activating signals [35]. These include the receptors 2B4 [36], NTB-A [37], DNAM-1 [38] and NKp80 [39].

Both ITAM-dependent signaling and DAP10-dependent signaling of NK activating receptors converge on a common NK cell cytotoxicity pathway. This common pathway involves the activation of Rac, which sequentially leads to activation of the

Figure 1-3. Outcome of NK cell response is dependent on the balance between activating and inhibitory signals.
According to the 'dynamic equilibrium' concept, the balance of activating and inhibitory signals ultimately decides the fate of the target cell.

Figure 1-4. Sequential steps involved in the NK lysis of target cells.

Three steps involved in the lysis of target cells by NK cells. 1) Adhesion mediated by the adhesion receptors and ligands results in the formation of immunosynapse. 2) Engagement of activation and inhibitory receptors with their respective ligands, integration of signals and NK cell activation. 3) The activated NK cell undergoes granule exocytosis resulting in target cell death.

mitogen activated protein kinase (MAPK), extracellular signal regulated kinase (ERK) and mitogen activated or extracellular signal regulated protein kinase kinase (MEK) [40] resulting in the redistribution of intracytoplasmic granules and their release. Thus signaling by co-receptors like DNAM-1 (ITAM-dependent) can provide a synergistic signal to NKG2D signaling which is mediated through DAP10.

Inhibition by ITIM bearing inhibitory receptors of activating signaling is saturable and proportional to the magnitude of engagement of both types of receptors by their cognate ligands. When ITIM bearing receptors engage their ligands, the tyrosine residue is phosphorylated, probably by a Src family kinase, resulting in the recruitment of the lipid phosphatase SHIP-1 or the tyrosine phosphatases SHP-1 or SHP-2 [41]. The inhibitory NK receptors recruit these phosphatases to the interface between the NK cell and its prospective target cell, and these tyrosine phosphatases suppress NK cell responses by dephosphorylating the protein substrates of the tyrosine kinases linked to activating NK receptors.

Inhibitory and activating receptors act in concert and co-aggregation between activating and inhibitory receptors is required for inhibition to occur [42]. A dynamic equilibrium is set between the “strength” of activating and inhibitory signals and is relayed by the level of tyrosine phosphorylation of critical signaling components (like Vav1) that can serve as targets for both Syk-family protein tyrosine kinase and SHP-1 or SHP-2 protein phosphatases [43].

In our study, we focused on the NKG2D pathway because unlike NCRs that are expressed at different surface densities in different NK cells, the expression of NKG2D is relatively homogeneous [44]. Both NCR^{bright} and NCR^{dull} NK clones show comparable surface densities of NKG2D [45, 46]. Also till date, tumor ligands for the NCRs remain undefined except for B7-H6 which has been recently identified as a ligand for NKp30 [47].

The NKG2D Pathway

Natural Killer Group 2 member D (NKG2D) was identified in a screen for genes that are preferentially expressed by human NK cells [48, 49]. Interest in NKG2D was prompted by a curiosity in identifying activating receptors used by NK cells to recognize target cells. In humans, NKG2D is expressed on NK cells, CD8⁺ T cells and $\gamma\delta$ T cells [34, 50, 51]. NKG2D is encoded by a gene on human chromosome 12 mapping within the natural killer (NK) gene complex. This receptor belongs to the type II C-type lectin like family of transmembrane proteins [52]. In humans, NKG2D is expressed as a homodimer in association with its adaptor molecule DNAX activating protein of 10kDa (DAP10) [53] (**Figure 1-5**). Each NKG2D homodimer assembles on the cell surface with two dimers of DAP10, thereby forming a hexameric structure, which reduces the threshold of ligand levels required for inducing signal transduction [54]. The association of NKG2D with DAP10 is necessary for signaling and considered important for its cell surface expression. DAP10 is a type I transmembrane protein with conserved aspartic acid residues in its transmembrane region that interacts with a positively charged arginine residue in the transmembrane region of NKG2D [55]. Engagement of NKG2D in human NK cells results in tyrosine phosphorylation of DAP10 and coupling of the receptor to the phosphatidylinositol-3-kinase (PI3K)/ growth factor receptor bound protein 2 (Grb2)-Vav pathway [56]. The PI3K pathway plays a critical role in regulating NK cytotoxicity by promoting perforin and granzyme B movement toward target cells and DAP10-mediated cytokine production [40, 57].

NKG2D ligands are diverse proteins that are structurally similar to MHC class I molecules (**Figure 1-5**). In humans, NKG2D ligands fall into two families- the MHC class I chain related gene A (MICA) and B (MICB) family and the cytomegalovirus UL-16 binding protein (ULBP) family comprising of ULBP1-ULBP3 proteins. First evidence of a protein that binds to NKG2D came from studies showing that a soluble form of MICA binds to various lymphocyte subsets. A monoclonal antibody blocking the interaction was subsequently shown to bind NKG2D [34]. Further analysis showed that MICB- a close relative of MICA (84% identical) also binds NKG2D [58]. No functional homologues of MICA and MICB are present in mice. MICs are highly glycosylated transmembrane proteins possessing the characteristic MHC class I like $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains [59, 60]. Despite the presence of the $\alpha 3$ domains, MICA and MICB do not associate with $\beta 2$ microglobulin or require $\beta 2$ microglobulin for their surface expression. MICs are highly polymorphic with more than 50 MICA and 13 MICB alleles so far reported [61].

A distinct approach identified three related human proteins that bind to the UL16 protein of human cytomegalovirus (HCMV). UL16 was studied because it was a candidate virus protein involved in immune escape. ULBP1 was first identified on the basis of its binding to the HCMV glycoprotein UL16. Subsequent database searches for homologues of ULBP1 led to the identification of additional ULBP members. ULBPs also contain $\alpha 1$ and $\alpha 2$ domains but lack the $\alpha 3$ domain and are expressed as glycosylphosphatidylinositol (GPI) anchored proteins [62, 63]. Notably, the MICs and ULBPs only share 20-25% sequence homology [64].

Figure 1-5. NKG2D receptor and ligands in humans.

Illustration of the human NKG2D receptor and its ligands. Also depicted is the classical MHC class I molecules.

Given that the NKG2D ligands vary in structure and sequence, it is pertinent to wonder whether all ligands are equally able to bind to NKG2D and invoke an activating signal in immune cells. The binding of NKG2D to its various ligands is generally of high affinity than many immunoreceptor ligand interactions [65]. Three dimensional structure analyses indicate that ligand binding to NKG2D is focused on key tyrosine residues that are rigidly held in the NKG2D structure. Crystal structure studies of human NKG2D in complex with MICA and ULBP3 reveal that NKG2D binds to the $\alpha 1/\alpha 2$ helices of the ligands. Notably, most of the receptor amino acid residues that dominate binding to different ligands are the same and several of the contact residues between different ligands are conserved, especially those that contribute most of the binding energy. Therefore, despite differences in the amino acid sequences of different NKG2D ligands, they interact with the receptor similarly, and the receptor does not seem to undergo marked conformational changes (induced fit) to accommodate different ligands. Rather binding of NKG2D with diverse ligands is explained as 'rigid adaptation', in which a rigid binding site on NKG2D makes diverse interactions with a series of structurally distinct ligand surfaces by utilizing a core set of residues capable of multifarious bonds [66]. Binding studies of human NKG2D with MICA reveal that each NKG2D homodimer can only bind with a single monomeric ligand suggesting that NKG2D can bind to only one ligand at a time. [67, 68].

The NKG2D ligands are encoded by genes in the hosts' own genome i.e. they are self proteins as opposed to foreign antigens. Moreover, they are either absent or present at low levels on normal healthy cells but their expression is often upregulated in pathological conditions like infection or tumor transformation. Therefore recognition of NKG2D ligands by NKG2D is referred to as 'induced-self' recognition. Although NKG2D ligands are often upregulated as a result of cellular stress, infection or tumorigenesis, the mechanisms that regulate their expression are still ill-defined. Several studies indicate that the regulation of individual NKG2D ligands differs substantially in particular tumors or infected cells, suggesting that the diverse NKG2D ligands do not simply reflect the redundant expression of molecules with the same function.

The first insight into the regulation of NKG2D ligands came from analysis of the promoters of MICA and MICB, which were found to resemble the promoter of heat shock protein (HSP)70 [69]. Accordingly, expression of MICA is induced by heat shock [59]. On the basis of sequence analysis, investigators have shown that heat shock elements may also exist in some ULBP genes but it hasn't been demonstrated that these genes are regulated by heat shock [70]. It has also been proposed that NF- κ B may stimulate MICA transcription and an NF- κ B binding site in intron 1 of the MICA gene has been described [71, 72]. Studies of MICA, MICB and ULBP1 genes have identified potential binding sites for Sp family transcription factors [69, 73]. In addition, the DNA damage pathway has also been implicated in induction of NKG2D ligands [74, 75]. DNA damaging agents activate ATM and ATR thereby activating the DNA damage response resulting in ligand induction. Thus, DNA damage response, which arrests the cell cycle and enhances DNA repair functions, or triggers apoptosis, also alerts the immune system in response to potentially dangerous cells through the activation of NKG2D. However,

the mechanism of regulation of NKG2D ligands by the DNA damage response still remains poorly understood [76].

There are also some reports of micro RNA mediated regulation of NKG2D ligand expression. Initially, HCMV and other virus encoded micro RNA's were shown to downregulate expression of MICA, MICB or ULBP3 [77-79]. However, subsequently Stern-Ginossar *et al* showed that certain cellular micro RNA's can also target the MIC transcripts. They reported that cellular micro RNAs maintain threshold levels of MICA and MICB in normal cells while transcriptional induction of the ligand genes can overcome this buffering capacity of micro RNAs [80]. Some studies on post translational regulation of NKG2D ligands have also been reported but primarily in the mouse system. Nice *et al* reported that the mouse NKG2D ligand MULT1 is polyubiquitinated and degraded in unstressed cells while in cells subjected to heat shock or UV irradiation MULT1 was less ubiquitinated and expressed on the cell surface [81, 82]. Although there is no direct evidence yet of such regulation of human NKG2D ligands, the intracellular sequestration of MICA in melanoma cells may perhaps be due to a similar or related mechanism [83]. Post translational regulation of MICs has been reported in the context of viral infections. Kaposi's sarcoma-associated herpesvirus (KSHV) encoded E3 ubiquitin ligase K5 can downregulate cell surface MICA and MICB. However, unlike MULT1, ubiquitination of MICA resulted in redistribution of MICA from the plasma membrane but not its degradation [84].

One of the most important findings in NKG2D ligand regulation is that ligands are proteolytically cleaved from the cell surface or excreted in exosomes. Soluble forms of MICs and ULBPs have been observed in cancer patients and cancer cell culture supernatants [85-90]. The presence of soluble ligands may act as indicators of poor prognosis in cancer patients [91]. Shedding of ligands was shown to be due to cleavage of extracellular domain of NKG2D ligands by matrix metalloproteinases (MMPs) [86, 89]. Also, the membrane associated disulphide isomerase endoplasmic reticulum protein 5 (ERP5) was shown to be responsible for proteolytic shedding of MICA [92]. In one study, ULBP3 in tumor cell culture supernatants was associated with exosomes while ULBP2 was primarily in the cleaved form [93]. Shedding of NKG2D ligands can result in lowered NK susceptibility of tumor cells due to lower surface expression of the ligands [90]. Although, some studies also report downmodulation of the NKG2D receptor in the presence of soluble ligands [85, 93], some others propose contrasting observations [89, 94, 95]. Taken together, although there is some understanding of how NKG2D ligands are regulated, a lot is still unknown. Increased knowledge of the differential expression and regulation of NKG2D ligands will contribute to our understanding of tumor immunosurveillance and help in therapeutic interventions that induce NKG2D mediated immune responses.

The NKG2D system has evolved to function as a sensory system to alert the immune system in response to infections and tumors. Initially, Bauer *et al* reported that target cells ectopically expressing NKG2D ligands are efficiently eliminated by NKG2D expressing immune cells [34, 96, 97]. These observations were subsequently made *in vivo* as well [26, 27]. A noteworthy observation of NKG2D in tumor immunity was

provided by NKG2D neutralization experimental models. When mice were injected with antibody to neutralize NKG2D, they showed an increased susceptibility to carcinogen methylcholanthrene (MCA) induced fibrosarcoma in comparison to the isotype control treated mice [98]. Furthermore, a critical role of NKG2D in tumor immunosurveillance *in vivo* was proposed by Guerra *et al.* They reported that NKG2D deficient mice when crossed to TRAMP mice that spontaneously develop prostrate adenocarcinoma showed a higher incidence of aggressively arising tumors in comparison to the control mice [99]. In addition to tumor surveillance, NKG2D has also been implicated in pathogen immunity [62].

The interesting promiscuous nature of NKG2D also deserves a mention. Most NK receptors bind to one or two proteins but NKG2D is a notable exception to this rule. Although the exact reason for ligand diversity for a conserved receptor isn't known, there are several speculative theories. One of the notions is that selection pressure by viruses has led to the diversity in NKG2D ligands. Infection of cells by viruses particularly HCMV, Influenza A and Epstein Barr virus has shown to induce NKG2D ligand expression [100-102]. However, HCMV deploy immunoevasins that prevent the surface expression of NKG2D ligands and help escape NK killing. Interestingly however, each immunoevasin only has limited capacity to bind NKG2D ligands. For example, the HCMV protein UL16 can intracellularly sequester MICB, ULBP1 and ULBP2 but not MICA and ULBP3 [103-105]. In this scenario, having multiple NKG2D ligands is therefore beneficial to the host to alert its immune system to viral infection. Competition with viruses, thus presents an attractive theory for the presence of NKG2D ligand diversity. Another theory is the differential binding affinities of various ligands to NKG2D. Theoretically, the expression of a lower affinity ligand would not invoke a powerful immune response and vice versa. However, these differences have not yet been shown to have a functional impact on NK responses [106]. Having so many ligands with varying binding affinities would allow for greater flexibility in the decision of the effector NK cells to activate or not, depending on the activation threshold set by signals from the engagement of other receptors. A third theory is that malignant transformation is a drive for NKG2D ligand diversity. From our observations and others, expression of different NKG2D ligands was seen to be under the control of different cancer related stress response pathways [107-109]. Possessing multiple NKG2D ligands that are regulated differently would thus provide the host with a relatively fail-safe mechanism for alerting the immune system.

Clinical Significance of NK Cells

Surgery, chemotherapy and ionizing radiation have been used for decades as primary treatment strategies to eliminate tumors in patients. However, the development of resistance to drugs or radiation has led to significant tumor relapse. Hence the past few decades have focused on therapies that harness the power of the immune system. Tumor immunotherapy using various therapeutic agents including cytokines, monoclonal antibodies, vaccines, adoptive cell transfers has become an attractive tool for the treatment of malignancies [110, 111]. Cellular therapy with NK cells is increasingly

gaining importance and till date a number of clinical trials have tapped the beneficial anti-cancer potential of NK cells.

Evidence of the anti-cancer effects of NK cells came from an 11 year follow up study that showed that patients with low NK cell cytotoxicity were associated with a greater risk of cancer [112]. Various other groups reported that high levels of tumor infiltrating NK cells are associated with favorable tumor outcome in cancer patients [113]. The greatest revelation of NK cells in tumor control was shown in the context of allogeneic hematopoietic stem cell transplantation (HSCT). In 2002, Ruggeri *et al* demonstrated a significant long term benefit and prevention of leukemia relapse for haploidentical mismatched transplant recipients suffering from acute myeloid leukemia (AML) [114]. T cell depleted donor grafts containing alloreactive NK cells improved the overall survival of patients in remission by mediating an enhanced graft versus leukemia (GVL) effect and significantly reducing graft versus host disease (GVHD). The decrease in GVHD was explained by donor NK cell alloreactivity against host antigen presenting cells (dendritic cells) that prevents presentation of host antigens to donor graft T cells [115, 116]. In addition, non-transformed tissues generally do not express ligands for NK activating receptors, hence alloreactive NK cells do not cause GVHD [117].

Outside the context of allogeneic HSCT, infusion of allogeneic NK cells has also been explored as a means of cancer therapy. Several protocols of clinical grade NK cells purification and expansion are now validated and studies have shown that the infusion of NK cells is safe in humans [118-124]. However, no firm conclusions can be made regarding the clinical benefits of these NK cell infusions as the clinical efficacy of these NK therapies has been modest [125]. Nevertheless available data indicate that NK infusions are safe and can generate anti-tumor responses and long term remission in some patients. NK cells also express CD16 on their surface which enables them to recognize antibody coated target cells and trigger NK mediated ADCC. Strong evidence supporting role for NK mediated ADCC comes from anti-CD20 (Rituximab) treated non-Hodgkin's lymphoma patients and anti-HER2 (Trastuzumab/Herceptin) treated metastatic breast cancer and gastric carcinoma patients [126-129]. Additionally, potential use of NK cell lines as a source of therapeutic allogeneic NK cells has also been reported. NK92 cells have been demonstrated to be safe and with potentially beneficial anti-tumor effects. It has received FDA approval for testing in patients with advanced malignant melanoma and renal cell carcinoma and is currently the only NK cell line that has entered clinical trials [130-133].

In addition, genetically modified NK cells expressing chimeric antigen receptors (CARs) has also been reported by various groups. Successful expression of CARs against CEA, CD33, CD20, and Her2/neu has been shown in the context of NK cell lines where they improved NK responses *in vitro* and *in vivo* [134-137]. Similar observations have been reported with primary NK cells as well [138, 139]. Imai *et al* successfully demonstrated that NK cells from B-ALL patients genetically modified to express CD19-CAR efficiently killed autologous leukemic cells [140].

A proliferation of studies is currently exploiting the immunotherapeutic potential of NK cells for cancer treatment. Although the results of these NK based immunotherapeutic strategies to date are promising in experimental systems, their clinical efficacy in humans has been modest, presumably due to tumor escape mechanisms. With a greater understanding of NK cell recognition of target cells, treatment strategies are now being focused on modulation of NK cell receptors systems. NKG2D, being a major activating receptor on NK cells has been studied in the clinical setting as well. The importance of NKG2D has come from correlative observations of impaired NKG2D function with disease stages in cancer patients. For example, Wu *et al* reported that defective NKG2D mediated NK function correlated with cancer stages in prostate cancer patients [141]. Similar observations were made in multiple myeloma, colon cancer and glioma patients as well [88, 142, 143]. In a clinical trial involving vaccination with irradiated, granulocyte-macrophage colony stimulating factor (GM-CSF)-secreting tumor cells and antibody blockade of cytotoxic T lymphocyte-associated antigen 4 (anti CTLA-4) for melanoma therapy, patients who generated anti-MICA antibodies during the therapy showed significantly better clinical outcomes than those who did not [144]. The beneficial effect was shown to act through antibody antagonizing soluble MICA induced suppression of NK and CD8 T cell responses.

In addition, a new and interesting mechanism to utilize the NKG2D mediated immunity in tumor therapy has been reported. This strategy involves expression of NKG2D-CAR on T cells or NK cells for adoptive cell therapy. The chimeric NKG2D is not downregulated by soluble NKG2D ligands and can also overcome inhibitory signaling. This approach has been demonstrated to be very effective in various experimental models [145-149]. NKG2D-CAR engineered T cells are currently in phase I clinical trials for the treatment of ovarian cancer patients. Recently, Chang *et al* reported the expression of NKG2D-CAR on activated NK cells and demonstrated their anti-cancer potential *in vitro* and *in vivo* [150]. A few therapies utilizing bispecific protein to target NKG2D or NKG2D ligands have also shown therapeutic potential. A ULBP2-BB4 bispecific protein increased susceptibility of multiple myeloma cell lines to NK mediated cytotoxicity and enhanced the elimination of a xenograft tumor when combined with adoptive transfer of PBMCs [151]. It was shown that ULBP2 engaged the NKG2D receptor while BB4 bound CD138, which is overexpressed on multiple myeloma cells.

Collectively, NK cells as tools for immunotherapy in cancer have shown promise in the clinic. However, the clinical efficacy of these NK based therapies can be further augmented by modulating receptor pathways such as NKG2D. The ability to manipulate not only the balance of activating and inhibitory receptors on NK cells but also their cognate ligands and the NK sensitivity of tumor cells, opens new perspectives in NK cell based immunotherapy. Thus, further benchside research focused on understanding mechanisms of NKG2D mediated NK resistance is needed to get a clearer picture of the clinical significance of this pathway that can eventually help in designing more effective NK therapies.

CHAPTER 2. HYPOTHESES AND AIMS

Problem at Hand

NK transplantation is being increasingly used for the treatment of chemo-refractory cancers. Although various clinical trials have evaluated the safety and efficacy of NK based cancer therapies, the results were conflicting. While the safety of NK therapies has been firmly established, the clinical effectiveness has been modest. One of the reasons for the inconsistent clinical usefulness of NK therapies might be variable NK evasion strategies employed by tumor cells. The success of NK therapies relies heavily on the NK susceptibility of cancer cells. However, till date the prevalence and mechanisms of NK resistance has not been well elucidated. Therefore a better understanding of the predominance and mechanisms of NK resistance is needed for designing more effective NK therapies in the future.

Central Hypothesis

NKG2D is a major activating receptor on NK cells. Hence, in our study we focused on this receptor pathway and examined the NK resistance mechanisms employed by tumor cells. We hypothesized that NKG2D is the primary pathway for tumor cell recognition; thereby, susceptible tumors activate NK cell through NKG2D, whereas resistant tumors evade NKG2D recognition.

Specific Aims

Aim 1- To determine the prevalence of NK cell susceptibility in cancer and the role of NKG2D. NK susceptibility was evaluated in a spectrum of cancer cell lines and the role of NKG2D in cancer was investigated by determining the surface expression of NKG2D ligands and their contribution to NK susceptibility. Furthermore, correlation studies were undertaken to test the hypothesis that NK susceptibility directly correlated with the level of NKG2D ligands expressed by tumor cells.

Aim 2- To identify the molecular mechanisms of NK resistance, if any. The two hypothetical mechanisms included a) the absence of NKG2D activation, or b) the presence of NKG2D activation but overridden by inhibitory signals.

CHAPTER 3. MATERIALS AND METHODS

Cancer Cell Lines

K562, U937, SEM, RS4;11, RPMI 6666, Hs 445, Daudi, HCT116, HeLa, CAL 27, RD and 293T cells were obtained from American Type Culture Collection (ATCC). Jurkat, MOLT-4, HDLM-2, L-428, L-540, L-591 and MV4-11 were purchased from the German Resource Center for Biological Material (DSMZ, Braunschweig, Germany). The cell lines NB-1691 (from Dr. Peter Houghton, Center for Childhood Cancer, Nationwide Children's Hospital Columbus, OH) and CHLA-255 (from Dr. C. Patrick Reynolds, Children's Hospital of Los Angeles, Los Angeles, CA) were established from patients with different metastatic sites, stages and N-myc amplification. NKL cell line was a kind gift from Dr. Francisco Borrego, NIAID/NIH, Rockville, MD. YT-Indy cell line was a generous gift from Dr. Zacharie Brahmi, Indiana University, Indianapolis, IN. All cell lines were cultured as recommended and passaged with no more than 3 months. Routine authentication was conducted by typical morphology observation.

Cell Purification

Peripheral blood mononuclear cells (PBMC) were collected from healthy donors and from pediatric leukemia patients with approval from Institutional Review Board and written informed consent from donors, patients, or legal guardians. Briefly, the specimens were layered over Ficoll-Hypaque gradient (GE Healthcare, Piscataway, NJ) and centrifuged at 2,300 rpm for 20 minutes. The light-density fraction was collected, washed, and used immediately. NK cells were enriched from healthy donor PBMCs using an NK Cell Isolation Kit (Miltenyi, Germany) according to the manufacturer's instructions. The purity of the NK cells was more than 95% in all experiments as determined by flow cytometry. Enriched NK cells were activated overnight with 10U/mL of recombinant interleukin-2 (R&D Systems, Minneapolis, MN) in all experiments unless indicated otherwise.

Phenotypic Analysis by Flow Cytometry

The following antibodies (from commercial suppliers) were used for phenotypic analysis: phycoerythrin (PE)-conjugated anti-MICA (159227) and anti-ULBP2 (165903); fluorescein isothiocyanate (FITC)-conjugated anti-ULBP1 (170818) and anti-ICAM-1 (84H10); allophycocyanin (APC)-conjugated anti-CD56 [N901(NKH-1)] and anti-MICB (236511); Alexa fluor 488-conjugated anti-PI-9 (7D8); phycoerythrin-Texas red-conjugated anti-CD3 (UCHT1) and unconjugated anti-ULBP3 (166510). Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Flow cytometry analyses were conducted with LSRII or Accuri C6 flow cytometers (both from BD Biosciences) and the data were analyzed with FlowJo V10 (Tree Star, Ashland, OR).

Retroviral Constructs and Transduction of Cells

Full length MICA*001 was amplified by PCR from HCT116 cell line and cloned into pMSCV-IRES-GFP retroviral vector (obtained from vector lab at St.Jude). Retroviral supernatants were generated by co-transfecting 293T cells with plasmids encoding VSV gag/pol and env and pMSCV retroviral constructs using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. After 48 hours of transfection, viral supernatant was harvested and used for transducing RS4;11, Daudi and NB-1691 cell lines by spinoculation. Virus supernatants were mixed with cell pellets and centrifuged at 3900 rpm for 90 mins at 32°C in the presence of 8 ug/mL polybrene (Sigma, St.Louis, MO). Stable cell lines were established by sorting for GFP and MICA double positive cells by flow cytometry.

NK Cytotoxicity Assay by BATDA

Target cells, including those from cell lines and primary patient samples, were labeled with DELFIA BATDA reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA) according to the manufacturer's instructions. After labeling, the cells were washed and then co-cultured with effector cells at 20:1 ratio (effector to target) for 2 hours at 37°C. Cell supernatants were then mixed with DELFIA Europium solution, and the fluorescence was measured using a Wallac Victor 2 Counter Plate Reader (both from Perkin Elmer Life and Analytical Sciences). Specific lysis was calculated by a formula: (experimental lysis count - spontaneous lysis count) / (maximum lysis count [by lysis buffer] - spontaneous lysis count) times 100%. In HLA inhibition experiments, target cells were coated with 10 ug/mL anti-pan-major histocompatibility complex (MHC; LEAF clone W6/32, BioLegend, San Diego, CA) antibody for 30 minutes at 37°C prior to co-culture with the effector cells. For NKG2D blocking assays, primary NK cells were coated with 20 ug/ml anti-NKG2D blocking antibody (clone 1D11, BD Biosciences) for 30 minutes at 37°C prior to co-culture with the target cells.

Drug Treatments

Cell lines were treated with various concentrations of histone deacetylase inhibitor sodium valproate (Depacon, Hikma Farmaceutica, Portugal). MICA expression was observed 2 days after sodium valproate treatment for all cell lines except NB-1691 and CHLA-255 that were analyzed 4 days post treatment. For treatment with proteasome inhibitor Bortezomib (Velcade, Millenium Pharmaceuticals, Cambridge, MA), various concentrations were used and MICA was analyzed 16 hours after treatment. The optimum concentration for both drugs was determined by analysis of cell viability using trypan blue staining.

Quantification of MICA Transcripts by Real-Time PCR

Total RNA was isolated from cell lines before and after sodium valproate treatment using the RNeasy Plus kit (Qiagen, Valencia, CA). Two µg of total RNA was reverse transcribed using the cDNA synthesis Vilo kit (Invitrogen) according to the manufacturer's protocol. Real-time PCR analysis was performed using ABI 7900HT sequence detection and a Fast SYBR Green PCR master mix system (Applied Biosystems, Foster City, CA) according to the manufacturer's specification. Samples (50 ng of cDNA per well) were performed in duplicate in 20µl reactions with 200nM primers. The real-time PCR cycling conditions were 95°C for 20 sec; 40 cycles at 95°C for 1 sec and 60°C for 20 sec; and then extension at 72°C for 3 mins followed by a dissociation phase. GAPDH was used as an internal control. Primers used were MICA forward 5'-ACA ATG CCC CAG TCC TCC AGA-3', MICA reverse 5'-ATT TTA GAT ATC GCC GTA GTT CCT-3', GAPDH forward 5'-GCC ATC AAT GAC CCC TTC ATT-3' and GAPDH reverse 5'-TTG ACG GTG CCA TGG AAT TT-3'. MICA mRNA expression was normalized with that of GAPDH and compared to untreated cells.

CD107a Degranulation Assay

NK cells pretreated overnight with 10 U/mL of IL-2 were cocultured with target cells at a ratio of 1:2 in the presence of FITC-conjugated anti-CD107a antibody (H4A3, BD Biosciences). Monensin (BD Biosciences) was added to the cells 1 hour after coculture. After 4 hours, the cells were harvested and stained with anti-CD3 and anti-CD56 antibodies to determine the percentage of CD107a⁺ NK cells by flow cytometry. Anti-NKG2D and anti-HLA-ABC antibodies were also used in some experiments.

ELISA

For detection of soluble MICA (sMICA), plates were coated overnight with capture anti-MICA monoclonal antibody AMO1 (BamOmaB, Germany) at 2 µg/ml in PBS, blocked with 3% BSA for 2 hours at 37°C and washed with buffer consisting of 1X TBS + 0.1% Tween 20 (TBST). Afterward 100 uL of standard (recombinant human MICA-Fc chimera, R&D Systems) or samples (72 hour cell culture supernatants) diluted in 3% BSA + 0.1% TBST were added to the plates and incubated for 2 hours at room temperature. After incubation, plates were washed and the detection antibody BAMO3 (BamOmaB) at 1 µg/ml in 3% BSA + 0.1% TBST was added for 2 hours at 37°C. Plates were then washed and anti-mouse IgG2a-HRP (SouthernBiotech, Birmingham, AL) diluted 1:8000 in 3% BSA + 0.1% TBST was added for 1 hour at room temperature. Plates were then washed and developed using the Tetramethylbenzidine Peroxidase Substrate System (eBioscience, San Diego, CA). The absorbance was measured at 405 nM using a Wallac Victor 2 Counter Plate Reader.

Immunoprecipitation and Western Blotting

MICA immunoprecipitation was carried out using the Pierce Classic IP kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Lysates were prepared by re-suspending cell pellets in IP lysis buffer (25mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% NP-40, 5% glycerol, pH 7.4) with protease inhibitor (Sigma). Cells were lysed at 4°C for 5 min and centrifuged at $13,000 \times g$ for 10 min. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. Cell lysates containing 800ug of protein were pre-cleared using the control agarose resin. Pre-cleared lysates were incubated with 6µg anti-MICA/B antibody BAMO3 (MBL International Corporation, Woburn, MA) or 6ug mouse IgG2a isotype control (Abcam, Cambridge, MA) at 4°C overnight. Immune complexes were captured using Protein A/G Plus Agarose, washed with IP lysis buffer and eluted in sodium dodecylsulfate gel electrophoresis (SDS-PAGE) sample buffer. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were kept in blocking buffer (LI-COR, Lincoln, NE) for 1 hour at room temperature, incubated with goat polyclonal anti-human MICA antibody (AF1300) overnight at 4°C followed by IRDye 680LT conjugated donkey anti-goat IgG (LI-COR). Following incubation, membranes were washed and scanned at 700 nm with the Odyssey infrared imaging system (LI-COR).

Immunofluorescence Confocal Microscopy

Cells were fixed in 3.7% paraformaldehyde in PBS for 15 mins and permeabilized in 0.1% Triton X-100 for 10 mins. After blocking in PBS with 3% BSA and 0.02% Triton X-100, cells were incubated with MICA antibody AMO1 (BamOmaB) diluted 1:100 in PBS with 3% BSA for 1 hour. Unbound primary antibody was washed out by rinsing three times in PBS. Bound antibody was detected with AlexaFluor 594 goat anti-mouse IgG (Molecular Probes, Grand Island, NY) diluted 1:200 in PBS with 3% BSA. After unbound secondary antibody was washed out by rinsing three times in PBS, cells were mounted on glass slides using Prolong Gold Antifade reagent with DAPI (Molecular Probes). For co-localization experiments, fixed and permeabilized cells were incubated with organelle markers diluted in blocking buffer for 1 hour after MICA staining. FITC labeled antibodies to EEA1 (diluted 1:100), GM130 (diluted 1:10) and LAMP1 (diluted 1:10) were obtained from BD Biosciences and unconjugated anti-calnexin antibody (diluted 1:200) was obtained from Abcam. Bound calnexin antibody was detected by FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). All procedures were performed at room temperature. Images were acquired on a Zeiss 510 Meta multi-photon confocal microscope (Zeiss, Oberkochen, Germany). Analysis was performed using LSM510 META software (Zeiss).

Statistical Analysis

In all experiments, statistical significance was calculated using student's t-test. For cluster plot analysis, mean ligand expression score and mean lysis were calculated as below:

Mean ligand expression score

- Expression = Ligand mean fluorescence intensity (MFI) - background MFI
- Expression mean = natural log (Mean (expression in experiment 1+ expression in experiment 2+ expression in experiment 3) +1)
- Expression mean weight = Expression mean X weight* for each ligand
- Mean ligand expression score = sum of Expression mean weight within a cell line

*Weight was calculated as spearman correlation coefficients between each ligand expression and corresponding percentage of cell lysis (**Table 3-1**).

Mean lysis

$$\text{Mean lysis} = \text{natural log} (\text{mean (lysis at experiment 1) + (lysis at experiment 2) + (lysis at experiment 3) +1})$$

After determining the mean ligand expression score and mean lysis, K-means clustering method was applied to the data from 20 cancer cell lines. In this algorithm, “n” observations are partitioned into “k” clusters in which each observation belongs to the cluster with the nearest mean. Mathematically, analysis of x variables presents 2^x possibilities. Since 2 variables were analyzed, “k” was fixed at 4 in our cluster analysis. The 4 phenotypes hypothesized are shown in **Table 3-2**.

Table 3-1. Weight for each NKG2D ligand

NKG2D ligand	Weight
MICA	0.2616643
MICB	0.5038257
ULBP1	0.5758988
ULBP2	0.4181352
ULBP3	0.1212418

Table 3-2. Four possible phenotypes in our cancer cell spectrum

Group	Level of NKG2D ligand expression	NK susceptibility
1	High	High
2	Low	Low
3	High	Low
4	Low	High

CHAPTER 4. RESULTS

Heterogeneity in NK Susceptibility and NKG2D Ligand Expression in Cancer Cell Lines

We first sought to determine the susceptibility of cancer cell lines to NK cell lysis. Resting primary NK cells from 3 healthy donors were co-cultured with different target cancer cells in an effector:target ratio of 20:1. A clear heterogeneity was observed in the cancer cell susceptibility to NK cells. Only one third of the cancer cell lines were susceptible to NK killing (defined as specific lysis > 10%, **Figure 4-1A**). Leukemic cell lines U937, K562, and Jurkat had a high percentage of specific lysis while another leukemia cell line MOLT-4, lymphoma cell line L-428 and colon carcinoma cell line HCT116 were moderately NK sensitive. Two thirds of the cancer cell lines exhibited resistance to NK killing with less than 5% specific lysis.

We determined the surface expression of NKG2D ligands MICA, MICB, ULBP1, ULBP2 and ULBP3 on the cancer cell lines. By flow cytometry we quantified surface expression in terms of mean fluorescence intensity (MFI). The ligand expression was found to be heterogeneous in our cancer cell line spectrum (**Figure 4-1B, C, D, E and F**). For MICA, high levels of expression were observed on HeLa, RD, L-428 and K562 and minimal levels on the other 16 cell lines (**Figure 4-1B**). For MICB, high level of expression was observed on K562 and low levels on Jurkat, HeLa and MOLT-4 cell lines (**Figure 4-1C**). For ULBP1, high expression was observed on MV4-11 and low levels on U937, Jurkat, K562, HCT116 and HDLM-2 (**Figure 4-1D**). For ULBP2, high expression was seen on HeLa, Jurkat, and CAL 27, and low levels on HDLM-2, HCT116, L-428, K562, U937 and MOLT-4 (**Figure 4-1E**). Lastly, for ULBP3, very high expression was observed on L-428, moderate expression on RD, HDLM-2, HCT116, and CAL 27, and low levels on K562, HeLa, L-540, MV4-11 and Daudi (**Figure 4-1F**). Collectively, the top 5 cell lines that had the highest cumulative levels of the 5 NKG2D ligands were K562, HeLa, Jurkat, L-428 and HCT116.

Level of NKG2D Ligand Expression Usually (but Not Always) Correlates with NK Susceptibility

In order to determine whether NKG2D ligand expression is directly proportional to NK susceptibility of tumor cells, the correlation between the 2 parameters was analyzed by K-means clustering algorithm and Wilcoxon rank-sum tests. Cluster analysis revealed 3 phenotypic clusters (**Figure 4-2**). Group 1 phenotypic cluster included U937, K562, Jurkat, MOLT-4, HCT116 and L-428. Compatible with our hypothesis that the higher the level of NKG2D ligand expression, the greater the susceptibility to NK cells, these group 1 cancer cell lines had abundant NKG2D ligand expression and high susceptibility to NK cells. Also in line with the hypothesis are the group 2 cancer cells with low NKG2D ligand expression and low NK susceptibility. This group included the

Figure 4-1. Heterogeneity in NK susceptibility and NKG2D ligand expression of cancer cell lines.

(A) Percentage lysis of 20 cancer cell lines by resting primary NK cells co-cultured for 2 hr at E:T ratio of 20:1. Data represents mean lysis $\pm$ SEM from 3 independent experiments. Surface expression of (B) MICA, (C) MICB, (D) ULBP1, (E) ULBP2 and (F) ULBP3 on 20 cancer cell lines was determined by flow cytometry and expressed as mean fluorescence intensity (MFI). Data represents mean $\pm$ SEM of from 3 independent experiments.

Figure 4-2. Level of NKG2D ligand expression usually (but not always) correlates with NK susceptibility.

(A) Cluster plot of four distinct phenotypes in the cancer cell spectrum. Level of NKG2D ligand expression represents mean ligand expression score as stated in materials and methods. Level of NK susceptibility denotes mean lysis. (B) Mean ligand expression score for groups 1, 2 and 3. (C) Mean percentage of lysis for groups 1, 2 and 3. Bars represent median values. Data are the mean $\pm$ SEM from 3 independent experiments. **, $p < 0.01$, *** $p < 0.001$ as compared with group 1.

cell lines NB-1691, CHLA-255, RS4;11, SEM, L-540, L-591, Hs 445, RPMI 6666 and Daudi. In contrast to the above 2 groups, group 3 cell lines had an unexpected phenotype of low NK sensitivity despite a high NKG2D ligand expression. Group 3 included HeLa, MV4-11, RD, CAL 27 and HDLM-2. Notably, none of the cell lines in our cohort exhibited a group 4 phenotype i.e. low NKG2D ligand expression and high NK susceptibility, supporting our primary hypothesis that NKG2D ligand expression is essential (though at times insufficient) for NK cell recognition.

NK Sensitivity of Group 1 Cells Involves the NKG2D Pathway

To validate that the high NK susceptibility of group 1 cells was due to the presence of NKG2D ligands on their surface, we performed NK cytotoxicity assay in the presence of NKG2D blocking antibody. Primary resting NK cells were incubated with BATDA labeled group 1 cell lines as targets in an effector: target ratio of 20:1 in the presence or absence of NKG2D blocking antibody. We observed a significant reduction in the NK lysis of group 1 cancer cells when the NKG2D receptor was blocked on NK cells (**Figure 4-3**). More than 50% loss of specific lysis was seen in the presence of the NKG2D blocking antibody, suggesting that the NKG2D pathway contributed significantly to the NK sensitivity of the group 1 cancer cells.

Pediatric Leukemia Patients Show a Group 2 rather than Group 1 Phenotype

Since NK transplantation is commonly used for the treatment of childhood leukemia, we hypothesized that pediatric leukemia exhibit a group 1 phenotype. Surprisingly, negligible levels of surface NKG2D ligands were observed on 8 patient samples when compared to K562 (**Figure 4-4**). One of the patient samples (represented as ▲) showed low level of surface expression of MICA, ULBP1, ULBP2 and ULBP3 with low % lysis. Another patient sample (represented as ○) expressed high level of ULBP3 on the cell surface with NK lysis almost comparable to that of K562. As expected, the 8 leukemia samples that did not express NKG2D ligands on their cell surface exhibited NK resistance. Taken together, the majority (90%) of pediatric leukemia patients exhibited a group 2 phenotype with NK resistance and absence of NKG2D ligands, and only 1 patient (10%) showed a group 1 phenotype (high NK susceptibility and NKG2D ligand expression).

Ectopic Expression of MICA Overcomes NK Resistance

In order to test our hypothesis that the absence of NKG2D ligand expression was solely responsible for NK resistance in the group 2 cells, we ectopically expressed MICA on MLL cell line RS4;11, lymphoma cell line Daudi and neuroblastoma cell line NB-1691. We created stable MICA expressing lines via retrovirus transduction and confirmed surface expression of MICA on the three cell lines 72 hours post transduction by flow with the cytometry (**Figure 4-5A**). Using these cells lines as targets, we performed NK

Figure 4-3. NK cytotoxicity of group 1 cells involves the NKG2D pathway. Mean percentage specific lysis of group 1 cell lines by primary resting NK cells in the absence (□) or presence (■) of anti-NKG2D blocking antibody. Effector and targets were cocultured at 20:1 ratio and lysis was evaluated by BATDA assay. Data are mean % specific lysis $\pm$ SEM from 3 independent experiments. *, $p < 0.05$ when compared to untreated cells.

Figure 4-4. NKG2D ligand expression and NK susceptibility of leukemia patients. Surface expression of NKG2D ligands (A) MICA, (B) MICB, (C) ULBP1, (D) ULBP2 and (E) ULBP3 on K562 (●) and leukemia patient samples (■). Each (●) represents one experimental data point for K562. The outliers in patient samples are represented as (▲) and (○). Bars represent median mean fluorescence intensity of NKG2D ligands. (F) Mean percentage lysis of K562 and patient blasts by NKL cell line at E:T ratio 20:1. Bars represent median lysis. *** $p < 0.001$.

Figure 4-5. Ectopic expression of MICA overcomes NK resistance in group 2 cancer cells.

(A) Histogram of MICA surface expression on group 2 cancer cell lines RS4;11, Daudi and NB-1691 stably transduced with empty vector (black line) or full length MICA (red line). Isotope control was presented as shaded histograms. (B) Cytotoxicity of NKL cell line against MICA transduced cell lines (■) and empty vector transduced cell lines (○). Data represents mean % lysis $\pm$ SEM from 3 independent experiments. *, $p < 0.05$ compared to cell line transduced with empty vector.

cytotoxicity assays using the NKG2D dependent effector cell line NKL. NK cytotoxicity assays revealed a significantly higher lysis of the MICA expressing cell lines when compared to the vector alone controls (**Figure 4-5B**).

Low Levels of MICA Surface Expression in Group 2 Cancer Cells Were in Part due to Transcription Silencing

To understand the mechanism of low MICA surface expression in group 2 cancer cells, we determined the mRNA levels by real time PCR. We found significantly lower levels of MICA transcripts in the group 2 cell lines when compared to group 1 cell line K562 (**Figure 4-6**). Notably, the neuroblastoma cell lines NB-1691 and CHLA-255 had no detectable MICA mRNA. Among the group 2 cancer cell lines, lymphoma line L-591 had the highest levels of mRNA transcripts, compatible with the highest surface protein expression among this group of cell lines.

Histone Deacetylase Inhibitors Can Upregulate MICA mRNA and Surface Expression in Neuroblastoma Cell Lines

Epigenetic mechanisms like histone deacetylation have been implicated in repression of gene transcription [152]. Altered histone deacetylase (HDAC) activity has been identified in several cancers [153, 154]. Hence, we investigated whether histone deacetylation was responsible for the low levels of MICA mRNA in the group 2 cancer cell lines. We observed a significant upregulation in MICA mRNA in the neuroblastoma cell lines NB-1691 and CHLA-255 post treatment with histone deacetylase inhibitor (HDACi) sodium valproate (**Figure 4-7A**). Flow cytometry showed a corresponding increase in MICA expression on the neuroblastoma cell lines (**Figure 4-7B**). To investigate NK susceptibility of the HDACi treated cells, we performed NK cytotoxicity assay on NB-1691 cells four days post treatment with 5mM sodium valproate. HDACi treated NB-1691 cells showed significantly higher NK lysis as compared to the untreated cells. The increase in NK susceptibility was completely abolished in the presence of anti-NKG2D blocking antibody (**Figure 4-7C**).

Histone Deacetylase Inhibitor Treatment Only Marginally Upregulated MICA mRNA and Surface Protein in the Leukemia and Lymphoma Group 2 Cancer Cells

For the resistant leukemia and lymphoma cell lines in our group 2 cluster, we determined if HDAC inhibitor treatment could upregulate MICA expression as in neuroblastoma. We observed only a marginal increase in the transcript levels in most of the cell lines, except Hs 445 and Daudi, 48 hours after sodium valproate treatment (**Figure 4-8A**). In accordance with the mRNA increase, only Daudi and Hs 445 showed slight increase in surface MICA expression post HDAC inhibitor treatment while the remaining group 2 lines showed negligible increase (**Figure 4-8B**). This marginal

Figure 4-6. Low level of MICA transcripts in group 2 cancer cells.

MICA mRNA expression in group 2 cancer cells (■) normalized to GAPDH and relative to K562 (□, taken as 1). Data are mean $\pm$ SEM from 3 independent experiments.

Figure 4-7. HDAC inhibitor treatment upregulates MICA mRNA and protein levels on neuroblastoma cell lines and increases their susceptibility to NK cells. (A) Relative MICA mRNA levels in untreated (white) and 5mM treated NB-1691 (black) or 2.5mM treated CHLA-255 (black) four days after treatment. Data are mean mRNA expression $\pm$ SEM in treated cells compared to untreated cells from 3 independent experiments. (B) Histograms of surface MICA on untreated (black) and sodium valproate treated (red) neuroblastoma cells. Isotype control was presented as shaded histograms. Data are representative of 4 experiments. (C) Cytotoxicity of primary NK cells against untreated (white), sodium valproate treated (black) NB-1691 cells and sodium valproate treated NB-1691 cells in the presence of anti-NKG2D blocking antibody (grey). Data are mean % lysis $\pm$ SEM from 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$.

Figure 4-8. Effect of HDAC inhibitor treatment on MICA mRNA and protein levels in the remaining group 2 cancer cells.

(A) Relative MICA mRNA levels in untreated (white) and sodium valproate treated (black) group 2 cancer cells. Data are mean MICA mRNA $\pm$ SEM in the treated cells compared to untreated cells from 3 independent experiments. (B) Histograms of MICA surface expression on untreated cells (black line) and sodium valproate treated cells (red line) 48 hours after treatment. Isotype control was presented as shaded histogram. Data are representative of 4 independent experiments. (C) Cytotoxicity of primary NK cells against untreated (white), sodium valproate treated (black) and sodium valproate treated group 2 cells in the presence of anti-NKG2D blocking antibody (grey). Data are mean % lysis $\pm$ SEM from 3 independent experiments. *, $p < 0.05$.

A

B

C

upregulation in MICA was mirrored by a marginal upregulation in the NK lysis of the group 2 cancer cells (**Figure 4-8C**).

Group 2 Cells Express Low Levels of Intracellular MICA

To determine whether the low mRNA quantity in group 2 leukemia and lymphoma lines corresponded with low levels of intracellular MICA protein, we determined the amount of intracellular MICA using confocal microscopy (**Figure 4-9**). As expected, the level of intracellular MICA protein was much lower in the group 2 cells than in the group 1 cell line K562. In K562, relatively large amount of surface and intracellular MICA could be visualized.

Shedding of MICA Is Not Responsible for the Absence of Surface MICA in Group 2 Cancer Cell Lines

Having observed low amount of intracellular and surface MICA in our group 2 cluster, we investigated whether shedding of NKG2D ligands collaborated with transcription silencing [85, 93]. In all group 2 cell lines, we could not detect soluble MICA in the culture supernatants by ELISA (**Figure 4-10**). Collectively, these results indicate that shedding of MICA did not contribute to the low surface levels in our group 2 cancer cell lines, but rather the mechanism is related to transcription repression.

The Small Amount of MICA in Group 2 Cells Is Retained in the Endoplasmic Reticulum

With high resolution confocal microscopy we next investigated the subcellular localization of MICA with markers for endoplasmic reticulum (calnexin), golgi complex (GM130), lysosomes (LAMP1) and endosomes (EEA1). Co-localization of MICA (shown in red) with the ER marker calnexin (shown in green) was observed (yellow staining) in all group 2 cell lines including RS4;11, Hs 445 and RPMI 6666. In addition, co-localization of MICA with calnexin was also observed in an AML patient sample that was negative for cell surface expression of MICA (**Figure 4-11A**). Clear points of co-localization (indicated by white arrows) were observed in a single cell image of RS4;11 (**Figure 4-11B**). Confocal microscopy in RS4;11 cells with other subcellular organelle markers revealed no significant co-localization of MICA with the golgi complex marker GM130 (**Figure 4-11C**), early lysosome marker LAMP-1 (**Figure 4-11D**) and early endosome marker EEA1 (**Figure 4-11E**).

Treatment with Proteasome Inhibitor Increases Surface Expression of MICA

With knowledge of MICA being retained in the ER, we determined whether their surface expression could be restored using proteasome inhibitor bortezomib. MICA

Figure 4-9. Presence of intracellular MICA protein in group 2 cells. Immunofluorescence images of differential interference contrast (DIC), DAPI (blue), MICA (red) and merge of the blue and red channels in group 1 cell line K562 and the indicated group 2 lines. Results are representative of 3 independent experiments.

Figure 4-10. MICA is not shed by group 2 cancer cell lines.

Level of soluble MICA in the supernatants of group 2 cell lines. Data represent mean soluble MICA (pg/mL) $\pm$ SEM from 2 independent experiments. Red line represents the background level from medium alone control. The detection limit of the ELISA was 50 pg/mL to 6250 pg/mL.

Figure 4-11. MICA is retained in the endoplasmic reticulum.

(A) Confocal microscopy images showing co-localization of MICA (red) with ER marker calnexin (green) in the indicated group 2 cell lines and in a patient sample. Results are representative of 2 independent experiments. (B) Single cell image RS4;11 cell showed MICA co-localization with calnexin. White arrows show points of colocalisation. Confocal microscopy images showing no significant co-localization of MICA with other compartment markers including (C) Golgi marker GM130; (D) lysosome marker LAMP1 and (E) early endosome marker EEA1 in RS4;11 cells.

Figure 4-11. (Continued).

immunoprecipitation revealed an increase in intracellular MICA in group 1 cell line HCT116 and group 2 cell lines L-591 and RS4;11, which in turn increased the surface expression of MICA (**Figures 4-12A and B**). The effect of bortezomib was independent of the background expression of MICA since it upregulated MICA in the group 1 HCT116 cells that show surface MICA and also on the group 2 cells that have low to negligible levels of surface MICA. We further investigated whether the increase in surface MICA could increase NK susceptibility. NK cytotoxicity assays revealed that L-591 cells had a significantly higher NK lysis post bortezomib treatment. To validate that the augmented susceptibility was due to the NKG2D pathway, we neutralized the receptor on NK cells using anti-NKG2D blocking antibody. When anti-NKG2D blocking antibody was applied, a significant reduction in NK susceptibility of L-591 cells was observed (**Figure 4-12C**).

NK Resistance of Group 3 Cell Lines Was Not due to PI-9 Overexpression

We then investigated the mechanisms of NK resistance in the group 3 cell lines despite high level of NKG2D ligand expression. It is well known that tumors can resist cytotoxic T lymphocyte mediated killing by expression of the serine protease inhibitor protease inhibitor 9 (PI-9) which inactivates granzyme B [155]. Hence, we considered whether overexpression of protease inhibitor 9 was a possible mechanism for NK resistance in our group 3 cell lines. The NK resistant group 3 cancer cells showed similar levels of PI-9 expression as the NK sensitive group 1 cells (**Figure 4-13**). Positive control YT-Indy NK cells showed high levels of intracellular PI-9 by flow cytometry. This indicated that overexpression of PI-9 was not a contributing factor to the NK resistance in the group 3 cells.

KIR Inhibition Overrides NKG2D Activation in Group 3 Cells

According to the rheostat concept, inhibitory signals overriding the activating signals can lead to NK resistance of tumors [24, 156]. To determine if this was indeed the mechanism of resistance in our group 3 cells, we examined the expression level of KIR inhibitory ligands HLA-ABC on our group 3 cell lines. All cancer cell lines showed high level of KIR inhibitory ligands on the cell surface (**Figure 4-14A**). When we performed NK cytotoxicity assays with primary NK cells against the group 3 lines in the absence or presence of anti-HLA-ABC blocking antibody, blockade of the inhibitory signals resulted in a significant increase in NK susceptibility of HeLa, HDLM-2, CAL 27, and MV4-11. When saturating amounts of the anti-NKG2D blocking antibody was applied, the augmented NK sensitivity was considerably reduced suggesting that the increased NK susceptibility was due to the activating signals from the NKG2D pathway in K562 and HDLM-2 (**Figure 4-14B**). However, RD rhabdomyosarcoma cells did not show increased NK sensitivity in the presence of the anti-HLA antibody.

Figure 4-12. Treatment with proteasome inhibitor increases surface expression of MICA.

(A) MICA immunoprecipitation showing MICA levels in the absence (-) or presence (+) of bortezomib 16 hours after treatment in the indicated cell lines. Data are representative of 2 experiments. (B) Histograms of surface MICA expression in untreated cells (black line) and bortezomib treated cells (red line). Isotype control was presented as shaded histograms. Data are representative of 3 experiments. (C) NK susceptibility of untreated or 25nM bortezomib treated L-591 cells in the presence of isotype control (white) or anti-NKG2D antibody (black). Data are mean % lysis $\pm$ SEM from 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$.

Figure 4-13. No significant difference in PI-9 levels between group 1 and group 3 cell lines.

Bars represent mean PI-9 MFI in group 1 and group 3 cell lines. Data are mean MFI $\pm$ SEM from 3 independent experiments. Positive control is YT-Indy cells.

Figure 4-14. KIR inhibition overrides NKG2D activation in group 3 cells.

(A) Expression of inhibitory ligands HLA-ABC (black line) on the indicated group 3 cell lines. Isotype control was presented as shaded histogram. Data are representative of 2 independent experiments. (B) NK susceptibility of group 3 cells lines untreated (blue line), treated with anti- HLA-ABC blocking antibody (red line), treated with anti-HLA blocking antibody+anti-NKG2D blocking antibody (green line) to NK cells at E:T ratios 5:1, 10:1, 20:1. Data are mean % lysis $\pm$ SEM from 3 independent experiments.

*, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$.

RD Cells Cannot Degranulate NK Cells

Since blockade of KIR-HLA interaction did not augment NK sensitivity of RD cells, we determined whether RD cells might at least activate NK cells through NKG2D. We observed that the NK sensitive group 1 cell line K562 could trigger degranulation in 42% of NK cells while the resistant group 3 lines HDLM-2 and RD could trigger degranulation in less than 10% of NK cells. In the presence of anti-HLA-ABC blocking antibody, K562 did not show an increase on CD107a⁺ NK cells as it lacks MHC class I on its surface; however HDLM-2 showed a significant increase in CD107a⁺ NK cells. In contrast, RD cells showed no increase in NK degranulation. When saturating amounts of anti-NKG2D antibody was applied, the percentage of CD107a⁺ NK cells was significantly reduced in K562 and HDLM-2 cell lines' suggesting that NK activation was through the NKG2D pathway. No significant loss of NK degranulation was observed in RD cells (**Figure 4-15**). Taken together, these data suggested that the resistance of RD cells was not due to KIR-HLA inhibition, but inability to activate NKG2D despite high levels of NKG2D ligand expression.

NK Resistant RD Cells Expressed Low Levels of ICAM-1

We then hypothesized that the NK resistance in RD cell line might be due to low levels of adhesion molecule ICAM-1, which is essential for immune-synapse formation. Flow cytometry revealed that ICAM-1 levels on the RD cells are indeed significantly lower than other group 3 cell lines (**Figure 4-16**).

NKG2D Pathway Can Be Modulated in Patient Samples

We then extended our investigation from cancer cell lines to fresh patient samples. We carried out analyses on two representative pediatric leukemia patient samples. Blasts from a patient with MLL rearranged leukemia (*patient 001*) had low NKG2D ligand expression on cell surface representing group 2 cells, while those from an AML patient (*patient 002*) had high NKG2D ligand expression with low NK susceptibility representing group 3 cells (**Figure 4-17A and E**).

Based on our previous findings on the NK resistance mechanisms employed by group 2 cell lines, we determined whether the NKG2D ligand expression on the patient *001* blasts could be modulated by treatment with HDACi sodium valproate and proteasome inhibitor bortezomib. Treatment of *patient 001* blasts with sodium valproate or bortezomib resulted in a marked increase in the surface MICA and ULBP2 ligands while MICB and ULBP1 showed no change in surface expression. MICA and ULBP2 showed a higher increase in the sodium valproate treated blasts when compared to the bortezomib treated blasts (**Figure 4-17A and B**). We observed a 2.1 fold increase in the percentage of degranulated (CD107⁺) NK cells post treatment with sodium valproate using the CD107 assay and a 1.4 fold increase in CD107⁺ NK cells post bortezomib treatment of the patient blasts (**Figure 4-17C top panel**).

Figure 4-15. RD cells cannot degranulate NK cells.

Percentage of CD107a⁺ NK cells in the presence of target cells alone (white), target cells coated with anti-HLA-ABC (black), target cells coated with anti-HLA-ABC and NK cells coated with anti-NKG2D (grey). Data are mean percentage of CD107a⁺ NK cells $\pm$ SEM from 2 independent experiments. *, $p < 0.05$.

Figure 4-16. Low ICAM-1 expression on RD cells.

Surface expression of ICAM-1 on group 3 cell lines. Data are mean MFI $\pm$ SEM from 2 independent experiments.

Figure 4-17. Modulation of NKG2D pathway in pediatric leukemia samples.
(A) Surface expression of the indicated NKG2D ligands on *patient 001* blasts before (black line) and after 48 hour treatment with 2.5mM sodium valproate (red line). **(B)** Surface expression of the indicated NKG2D ligands on untreated *patient 001* blasts (black line) and after 16 hour treatment with 10nM bortezomib (red line). Isotype controls were presented as shaded histograms. **(C)** NK degranulation in untreated and drug treated *patient 001* blasts in the absence or presence of anti-NKG2D antibody indicated as percentage of CD107 positive cells. **(D)** NK susceptibility of untreated or drug treated *patient 001* blasts at 20:1 E:T ratio in the absence (white) or presence (black) of anti-NKG2D antibody. **(E)** Surface NKG2D ligand expression on *patient 002* blasts. **(F)** Susceptibility of *patient 002* blasts to primary NK cells at 20:1 E:T ratio. Data are mean % lysis $\pm$ SEM from 3 different donors. *** $p < 0.001$.

D

E

F

Figure 4-17. (Continued).

The significant reduction in NK degranulation in the presence of blocking anti-NKG2D antibody validated that the increase in NK activation post drug treatment was due to the NKG2D pathway (**Figure 4-17C bottom panel**). Higher NK susceptibility of the sodium valproate treated and bortezomib treated blasts was observed when compared to the untreated cells. The bortezomib treated blasts were however killed to a lesser extent when compared to the HDACi treated blast cells (**Figure 4-17D**). The percentage of NK degranulation and susceptibility of *patient 001* blasts was in accordance with the level of increase in surface MICA and ULBP2 post treatment with HDACi and bortezomib.

Blasts from *patient 002* had a high NKG2D ligand expression on their surface (**Figure 4-17E**) and showed only 10% specific lysis, indicating a NK resistant group 3 phenotype. Based on our previous observations of KIR inhibition overriding NKG2D activation, we treated *patient 002* blasts with pan anti-HLA-ABC blocking antibody. In the presence of the HLA blocking antibody, a significant increase in the NK susceptibility of the blasts was observed. This increased NK lysis was also significantly diminished in the presence of the anti-NKG2D blocking antibody (**Figure 4-17F**).

CHAPTER 5. DISCUSSION

Despite intensive therapy and new chemotherapeutic agents, a high incidence of chemo-resistance is observed in hematological malignancies and solid tumors [157, 158]. Investigations in the past decade have focused on inventing better immune based therapies, given their non-cross resistance and less overlapping side effects with current therapies [159, 160]. NK cell transplantation is being increasingly used for treatment of chemo-resistant cancers, because it has the potential to provide anticancer effects without GVHD or organ toxicity [161]. However, NK resistance of cancers (if prevalent) would undermine the efficacy of NK transplantation. In this study we showed that 1) the majority of cancer cell lines and pediatric patient samples are resistant to NK cell lysis, 2) NK resistance is commonly due to lack of NKG2D activation or due to inhibitory signals overriding NKG2D activation, and 3) NKG2D pathway can be modulated to overcome NK resistance of cancer. In addition to demonstrating that NKG2D is the primary pathway in NK susceptible tumors, our comprehensive study also reinforces the key therapeutic paradigm that the NKG2D pathway can be manipulated to increase NK susceptibility of tumor cells thereby improving the outcomes of NK transplantation.

NK Resistance Is Predominant in Cancer

The occurrence of malignancies resulting via tumor escape may be due to defective NK cells or inherent features of tumor cells [162]. Elucidating these aspects regulating NK interaction with tumor cells is important to understand the role of NK cells in the control of cancer as well as their potential as a means of immunotherapy. Although various groups have investigated certain aspects of NK lysis of tumor cells [162-166], the prevalence and mechanism of NK resistance in cancer is still not well elucidated. In our study, we conducted a comprehensive analysis to determine the occurrence and frequency of NK resistance in cancer. Contrary to the above mentioned research groups that focused their study on a particular cancer type or limited number of cell lines, we analyzed NK susceptibility in a broad spectrum of 20 cancer cell lines representing various different cancer types including chronic myeloid leukemia (K562), T-cell acute lymphoblastic leukemia (MOLT-4, Jurkat), acute myeloid leukemia (U937), mixed lineage leukemia (SEM, RS4;11, MV4-11), Burkitt's lymphoma (Daudi), Hodgkin's lymphoma (L-540, L-591, Hs 445, RPMI 6666, L-428, HDLM-2), colon carcinoma (HCT116), rhabdomyosarcoma (RD), cervical carcinoma (HeLa), head and neck squamous carcinoma (CAL 27) and neuroblastoma (NB-1691, CHLA-255). Our results indicate a very high prevalence (70%) of NK resistance with majority of the cancer cell lines exhibiting less than 5% specific lysis. NK resistance was not associated with any particular cancer type. In fact, while some leukemia lines like K562, U937, Jurkat and MOLT-4 exhibited high NK susceptibility some others like MV4-11, SEM and RS4;11 showed NK resistance. Of the 6 lymphoma lines, only one cell line L-428 showed high NK lysis. Similarly, only one of 6 solid tumor cell lines (HCT116) was susceptible to NK killing. Collectively, NK resistance was commonly observed in both hematological malignancies and solid tumors.

Substantial Heterogeneity in NKG2D Ligand Expression and NK Susceptibility

We determined the surface expression of 5 NKG2D ligands on tumor cell lines and observed a clear heterogeneity in ligand expression. Our results show that MICB and ULBP1 were the least frequently expressed ligands (20-30%) while MICA, ULBP2 and ULBP3 were expressed on at least 40-50% of the cell lines. Our study while confirming the expression of NKG2D ligands on some standard cell lines [167-170] also demonstrates the pattern of NKG2D ligand expression on additional cell lines representing Hodgkin's lymphoma, rhabdomyosarcoma and neuroblastoma. Just as in the case of NK susceptibility, no specific pattern of ligand expression was observed within cell lines of a particular cancer type. For example, although both T-ALL cell lines, expressed NKG2D ligands on their surface, 4 of 5 ligands were expressed on Jurkat while MOLT-4 showed the presence of only 2. In the MLL rearranged leukemic cell lines, while MV4-11 showed the presence of surface NKG2D ligands, RS4;11 and SEM had negligible expression. Thus expression of NKG2D ligands was seen to vary among tumors of the same histotype. Collectively, we observed differences in the number of ligands expressed, type of ligands expressed and intensity of expression as quantified by MFI in our cancer cell lines. These factors could be responsible for the differences in their NK susceptibility.

In order to correlate NK susceptibility and NKG2D ligand expression, we analyzed data using the K-means clustering algorithm. Spearman correlation coefficients between each ligand expression and corresponding percentage of cell lysis showed no particular ligand to have significant positive correlation with NK susceptibility i.e. there was no selective advantage in the expression of a specific NKG2D ligand (**Table 3-1**). In fact, the diversity in the NKG2D ligand family clearly argues in favor of no particular ligand being more potent or favorable than the other in inducing NK susceptibility. Various research groups have clearly shown that the expression of any NKG2D ligand sensitizes tumor cells to NK killing and there are no known features that distinguish NKG2D ligands with respect to their function [34, 62].

Although NKG2D-mediated cytotoxicity of cancer cells is well known, the correlation between ligand expression and NK susceptibility has not been clearly established. The only paper addressing this was by Pende *et al* in 2002 where they reported that NKG2D mediated NK cytotoxicity strictly correlates with the expression levels and surface densities of its ligands on target cells [167]. However, their conclusions were based on qualitative analyses of ligand expression with NK cytotoxicity. For example, T cell lymphoma line H9 was characterized as ULBP2⁺ ULBP3⁺ and shown to have high NK susceptibility. In our study, we statistically analyzed the quantitative measure of NK lysis (in terms of % lysis) and NKG2D ligand expression (in terms of MFI) and performed a clustering algorithm to group our phenotypic observations. Cancer cells belonging to groups 1 and 2 showed that level of NKG2D ligand expression indeed correlates with NK susceptibility. However, our results also revealed a counterintuitive group 3 phenotype wherein cell lines exhibited NK resistance despite a high NKG2D ligand expression. This remarkable phenotype was seen in 25% of the cancer cell lines with no predominance of a particular cancer type.

Collectively, our results suggest that the level of NKG2D ligand expression usually (but not always) correlates with NK susceptibility of cancer.

Another striking observation was the absence of cancer cell lines representing a group 4 phenotype i.e. high NK susceptibility without the expression of NKG2D ligands. This was in accordance to our hypothesis that NKG2D is the primary pathway for tumor cell recognition. Because NKG2D-independent NK killing was not observed in our cancer cell spectrum, our data clearly indicated the importance of this pathway in cancer. Although NCR-dependent killing has been reported in various NKG2D ligand negative B-cell leukemic lines and neuroblastoma lines [157, 167, 171], it does not preclude the role of the NKG2D pathway in these cancers, provided these cell lines could also express NKG2D ligands with cell stress during transformation.

Furthermore, significant reduction in the lysis of the group 1 cancer cells by antibody blockade of NKG2D validated a substantial role of this pathway in NK susceptibility. Almost 80% reduction in NK lysis was observed in most of the group 1 cells, clearly indicating that NKG2D was the primary pathway responsible for NK sensitivity of these cells. The observation of approximately 20-30 % lysis in the presence of the NKG2D antibody, however, suggests either incomplete blockade by the antibody or that other activating signals may simultaneously contribute to the NK susceptibility of these lines. Synergistic activating signals may be provided by NCRs or co receptors like DNAM-1 and 2B4 [172-174].

Lack of NKG2D Activation Is Commonly Responsible for NK Resistance

Analysis of blasts from patient samples showed that the majority of the patient samples exhibit the NK resistant group 2 phenotype. Only one patient exhibited the group 1 phenotype with surface ULBP3 expression resulting in NK susceptibility. With strong evidence that cancer patients predominantly exhibit a group 2 phenotype, we created stable MICA expressing cell lines to determine whether the absence of NKG2D ligand expression was solely responsible for the NK resistance of our group 2 cancer cells. Our results indicate that transduction-mediated expression of MICA could increase the NK susceptibility of these resistant cell lines. This effect was not specific to a particular cancer type as similar observations were made in cell lines representing leukemia, lymphoma and neuroblastoma (**Figure 4-5**). Moreover, the dependency on the NKG2D pathway was validated by using the NKL cell line as effectors. NKL expresses high levels of NKG2D, but only minimal levels of NCRs and is therefore well suited to study NKG2D-mediated recognition [34].

In light of the above observations we focused our understanding on mechanisms employed by group 2 cancer cells to prevent surface expression of NKG2D ligands and designing strategies to overcome the repression of expression. Our results show that the majority of group 2 cancer cells express MICA mRNA. However, comparison of their mRNA levels with that of the group 1 cell line K562 revealed considerably lower level of MICA transcripts in all group 2 cell lines. In particular, no detectable mRNA was seen in

the neuroblastoma lines. These results indicated transcription repression of MICA in the group 2 cancer cells. To further probe into the mechanisms of gene silencing, we determined whether histone acetylation was responsible for the low mRNA levels. Chromatin remodeling through histone acetylation has been implicated in the transcriptional control of genes [175]. Valproic acid has been shown to relieve HDAC-dependent transcriptional repression by causing hyperacetylation of histones in cultured cells and *in vivo* [176]. Our findings clearly demonstrate that HDAC inhibitor sodium valproate induced MICA mRNA and protein in the group 2 cancer cells albeit at variable levels.

The neuroblastoma lines including NB-1691 and CHLA-255 showed a marked increase in MICA mRNA and protein after treatment with HDACi sodium valproate. Expression of surface MICA resulted in an augmented lysis by NK cells which was completely abolished by the NKG2D blocking antibody, thus validating the role of this pathway. Downregulation of NKG2D ligands in neuroblastoma lines and patients has been reported previously [167, 172, 177] but to our knowledge histone acetylation has never been implicated for the lack of surface expression of NKG2D ligands in neuroblastoma. Shedding of MICA has been associated with the lack of surface expression in neuroblastoma lines [177]. In our study we ruled out this possibility because soluble MICA was not detected in cell culture supernatants. Our study provides the first evidence of transcriptional repression of NKG2D ligands in neuroblastoma lines. In addition to MICA, other NKG2D ligands were also increased at the mRNA and surface protein levels in the neuroblastoma cell lines after treatment with HDACi sodium valproate (**Figure A-1**). Although it is well known that NK lysis of neuroblastoma cells may be mediated via the NCR and DNAM-1 pathways [157, 171, 178], our study presents an alternative pathway that is clinically feasible to increase NK susceptibility of these resistant tumors.

For the group 2 cell lines representing leukemia or lymphoma, treatment with sodium valproate resulted in marginal upregulation of MICA mRNA. This was mirrored by a minimal increase in MICA on the surface of these cells and marginal increase in NK susceptibility suggesting that MICA expression in these lines could only partially be rescued by histone acetylation. Possibly additional post transcriptional/ post translational mechanisms may be involved in the regulation of MICA. Importantly, although MICA expression was only marginally upregulated by sodium valproate, the NKG2D ligand ULBP2 was induced considerably at the mRNA and surface protein levels in majority of the group 2 cancer cells (**Figure A-2**). Similarly, studies have reported induction of different NKG2D ligands combined with varying degrees of induction when treated with HDAC inhibitors [179]. Armeanu *et al* reported that sodium valproate increases transcription of MICA/B without affecting ULBPs in hepatoma cells [107]. Diermayr *et al*, showed the upregulation of ULBP1 and MICA/B on primary AML cells [180] while Poggi *et al* reported the induction of MICA, ULBP2 and ULBP3 on primary AML cells by sodium valproate [181]. Lopez-Soto *et al* reported trichostatin A induces ULBPs but not MICs in epithelial cancer cells. This heterogeneity suggests that diverse molecular mechanisms are involved in the HDACi effect on the expression of different NKG2D ligands. Similarly, our results show that HDACi sodium valproate increases MICA

mRNA and surface protein with different reaction patterns in the investigated group 2 cell lines.

To investigate other mechanisms that might be responsible for the absence of surface MICA in the NK resistant leukemia and lymphoma group 2 cell lines, proteolytic shedding of MICA was explored [86]. Remarkably, under our experimental conditions, soluble MICA was not detected in the cell culture supernatants suggesting that an alternate mechanism was responsible for the absence of surface MICA. Intracellular sequestration of MICA was thus examined. By immunofluorescence microscopy, intracellular MICA was observed in the group 2 cells but in comparison to the group 1 cell line K562, the levels were considerably lower. In the group 1 cells, both surface and intracellular MICA was observed while in the group 2 cells it was only seen intracellularly. The low level of MICA protein was in accordance with the low levels of mRNA seen in these group 2 cell lines. Next, investigation of the subcellular localization of MICA by colocalisation markers and confocal microscopy demonstrated substantial co-localization of MICA with the ER marker calnexin in the group 2 cells and also an AML patient that lacked surface MICA. We could not detect co-localisation of MICA with markers for the golgi complex, endosomes or lysosomes. Intracellular retention of NKG2D ligands in the ER and *cis*-Golgi has been reported previously in the context of human CMV infection of fibroblasts [103, 105, 182, 183] but similar phenomenon is not well established in tumor cells. Our studies demonstrate intracellular sequestration of MICA in leukemia and lymphoma cells similar to observations made previously in melanoma [83].

Retention of MICA in the ER could be due to association to chaperones, presence of ER retention or retrieval sequences such as KDEL or KEDL, post translational modifications induced by ER quality control mechanisms such as unfolded protein response and ER associated degradation [184-188]. In fact, it was recently reported that tumor cell-associated carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1) causes intracellular retention of MICA/B in human tumor cells [189]. Also, MICA associates with specific chaperones such as ERp5 that cause cleavage of this ligand from cell surface of tumor cells [92]. Further studies are warranted to determine whether an association with these proteins is responsible for absence of surface MICA in the group 2 cells.

Aberrations in the proteasome system have been associated with a number of diseases including cancer [190]. Proteasome inhibitors such as bortezomib, have been licensed for therapy of multiple myeloma and are currently in trials to assess its efficacy for treatment of other malignancies [191]. Hence, to rescue the intracellularly retained MICA in our group 2 cell lines, proteasome inhibitor bortezomib was applied to the tumor cells. An increase in intracellular MICA was observed not only in group 2 cells but also group 1 cancer cells post treatment with Bortezomib. This suggested that proteasomal degradation of MICA was a normal physiological process and inhibiting this by bortezomib may be a simple therapeutic strategy to increase MICA protein. The increase in intracellular MICA protein resulted in augmented expression of MICA on the surface of cancer cells. Subsequently, we determined the functional significance of the

enhanced MICA surface expression on the group 2 cancer cells. An increased NK susceptibility was observed and the role of the NKG2D pathway was confirmed by anti-NKG2D blocking antibody which resulted in a significant abrogation of NK lysis of the treated cells.

Various groups have published conflicting data on induction of NKG2D ligand mRNA by bortezomib. Butler *et al* reported that bortezomib up-regulated ULBP1 mRNA but not that of the other ligands [170]. Gomez *et al* reported an increase in ULBP2 mRNA but not MICA/B [169] while Armeanu *et al* reported that MICA/B mRNA was up-regulated in response to Bortezomib [192]. The discrepancy in their findings may be due to different cell lines used in their studies. In our study, MICA mRNA was not significantly up-regulated after bortezomib treatment in the group 2 cancer cell lines (**Figure A-3**) suggesting that the increase in MICA protein was not due to an increased transcription of the gene. Furthermore, bortezomib has been previously shown to enhance NK lysis of primary tumors by downregulating surface expression of the inhibitory MHC class I molecules in myeloma cells [193]. However in our study, analysis of bortezomib treated primary leukemic patient blasts showed no significant change in the surface expression of HLA molecules (**Figure A-4**). Thus, our preliminary observations argue against downregulation of MHC class I after bortezomib treatment in agreement with data by Lundqvist *et al* [194].

Taken together, our data conclusively show that the absence of surface expression of MICA on the group 2 cells was due to regulation at transcriptional and post translational levels. Transcriptional repression resulting in low mRNA levels was the foremost mechanism for the absence of MICA on the surface. Although marginal levels of protein were observed in some cell lines, intracellular retention in the ER provided a secondary escape route. Treatment with clinically relevant modulators like HDACi sodium valproate and proteasome inhibitor bortezomib were able to increase MICA at the mRNA and/or protein levels, enhanced its surface expression and overcame NK resistance in the majority of cancer cell lines.

Various reports suggest that ligands are regulated somewhat differently by stress pathways and that different cellular stresses selectively trigger upregulation of expression of different sets of NKG2D ligands. This feature presumably allows the same receptor to stimulate a response in different contexts because of upregulation of distinct ligands. Although our study used MICA as a model to investigate the mechanism of NK resistance in the group 2 cell lines, other NKG2D ligands are also under transcriptional, post transcriptional or translational regulation and they respond differently to different cellular stresses. From our observations and others, transcriptional repression of MICB and ULBP genes was also observed. ULBP2 mRNA and surface expression was significantly upregulated by HDACi treatment while histone acetylation could only partially rescue MICB and ULBP1 (**Figures A-2, A-5 and A-6**). The differential effect of HDACi on the regulation of NKG2D ligands clearly suggests that additional mechanisms of downregulation may be involved in the regulation of MICB and ULBP1. In fact, Tang KF *et al* showed that DNA methyltransferase inhibitor 5-azacytidine could upregulate MICB by promoting DNA methylation [195]. Furthermore, results from our study on

MICA elucidate the possibility of a similar subcellular retention of other NKG2D ligands particularly MICB and ULBP1 in cancer cells. Further studies are warranted to test these hypotheses.

NKG2D Ligand Expression Does Not Necessarily Lead to NK Activation

In contrast to the group 1 and group 2 cells, the group 3 cancer cells had an intriguing phenotype of NK resistance despite a high NKG2D ligand expression. This novel phenotype, although seen in only 25% of our cancer cell lines, suggests the existence of mechanisms that can override NKG2D activation. In an effort to uncover the NK resistance mechanisms in group 3 cell lines, we determined whether intrinsic features such as PI-9 expression were responsible. It has been shown in mice and in several cancers that overexpression of PI-9 directly protects cells from apoptosis through granzyme B inhibition [155, 196, 197]. However, our data revealed no significant difference in the levels of PI-9 between the resistant group 3 cell lines and the NK susceptible group 1 cells, suggesting the role of other factors in conferring NK resistance.

Several studies have demonstrated that the cell surface expression of NKG2D ligands results in an increased susceptibility of MHC class I expressing tumors to NK cells, thereby suggesting that NKG2D mediated cytotoxicity can override KIR inhibitory signaling [26, 27, 34]. However, contrasting studies showed that engagement of inhibitory receptors was able to efficiently block NKG2D-mediated cytotoxicity [44, 198]. In our studies, group 1 cells with high NKG2D ligand expression and high NK susceptibility illustrated the former observation where NKG2D-mediated cytotoxicity was able to overcome inhibitory signaling. However, in the group 3 cells that were NK resistant despite a high NKG2D ligand expression, blockade of inhibitory MHC class I molecules resulted in an increased NK susceptibility suggesting that strong inhibitory signaling through KIR could override NKG2D activation in these cancers. This was in agreement with the observations of Pende *et al* [44], but in contrast to their data that was based solely on redirected killing assays, our results provide direct evidence on how tumor cells may evade NKG2D-mediated cytotoxicity in the natural setting. We also show that the increased NK lysis of the group 3 cancer cells after HLA blockade was driven by the NKG2D pathway since blockade of NKG2D resulted in a marked reduction in NK lysis. This observation further substantiates the role of the NKG2D pathway in conferring NK susceptibility to cancer cells. Interestingly, HLA blockade on group 1 cancer cells (high NKG2D ligand expression) did not further increase the NK susceptibility of these cell lines (**Figure A-7**).

Taken together, HLA blockade experiments with the group 1 and 3 cell lines clearly demonstrate the ‘dynamic equilibrium’ concept that regulates NK function. According to this model, NK cytotoxicity is regulated by a fine balance between activating and inhibitory signals such that if the balance tips towards inhibition, NK lysis will be inhibited and vice versa. The NK resistant group 3 cells represent the net balance tipped towards inhibition. However, when the balance is tipped towards activation by MHC blockade, they become susceptible to NK killing. In contrast, group 1 cells are

representative of the net balance tipped towards activation and hence their high NK susceptibility. Moreover, since the net activation has already exceeded the net inhibition in these cells, MHC blockade does not further augment their NK sensitivity. Although, mean NKG2D ligand expression scores for the group 1 and 3 cell lines were comparable, the discrepancies in their NK susceptibility post HLA blockade can be speculated to be due to differences in the expression of other activating ligands that may provide a synergistic activating signal to NKG2D activation. These include (but are not limited to) NCR ligands, ligands for other NK co-receptors and also the NKG2D ligands ULBP 4, 5 and 6 that were not included in our study due to lack of specific antibodies and reagents [44, 199, 200]. Another possibility for the differences observed between group 3 and group 1 cell lines may be the strength of the negative signal [201, 202] as unpublished data from ongoing studies in our lab demonstrates variable strength of inhibition by different HLA alleles.

Nevertheless, HLA blockade on the group 2 cells (lack NKG2D ligands) showed no increase in NK lysis of these cells, suggesting that the release of inhibition alone is not sufficient for NK lysis of tumors [24] (**Figure A-8**). An example of this situation may be represented by encounters between human erythrocytes and circulating peripheral blood NK cells. Although human red blood cells do not express the inhibitory MHC class I, NK cells do not attack them because erythrocytes lack ligands capable of engaging the activating NK cell receptors [24]. In our study, when MICA was ectopically expressed in the group 2 cancer cell lines, they were efficiently lysed by NK cells clearly illustrating that rather than the mere absence of inhibition, the presence of activation is absolutely required for NK lysis (**Figure 4-5**). This phenomenon is also clearly demonstrated by two MHC class I negative cancer cell lines K562 and Daudi. While both cell lines lack inhibitory ligands, K562 (group 1) is efficiently lysed by NK cells due to the presence of NKG2D activation ligands but Daudi (group 2) is NK resistant due to the absence of activating ligands.

Although NK resistance due to KIR inhibition overriding NKG2D activation was common among the group 3 cell lines, rhabdomyosarcoma line RD was an exception. Since the RD cells did not show increased NK lysis post treatment with HLA class I blockade, its ability to activate NK cells was evidently limited. The inability of RD cells to degranulate NK cells suggests the incapability of these cells to adhere to NK cells. Conjugate formation between NK cells and its targets is a prerequisite for NK cell activation and cytotoxicity [203]. Since strong adhesion to target cells mediated by the adhesion receptor-ligand pair of LFA-1 and ICAM-1 is required for lysis by NK cells [204, 205], we determined ICAM-1 levels on all group 3 cell lines and found low surface expression of ICAM-1 specifically on RD. It can be speculated that the low levels of ICAM-1 impairs NK:target conjugate formation resulting in NK resistance in this cell line. However, assays to enumerate formally the effector:target conjugates will be necessary to confirm this hypothesis. The low levels of ICAM-1 on RD cells may be due to shedding of this molecule as shown in several cancers [206-208] or micro RNA mediated repression [209].

Translational Significance of Our Findings

In the past 15 years many groups have reported the anti-cancer effects of NK cells in humans [114, 210-212]. Strong evidence of the clinical benefits of NK cells was provided by Imai K *et al* in 2000 wherein their prospective study of 3625 healthy adults in Japan with a median followup of 11 years, showed that individuals who had higher than baseline NK cytotoxicity of K562 cells were at a significantly lower risk of developing adult cancers [112]. A number of clinical trials with infusion of allogeneic NK cells in patients with malignant diseases have shown feasibility with no side effects [213]. Ruggeri *et al* [114, 214] and others [158, 215] have shown that allogeneic NK cells can mediate antileukemic effects against AML. However, in a study by Miller *et al*, only 5 of the 19 AML patients obtained remission after NK transplantation, [216]. Moreover, all the renal cancer patients in their study did not show a favorable response [216]. Similarly, another study involving 20 patients with recurrent ovarian or breast cancer showed that NK infusions were well tolerated but no tumor responses were observed [124]. Several protocols of clinical-grade NK cell purification and *in vitro* population expansion are now validated, and studies have shown that the infusion of allogeneic NK cells is safe in humans [118, 122, 123] but the efficacy of NK cell based tumor therapies has not yet been firmly established. Although the results of NK based immunotherapeutic treatments of cancer are promising in the experimental models, till date their clinical efficacy in human trials has been modest, presumably due to tumor escape by alteration of NK cell function and resistance to NK killing [217-221].

Our comprehensive study on hematological malignancies and solid tumors provides fundamental insights concerning the biological mechanisms responsible for the modest clinical efficacies of NK cell therapy. Specifically, we determined the prevalence of NK susceptibility in a wide spectrum of cancer cell lines representing various cancer types. Our observations of NK resistance in the cancer cell lines was further validated by leukemia patient samples that exhibited 90% NK resistance. Correlation studies on NKG2D ligand expression and NK cytotoxicity resulted in the observation of 3 phenotypic clusters suggesting that the level of NKG2D ligand expression usually (but not always) correlates with NK susceptibility. On applying our cell line findings to patient samples, we observed the group 2 phenotype to be predominant in pediatric leukemia. Furthermore, induction of NKG2D ligands by HDACi sodium valproate or proteasome inhibitor bortezomib was conducted on primary patient blasts from an MLL patient who exhibited a group 2 phenotype with absence of ligands and NK resistance. The increase in surface MICA and ULBP2 resulting in an increased NK cell degranulation and augmented NK sensitivity substantiated our findings from the group 2 cancer cell lines. Also, blockade of the inhibitory HLA-KIR interaction on AML blasts from a representative group 3 patient demonstrated a significant increase in the NK sensitivity of the patient blasts thereby confirming our group 3 cell line findings. Role of the NKG2D pathway in conferring NK susceptibility was established by NKG2D neutralizing antibodies that abrogated NK lysis of the blasts in both the group 2 and group 3 type patients. Taken together, results from our patient samples mirror our clustering model and suggest that NKG2D pathway can be modulated clinically. However, one caveat of our patient data is the inclusion of leukemia samples only. Based

on the limited clinical effectiveness observed in adoptive NK therapy of solid tumors till date [124, 222], we speculate similar results on NK resistance and NKG2D pathway modulation in primary solid tumor samples as well.

Additionally, our clustering plot can be used as a predictive model to determine the clinical efficacy of NK transplantation in patients (**Figure 5-1**). Hypothetically, by analyzing 2 parameters including NK susceptibility and NKG2D ligand expression, we can determine the likelihood of patient responses to NK cell therapy by grouping them into phenotypic clusters. For example, if analysis shows the patient blasts to express high surface NKG2D ligands and high NK susceptibility indicative of the group 1 phenotype, such a patient would represent an ideal candidate for NK cell therapy. Alternatively, if the patient shows a low NKG2D ligand expression and NK resistance indicative of the group 2 phenotype, NKG2D ligands would need to be induced to make this patient's cells susceptible to NK lysis. In order to make NK therapy a success in this patient, the NKG2D ligands would have to be modulated using agents such as HDACi sodium valproate and/or proteasome inhibitor bortezomib, thereby transforming this patient to a group 1 phenotype.

Furthermore, if analysis predicts a patient having the group 3 phenotype, blockade of KIR inhibitory signaling would be beneficial to improve NK susceptibility. In fact, a therapeutic KIR blocking antibody is currently in clinical trials for AML and multiple myeloma [219]. This monoclonal antibody 1-7F9, recognizes KIR2DL1, 2, and 3 and therefore blocks the inhibition imposed by virtually all MHC class I C alleles, allowing it to be tested in all patients irrespective of their KIR and HLA genotypes [223]. If this antibody proves successful in clinical trials, it would benefit our group 3 patients undergoing NK transplantation. Moreover, KIR inhibitory antibody may also be used in combination with NKG2D ligand inducers such as sodium valproate for further augmenting NK responses via the NKG2D pathway in group 2 cancer cells [104].

Our study conclusively shows that the NKG2D pathway can be manipulated to overcome NK resistance thereby improving the immunotherapeutic potential of NK cell therapy. Modulating this pathway can prove beneficial when used singly or as part of a multipronged approach to augment the function of NK cells, including the use of cytokine [224], 4-1BB ligand [140], chimeric antigen receptors [225] and KIR antibody [223]. Several groups have conducted *in vitro* and *in vivo* studies on immune cells transduced with NKG2D-CAR [146, 150, 225, 226]. The success of such immunotherapeutic strategies relies heavily on the presence of NKG2D ligands on the cancer cells. While group 1 and group 3 type cancer cells would be susceptible to lysis by these CAR expressing cells, the group 2 cells being ligand negative would be resistant. However, HDACi and/ bortezomib treatment may be applied prior to adoptive transfer of the NKG2D-CAR bearing cells to ensure clinical efficacy of such therapeutic strategies [225]. It has been reported that *ex vivo* expanded NK cells kill NK resistant neuroblastoma cell lines through the NCR and DNAM-I pathways [157]. Although effective tumor lysis was observed in this study, we propose that HDACi mediated NKG2D ligand induction on neuroblastoma lines may present an alternate and clinically

Figure 5-1. Model for determining strategies for NK therapy. Cluster plot demonstrating the possible outcomes of NK therapy based on NKG2D ligand expression and NK susceptibility.

expedient pathway to enhance tumor lysis. This may be particularly beneficial in donors that are represented by an NCR dull phenotype since NKG2D receptor is constitutively and homogenously expressed. In conclusion, the clinical potential of NK immunotherapy can be enhanced by modulation of the NKG2D pathway. In accordance to the findings of this study, research in our lab is currently focused on high throughput screening (HTS) strategies to identify clinically safe drugs capable of modulating the NKG2D pathway.

CHAPTER 6. CONCLUSION

Surgery, chemotherapy and ionizing radiation have been used for decades as primary treatments for cancers. However, the development of resistance to these approaches has led to significant incidence of tumor relapse. Immunotherapy for cancer has thus drawn interest given its non-overlapping toxicity with chemotherapy and radiation therapy and an apparent lack of long term toxicity. In particular, the clinical potential of the innate immune cells namely NK cells has been harnessed in the treatment of cancers because they are very good candidates for immunotherapy. NK cells demonstrate rapid response (cytotoxic in minutes without prior sensitization, high potency (tumoricidal rather than tumoristatic), broad specificity (easily differentiate normal cells from tumor cells but do not require different preparations for different tumor types), potential for optimal donor selection (lab assays), ease of preparation and administration, lack of side effects (no GVHD and organ toxicity) and complementarity to other therapeutic strategies (can be administered alongwith antibodies, cytokines, bone marrow transplantation or gene therapy) [160]. These exceptional qualities have led to the use of NK transplantation for treatment of hematological malignancies and solid tumors. However, observation of modest clinical efficacy underscores the need for a better understanding of the factors that undermine NK therapy.

In an effort to understand some of the reasons that hinder the beneficial effects of NK cellular therapies, our study comprehensively analyzed a spectrum of 20 cancer cell lines representing various different cancer types. Analysis of their NK susceptibility showed NK resistance to be prevalent. Since NKG2D is a major activating receptor on NK cells, we characterized the role of this pathway in cancer. Heterogeneity in NKG2D ligand expression was observed among the cancer cells. Correlation between NKG2D ligand expression and NK susceptibility led to the observation of group 1 cells (high NKG2D ligand expression, high NK susceptibility), group 2 cells (low ligand expression, low NK susceptibility), group 3 cells (high ligand expression, low NK susceptibility) thereby suggesting that ligand expression does not always correlate with NK susceptibility. Ectopic expression of MICA overcame NK resistance in the group 2 cancer cells suggesting that absence of surface NKG2D ligands was responsible for NK resistance. Further investigations of resistance mechanisms in the group 2 cancer cells led to the observations that they lacked surface expression of NKG2D ligands due to low mRNA and /or protein levels. Transcriptional repression was primarily responsible for absence of surface ligands. In cells that expressed some protein, ER retention of NKG2D ligands (particularly MICA) contributed to an additional NK evasion mechanism. However, treatment with HDACi sodium valproate or proteasome inhibitor bortezomib helped circumvent these regulatory mechanisms resulting in increased expression of NKG2D ligands and NK susceptibility. While the lack of NKG2D activation was responsible for the NK resistance of the group 2 cancer cells, NK resistance of group 3 cancer cells was due to inhibitory signals overriding NKG2D activation. Blockade of the inhibitory KIR-HLA interaction significantly augmented NK lysis in these cells.

Finally, analysis of fresh leukemia patient samples validated our phenotypic clusters and cell line findings. Taken together, our study conclusively shows that NKG2D evasion mechanisms are strongly prevalent in cancer; however the NKG2D pathway is amenable to therapeutic alteration. By using our clustering plot as a predictive model to determine the efficacy of NK therapy and by implementing therapeutic strategies to modulate this pathway, greater clinical benefit will be expected for NKG2D-mediated NK immunotherapy. Thus, NK transplantation holds considerable promise for future NK cell therapies in cancer.

LIST OF REFERENCES

1. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *European journal of immunology*. 1975;5:112-7.
2. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *European journal of immunology*. 1975;5:117-21.
3. Galy A, Travis M, Cen D, Chen B. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity*. 1995;3:459-73.
4. Zhang Y, Wallace DL, de Lara CM, Ghattas H, Asquith B, Worth A, et al. In vivo kinetics of human natural killer cells: the effects of ageing and acute and chronic viral infection. *Immunology*. 2007;121:258-65.
5. Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. *Blood*. 1990;76:2421-38.
6. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends in immunology*. 2001;22:633-40.
7. Farag SS, Caligiuri MA. Human natural killer cell development and biology. *Blood reviews*. 2006;20:123-37.
8. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood*. 2001;97:3146-51.
9. Trapani JA, Davis J, Sutton VR, Smyth MJ. Proapoptotic functions of cytotoxic lymphocyte granule constituents in vitro and in vivo. *Current opinion in immunology*. 2000;12:323-9.
10. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature*. 1994;369:31-7.
11. Trapani JA, Jans DA, Jans PJ, Smyth MJ, Browne KA, Sutton VR. Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis

- induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. *The Journal of biological chemistry*. 1998;273:27934-8.
12. Screpanti V, Wallin RP, Ljunggren HG, Grandien A. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *J Immunol*. 2001;167:2068-73.
 13. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nature medicine*. 2001;7:94-100.
 14. Taylor MA, Ward B, Schatzle JD, Bennett M. Perforin- and Fas-dependent mechanisms of natural killer cell-mediated rejection of incompatible bone marrow cell grafts. *European journal of immunology*. 2002;32:793-9.
 15. Caligiuri MA. Human natural killer cells. *Blood*. 2008;112:461-9.
 16. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "l'union fait la force". *Blood*. 2005;106:2252-8.
 17. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nature immunology*. 2004;5:1260-5.
 18. Morandi B, Bougras G, Muller WA, Ferlazzo G, Munz C. NK cells of human secondary lymphoid tissues enhance T cell polarization via IFN-gamma secretion. *European journal of immunology*. 2006;36:2394-400.
 19. Moretta A. The dialogue between human natural killer cells and dendritic cells. *Current opinion in immunology*. 2005;17:306-11.
 20. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319:675-8.
 21. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology today*. 1990;11:237-44.
 22. Tay CH, Szomolanyi-Tsuda E, Welsh RM. Control of infections by NK cells. *Current topics in microbiology and immunology*. 1998;230:193-220.
 23. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nature immunology*. 2008;9:503-10.
 24. Lanier LL. NK cell recognition. *Annual review of immunology*. 2005;23:225-74.

25. Lanier LL, Corliss B, Phillips JH. Arousal and inhibition of human NK cells. *Immunological reviews*. 1997;155:145-54.
26. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature*. 2001;413:165-71.
27. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98:11521-6.
28. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annual review of immunology*. 1987;5:223-52.
29. Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, et al. Receptors for HLA class-I molecules in human natural killer cells. *Annual review of immunology*. 1996;14:619-48.
30. Mandelboim O, Malik P, Davis DM, Jo CH, Boyson JE, Strominger JL. Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96:5640-4.
31. Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, et al. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *The Journal of experimental medicine*. 1998;188:953-60.
32. Cantoni C, Bottino C, Vitale M, Pessino A, Augugliaro R, Malaspina A, et al. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *The Journal of experimental medicine*. 1999;189:787-96.
33. Pende D, Parolini S, Pessino A, Sivori S, Augugliaro R, Morelli L, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *The Journal of experimental medicine*. 1999;190:1505-16.
34. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science*. 1999;285:727-9.
35. Moretta L, Bottino C, Pende D, Mingari MC, Biassoni R, Moretta A. Human natural killer cells: their origin, receptors and function. *European journal of immunology*. 2002;32:1205-11.

36. Sivori S, Parolini S, Falco M, Marcenaro E, Biassoni R, Bottino C, et al. 2B4 functions as a co-receptor in human NK cell activation. *European journal of immunology*. 2000;30:787-93.
37. Bottino C, Falco M, Parolini S, Marcenaro E, Augugliaro R, Sivori S, et al. NTB-A [correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *The Journal of experimental medicine*. 2001;194:235-46.
38. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *The Journal of experimental medicine*. 2003;198:557-67.
39. Vitale M, Falco M, Castriconi R, Parolini S, Zambello R, Semenzato G, et al. Identification of NKp80, a novel triggering molecule expressed by human NK cells. *European journal of immunology*. 2001;31:233-42.
40. Jiang K, Zhong B, Gilvary DL, Corliss BC, Hong-Geller E, Wei S, et al. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. *Nature immunology*. 2000;1:419-25.
41. Ravetch JV, Lanier LL. Immune inhibitory receptors. *Science*. 2000;290:84-9.
42. Blery M, Delon J, Trautmann A, Cambiaggi A, Olcese L, Biassoni R, et al. Reconstituted killer cell inhibitory receptors for major histocompatibility complex class I molecules control mast cell activation induced via immunoreceptor tyrosine-based activation motifs. *The Journal of biological chemistry*. 1997;272:8989-96.
43. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nature immunology*. 2008;9:495-502.
44. Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, et al. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *European journal of immunology*. 2001;31:1076-86.
45. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annual review of immunology*. 2001;19:197-223.
46. Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytotoxicity. *Immunology today*. 2000;21:228-34.

47. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *The Journal of experimental medicine*. 2009;206:1495-503.
48. Houchins JP, Yabe T, McSherry C, Miyokawa N, Bach FH. Isolation and characterization of NK cell or NK/T cell-specific cDNA clones. *The Journal of molecular and cellular immunology : JMCI*. 1990;4:295-304; discussion 5-6.
49. Houchins JP, Yabe T, McSherry C, Bach FH. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *The Journal of experimental medicine*. 1991;173:1017-20.
50. Sutherland CL, Chalupny NJ, Schooley K, VandenBos T, Kubin M, Cosman D. UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. *J Immunol*. 2002;168:671-9.
51. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8 α T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nature immunology*. 2001;2:255-60.
52. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nature reviews Immunology*. 2003;3:781-90.
53. Wu J, Song Y, Bakker AB, Bauer S, Spies T, Lanier LL, et al. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science*. 1999;285:730-2.
54. Garrity D, Call ME, Feng J, Wucherpennig KW. The activating NKG2D receptor assembles in the membrane with two signaling dimers into a hexameric structure. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:7641-6.
55. Gilfillan S, Ho EL, Cella M, Yokoyama WM, Colonna M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nature immunology*. 2002;3:1150-5.
56. Upshaw JL, Arneson LN, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nature immunology*. 2006;7:524-32.
57. Lanier LL. On guard--activating NK cell receptors. *Nature immunology*. 2001;2:23-7.

58. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96:6879-84.
59. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93:12445-50.
60. Xu B, Pizarro JC, Holmes MA, McBeth C, Groh V, Spies T, et al. Crystal structure of a gammadelta T-cell receptor specific for the human MHC class I homolog MICA. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108:2414-9.
61. Stephens HA. MICA and MICB genes: can the enigma of their polymorphism be resolved? *Trends in immunology*. 2001;22:378-85.
62. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity*. 2001;14:123-33.
63. Kubin M, Cassiano L, Chalupny J, Chin W, Cosman D, Fanslow W, et al. ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. *European journal of immunology*. 2001;31:1428-37.
64. Radosavljevic M, Cuillerier B, Wilson MJ, Clement O, Wicker S, Gilfillan S, et al. A cluster of ten novel MHC class I related genes on human chromosome 6q24.2-q25.3. *Genomics*. 2002;79:114-23.
65. Strong RK. Asymmetric ligand recognition by the activating natural killer cell receptor NKG2D, a symmetric homodimer. *Molecular immunology*. 2002;38:1029-37.
66. McFarland BJ, Strong RK. Thermodynamic analysis of degenerate recognition by the NKG2D immunoreceptor: not induced fit but rigid adaptation. *Immunity*. 2003;19:803-12.
67. Radaev S, Rostro B, Brooks AG, Colonna M, Sun PD. Conformational plasticity revealed by the cocrystal structure of NKG2D and its class I MHC-like ligand ULBP3. *Immunity*. 2001;15:1039-49.

68. Li P, Morris DL, Willcox BE, Steinle A, Spies T, Strong RK. Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA. *Nature immunology*. 2001;2:443-51.
69. Venkataraman GM, Suci D, Groh V, Boss JM, Spies T. Promoter region architecture and transcriptional regulation of the genes for the MHC class I-related chain A and B ligands of NKG2D. *J Immunol*. 2007;178:961-9.
70. Eagle RA, Traherne JA, Ashiru O, Wills MR, Trowsdale J. Regulation of NKG2D ligand gene expression. *Human immunology*. 2006;67:159-69.
71. Molinero LL, Fuertes MB, Girart MV, Fainboim L, Rabinovich GA, Costas MA, et al. NF-kappa B regulates expression of the MHC class I-related chain A gene in activated T lymphocytes. *J Immunol*. 2004;173:5583-90.
72. Lin D, Lavender H, Soilleux EJ, O'Callaghan CA. NF-kappaB regulates MICA gene transcription in endothelial cell through a genetically inhibitable control site. *The Journal of biological chemistry*. 2012;287:4299-310.
73. Lopez-Soto A, Quinones-Lombrana A, Lopez-Arbesu R, Lopez-Larrea C, Gonzalez S. Transcriptional regulation of ULBP1, a human ligand of the NKG2D receptor. *The Journal of biological chemistry*. 2006;281:30419-30.
74. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature*. 2005;436:1186-90.
75. Gasser S, Raulet DH. The DNA damage response arouses the immune system. *Cancer research*. 2006;66:3959-62.
76. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. *Annual review of immunology*. 2013;31:413-41.
77. Stern-Ginossar N, Elefant N, Zimmermann A, Wolf DG, Saleh N, Biton M, et al. Host immune system gene targeting by a viral miRNA. *Science*. 2007;317:376-81.
78. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. *Cell host & microbe*. 2009;5:376-85.
79. Bauman Y, Nachmani D, Vitenshtein A, Tsukerman P, Drayman N, Stern-Ginossar N, et al. An identical miRNA of the human JC and BK polyoma viruses targets the stress-induced ligand ULBP3 to escape immune elimination. *Cell host & microbe*. 2011;9:93-102.

80. Stern-Ginossar N, Gur C, Biton M, Horwitz E, Elboim M, Stanietsky N, et al. Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nature immunology*. 2008;9:1065-73.
81. Nice TJ, Coscoy L, Raulet DH. Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress. *The Journal of experimental medicine*. 2009;206:287-98.
82. Nice TJ, Deng W, Coscoy L, Raulet DH. Stress-regulated targeting of the NKG2D ligand Mult1 by a membrane-associated RING-CH family E3 ligase. *J Immunol*. 2010;185:5369-76.
83. Fuertes MB, Girart MV, Molinero LL, Domaica CI, Rossi LE, Barrio MM, et al. Intracellular retention of the NKG2D ligand MHC class I chain-related gene A in human melanomas confers immune privilege and prevents NK cell-mediated cytotoxicity. *J Immunol*. 2008;180:4606-14.
84. Thomas M, Wills M, Lehner PJ. Natural killer cell evasion by an E3 ubiquitin ligase from Kaposi's sarcoma-associated herpesvirus. *Biochemical Society transactions*. 2008;36:459-63.
85. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*. 2002;419:734-8.
86. Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. *J Immunol*. 2002;169:4098-102.
87. Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood*. 2003;102:1389-96.
88. Doubrovina ES, Doubrovin MM, Vider E, Sisson RB, O'Reilly RJ, Dupont B, et al. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *J Immunol*. 2003;171:6891-9.
89. Salih HR, Goehlsdorf D, Steinle A. Release of MICB molecules by tumor cells: mechanism and soluble MICB in sera of cancer patients. *Human immunology*. 2006;67:188-95.
90. Waldhauer I, Steinle A. Proteolytic release of soluble UL16-binding protein 2 from tumor cells. *Cancer research*. 2006;66:2520-6.

91. Paschen A, Sucker A, Hill B, Moll I, Zapatka M, Nguyen XD, et al. Differential clinical significance of individual NKG2D ligands in melanoma: soluble ULBP2 as an indicator of poor prognosis superior to S100B. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009;15:5208-15.
92. Kaiser BK, Yim D, Chow IT, Gonzalez S, Dai Z, Mann HH, et al. Disulphide-isomerase-enabled shedding of tumour-associated NKG2D ligands. *Nature*. 2007;447:482-6.
93. Fernandez-Messina L, Ashiru O, Boutet P, Aguera-Gonzalez S, Skepper JN, Reyburn HT, et al. Differential mechanisms of shedding of the glycosylphosphatidylinositol (GPI)-anchored NKG2D ligands. *The Journal of biological chemistry*. 2010;285:8543-51.
94. Hue S, Mention JJ, Monteiro RC, Zhang SL, Cellier C, Schmitz J, et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity*. 2004;21:367-77.
95. Dasgupta S, Bhattacharya-Chatterjee M, O'Malley BW, Jr., Chatterjee SK. Inhibition of NK cell activity through TGF-beta 1 by down-regulation of NKG2D in a murine model of head and neck cancer. *J Immunol*. 2005;175:5541-50.
96. Cerwenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH, et al. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity*. 2000;12:721-7.
97. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nature immunology*. 2000;1:119-26.
98. Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM, Hayakawa Y. NKG2D function protects the host from tumor initiation. *The Journal of experimental medicine*. 2005;202:583-8.
99. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity*. 2008;28:571-80.
100. Draghi M, Pashine A, Sanjanwala B, Gendzekhadze K, Cantoni C, Cosman D, et al. NKp46 and NKG2D recognition of infected dendritic cells is necessary for NK cell activation in the human response to influenza infection. *J Immunol*. 2007;178:2688-98.

101. Walsh KB, Lodoen MB, Lanier LL, Lane TE. NKG2D signaling and host defense after mouse hepatitis virus infection of the central nervous system. *Advances in experimental medicine and biology*. 2006;581:369-72.
102. Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. *Current opinion in immunology*. 2006;18:391-8.
103. Welte SA, Sinzger C, Lutz SZ, Singh-Jasuja H, Sampaio KL, Eknigk U, et al. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. *European journal of immunology*. 2003;33:194-203.
104. Benson DM, Jr., Bakan CE, Zhang S, Collins SM, Liang J, Srivastava S, et al. IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood*. 2011;118:6387-91.
105. Dunn C, Chalupny NJ, Sutherland CL, Dosch S, Sivakumar PV, Johnson DC, et al. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. *The Journal of experimental medicine*. 2003;197:1427-39.
106. Diefenbach A, Hsia JK, Hsiung MY, Raulet DH. A novel ligand for the NKG2D receptor activates NK cells and macrophages and induces tumor immunity. *European journal of immunology*. 2003;33:381-91.
107. Armeanu S, Bitzer M, Lauer UM, Venturelli S, Pathil A, Krusch M, et al. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer research*. 2005;65:6321-9.
108. Eisele G, Wischhusen J, Mittelbronn M, Meyermann R, Waldhauer I, Steinle A, et al. TGF-beta and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. *Brain : a journal of neurology*. 2006;129:2416-25.
109. Boissel N, Rea D, Tieng V, Dulphy N, Brun M, Cayuela JM, et al. BCR/ABL oncogene directly controls MHC class I chain-related molecule A expression in chronic myelogenous leukemia. *J Immunol*. 2006;176:5108-16.
110. Borghaei H, Smith MR, Campbell KS. Immunotherapy of cancer. *European journal of pharmacology*. 2009;625:41-54.
111. Weiner LM, Dhodapkar MV, Ferrone S. Monoclonal antibodies for cancer immunotherapy. *Lancet*. 2009;373:1033-40.

112. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet*. 2000;356:1795-9.
113. Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer*. 1997;79:2320-8.
114. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097-100.
115. Ruggeri L, Mancusi A, Perruccio K, Burchielli E, Martelli MF, Velardi A. Natural killer cell alloreactivity for leukemia therapy. *J Immunother*. 2005;28:175-82.
116. Ruggeri L, Capanni M, Mancusi A, Perruccio K, Burchielli E, Martelli MF, et al. Natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *International journal of hematology*. 2005;81:13-7.
117. Ruggeri L, Aversa F, Martelli MF, Velardi A. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. *Immunological reviews*. 2006;214:202-18.
118. Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. *Blood*. 2011;118:3273-9.
119. Miller JS, Klingsporn S, Lund J, Perry EH, Verfaillie C, McGlave P. Large scale ex vivo expansion and activation of human natural killer cells for autologous therapy. *Bone marrow transplantation*. 1994;14:555-62.
120. McKenna DH, Jr., Sumstad D, Bostrom N, Kadidlo DM, Fautsch S, McNearney S, et al. Good manufacturing practices production of natural killer cells for immunotherapy: a six-year single-institution experience. *Transfusion*. 2007;47:520-8.
121. Lundqvist A, McCoy JP, Samsel L, Childs R. Reduction of GVHD and enhanced antitumor effects after adoptive infusion of alloreactive Ly49-mismatched NK cells from MHC-matched donors. *Blood*. 2007;109:3603-6.
122. Barkholt L, Alici E, Conrad R, Sutlu T, Gilljam M, Stellan B, et al. Safety analysis of ex vivo-expanded NK and NK-like T cells administered to cancer patients: a phase I clinical study. *Immunotherapy*. 2009;1:753-64.

123. Bachanova V, Burns LJ, McKenna DH, Curtsinger J, Panoskaltsis-Mortari A, Lindgren BR, et al. Allogeneic natural killer cells for refractory lymphoma. *Cancer immunology, immunotherapy : CII*. 2010;59:1739-44.
124. Geller MA, Cooley S, Judson PL, Ghebre R, Carson LF, Argenta PA, et al. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy*. 2011;13:98-107.
125. Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. *Nature reviews Immunology*. 2007;7:329-39.
126. Dall'Ozzo S, Tartas S, Paintaud G, Cartron G, Colombat P, Bardos P, et al. Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration-effect relationship. *Cancer research*. 2004;64:4664-9.
127. Gluck WL, Hurst D, Yuen A, Levine AM, Dayton MA, Gockerman JP, et al. Phase I studies of interleukin (IL)-2 and rituximab in B-cell non-hodgkin's lymphoma: IL-2 mediated natural killer cell expansion correlations with clinical response. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2004;10:2253-64.
128. Alderson KL, Sondel PM. Clinical cancer therapy by NK cells via antibody-dependent cell-mediated cytotoxicity. *Journal of biomedicine & biotechnology*. 2011;2011:379123.
129. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *British journal of cancer*. 2006;94:259-67.
130. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. *Journal of hematotherapy & stem cell research*. 2001;10:535-44.
131. Klingemann HG. Natural killer cell-based immunotherapeutic strategies. *Cytotherapy*. 2005;7:16-22.
132. Malmberg KJ, Bryceson YT, Carlsten M, Andersson S, Bjorklund A, Bjorkstrom NK, et al. NK cell-mediated targeting of human cancer and possibilities for new means of immunotherapy. *Cancer immunology, immunotherapy : CII*. 2008;57:1541-52.
133. Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J, et al. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. *Cytotherapy*. 2008;10:625-32.

134. Schirrmann T, Pecher G. Human natural killer cell line modified with a chimeric immunoglobulin T-cell receptor gene leads to tumor growth inhibition in vivo. *Cancer gene therapy*. 2002;9:390-8.
135. Schirrmann T, Pecher G. Specific targeting of CD33(+) leukemia cells by a natural killer cell line modified with a chimeric receptor. *Leukemia research*. 2005;29:301-6.
136. Uherek C, Tonn T, Uherek B, Becker S, Schnierle B, Klingemann HG, et al. Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. *Blood*. 2002;100:1265-73.
137. Demirtzoglou FJ, Papadopoulos S, Zografos G. Cytolytic and cytotoxic activity of a human natural killer cell line genetically modified to specifically recognize HER-2/neu overexpressing tumor cells. *Immunopharmacology and immunotoxicology*. 2006;28:571-90.
138. Pegram HJ, Jackson JT, Smyth MJ, Kershaw MH, Darcy PK. Adoptive transfer of gene-modified primary NK cells can specifically inhibit tumor progression in vivo. *J Immunol*. 2008;181:3449-55.
139. Kruschinski A, Moosmann A, Poschke I, Norell H, Chmielewski M, Seliger B, et al. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:17481-6.
140. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood*. 2005;106:376-83.
141. Wu JD, Higgins LM, Steinle A, Cosman D, Haugk K, Plymate SR. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. *The Journal of clinical investigation*. 2004;114:560-8.
142. Crane CA, Han SJ, Barry JJ, Ahn BJ, Lanier LL, Parsa AT. TGF-beta downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients. *Neuro-oncology*. 2010;12:7-13.
143. Jinushi M, Vanneman M, Munshi NC, Tai YT, Prabhala RH, Ritz J, et al. MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:1285-90.

144. Jinushi M, Hodi FS, Dranoff G. Therapy-induced antibodies to MHC class I chain-related protein A antagonize immune suppression and stimulate antitumor cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103:9190-5.
145. Zhang T, Barber A, Sentman CL. Generation of antitumor responses by genetic modification of primary human T cells with a chimeric NKG2D receptor. *Cancer research*. 2006;66:5927-33.
146. Barber A, Zhang T, Megli CJ, Wu J, Meehan KR, Sentman CL. Chimeric NKG2D receptor-expressing T cells as an immunotherapy for multiple myeloma. *Experimental hematology*. 2008;36:1318-28.
147. Barber A, Zhang T, Sentman CL. Immunotherapy with chimeric NKG2D receptors leads to long-term tumor-free survival and development of host antitumor immunity in murine ovarian cancer. *J Immunol*. 2008;180:72-8.
148. Zhang T, Barber A, Sentman CL. Chimeric NKG2D modified T cells inhibit systemic T-cell lymphoma growth in a manner involving multiple cytokines and cytotoxic pathways. *Cancer research*. 2007;67:11029-36.
149. Barber A, Zhang T, DeMars LR, Conejo-Garcia J, Roby KF, Sentman CL. Chimeric NKG2D receptor-bearing T cells as immunotherapy for ovarian cancer. *Cancer research*. 2007;67:5003-8.
150. Chang YH, Connolly J, Shimasaki N, Mimura K, Kono K, Campana D. A chimeric receptor with NKG2D specificity enhances natural killer cell activation and killing of tumor cells. *Cancer research*. 2013;73:1777-86.
151. von Strandmann EP, Hansen HP, Reiners KS, Schnell R, Borchmann P, Merkert S, et al. A novel bispecific protein (ULBP2-BB4) targeting the NKG2D receptor on natural killer (NK) cells and CD138 activates NK cells and has potent antitumor activity against human multiple myeloma in vitro and in vivo. *Blood*. 2006;107:1955-62.
152. Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: causes and therapies. *Nature reviews Cancer*. 2001;1:194-202.
153. Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. *Journal of cellular physiology*. 2000;184:1-16.
154. Timmermann S, Lehrmann H, Polesskaya A, Harel-Bellan A. Histone acetylation and disease. *Cellular and molecular life sciences : CMLS*. 2001;58:728-36.

155. Medema JP, de Jong J, Peltenburg LT, Verdegaal EM, Gorter A, Bres SA, et al. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98:11515-20.
156. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nature reviews Immunology*. 2006;6:520-31.
157. Rujkijyanont P, Chan WK, Eldridge PW, Lockey T, Holladay M, Rooney B, et al. Ex vivo activation of CD56(+) immune cells that eradicate neuroblastoma. *Cancer research*. 2013;73:2608-18.
158. Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2010;28:955-9.
159. Mahoney DH, Jr., Starling KA. Immunotherapy in acute leukemias. Possible applications in children. *The American journal of pediatric hematology/oncology*. 1981;3:410-8.
160. Leung W. Immunotherapy in acute leukemia. *Seminars in hematology*. 2009;46:89-99.
161. Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol*. 2004;172:644-50.
162. Farnault L, Sanchez C, Baier C, Le Treut T, Costello RT. Hematological malignancies escape from NK cell innate immune surveillance: mechanisms and therapeutic implications. *Clinical & developmental immunology*. 2012;2012:421702.
163. Rossi AR, Pericle F, Rashleigh S, Janiec J, Djeu JY. Lysis of neuroblastoma cell lines by human natural killer cells activated by interleukin-2 and interleukin-12. *Blood*. 1994;83:1323-8.
164. Romanski A, Bug G, Becker S, Kampfmann M, Seifried E, Hoelzer D, et al. Mechanisms of resistance to natural killer cell-mediated cytotoxicity in acute lymphoblastic leukemia. *Experimental hematology*. 2005;33:344-52.
165. Lion E, Willemen Y, Berneman ZN, Van Tendeloo VF, Smits EL. Natural killer cell immune escape in acute myeloid leukemia. *Leukemia*. 2012;26:2019-26.

166. Pietra G, Manzini C, Rivara S, Vitale M, Cantoni C, Petretto A, et al. Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer research*. 2012;72:1407-15.
167. Pende D, Rivera P, Marcenaro S, Chang CC, Biassoni R, Conte R, et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer research*. 2002;62:6178-86.
168. Chan WK, Kung Sutherland M, Li Y, Zalevsky J, Schell S, Leung W. Antibody-dependent cell-mediated cytotoxicity overcomes NK cell resistance in MLL-rearranged leukemia expressing inhibitory KIR ligands but not activating ligands. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012;18:6296-305.
169. Vales-Gomez M, Chisholm SE, Cassady-Cain RL, Roda-Navarro P, Reyburn HT. Selective induction of expression of a ligand for the NKG2D receptor by proteasome inhibitors. *Cancer research*. 2008;68:1546-54.
170. Butler JE, Moore MB, Presnell SR, Chan HW, Chalupny NJ, Lutz CT. Proteasome regulation of ULBP1 transcription. *J Immunol*. 2009;182:6600-9.
171. Sivori S, Parolini S, Marcenaro E, Castriconi R, Pende D, Millo R, et al. Involvement of natural cytotoxicity receptors in human natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines. *Journal of neuroimmunology*. 2000;107:220-5.
172. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood*. 2005;105:251-8.
173. Verhoeven DH, de Hooge AS, Mooiman EC, Santos SJ, ten Dam MM, Gelderblom H, et al. NK cells recognize and lyse Ewing sarcoma cells through NKG2D and DNAM-1 receptor dependent pathways. *Molecular immunology*. 2008;45:3917-25.
174. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood*. 2006;107:159-66.
175. Attwood JT, Yung RL, Richardson BC. DNA methylation and the regulation of gene transcription. *Cellular and molecular life sciences : CMLS*. 2002;59:241-57.

176. Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *The EMBO journal*. 2001;20:6969-78.
177. Raffaghello L, Prigione I, Airolidi I, Camoriano M, Levreri I, Gambini C, et al. Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma. *Neoplasia*. 2004;6:558-68.
178. Castriconi R, Dondero A, Corrias MV, Lanino E, Pende D, Moretta L, et al. Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer research*. 2004;64:9180-4.
179. Skov S, Pedersen MT, Andresen L, Straten PT, Woetmann A, Odum N. Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3-dependent expression of MHC class I-related chain A and B. *Cancer research*. 2005;65:11136-45.
180. Diermayr S, Himmelreich H, Durovic B, Mathys-Schneeberger A, Siegler U, Langenkamp U, et al. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. *Blood*. 2008;111:1428-36.
181. Poggi A, Catellani S, Garuti A, Pierri I, Gobbi M, Zocchi MR. Effective in vivo induction of NKG2D ligands in acute myeloid leukaemias by all-trans-retinoic acid or sodium valproate. *Leukemia*. 2009;23:641-8.
182. Wu J, Chalupny NJ, Manley TJ, Riddell SR, Cosman D, Spies T. Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein. *J Immunol*. 2003;170:4196-200.
183. Ashiru O, Bennett NJ, Boyle LH, Thomas M, Trowsdale J, Wills MR. NKG2D ligand MICA is retained in the cis-Golgi apparatus by human cytomegalovirus protein UL142. *Journal of virology*. 2009;83:12345-54.
184. Andres DA, Rhodes JD, Meisel RL, Dixon JE. Characterization of the carboxyl-terminal sequences responsible for protein retention in the endoplasmic reticulum. *The Journal of biological chemistry*. 1991;266:14277-82.
185. Lewis MJ, Pelham HR. Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell*. 1992;68:353-64.
186. Nilsson T, Warren G. Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. *Current opinion in cell biology*. 1994;6:517-21.

187. Bukau B, Weissman J, Horwich A. Molecular chaperones and protein quality control. *Cell*. 2006;125:443-51.
188. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell*. 2000;101:249-58.
189. Chen Z, Chen L, Baker K, Olszak T, Zeissig S, Huang YH, et al. CEACAM1 dampens antitumor immunity by down-regulating NKG2D ligand expression on tumor cells. *The Journal of experimental medicine*. 2011;208:2633-40.
190. Pagano M, Benmaamar R. When protein destruction runs amok, malignancy is on the loose. *Cancer cell*. 2003;4:251-6.
191. Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. *Annual review of medicine*. 2006;57:33-47.
192. Armeanu S, Krusch M, Baltz KM, Weiss TS, Smirnow I, Steinle A, et al. Direct and natural killer cell-mediated antitumor effects of low-dose bortezomib in hepatocellular carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008;14:3520-8.
193. Shi J, Tricot GJ, Garg TK, Malaviarachchi PA, Szmania SM, Kellum RE, et al. Bortezomib down-regulates the cell-surface expression of HLA class I and enhances natural killer cell-mediated lysis of myeloma. *Blood*. 2008;111:1309-17.
194. Lundqvist A, Abrams SI, Schrupp DS, Alvarez G, Suffredini D, Berg M, et al. Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer research*. 2006;66:7317-25.
195. Tang KF, He CX, Zeng GL, Wu J, Song GB, Shi YS, et al. Induction of MHC class I-related chain B (MICB) by 5-aza-2'-deoxycytidine. *Biochemical and biophysical research communications*. 2008;370:578-83.
196. Ray M, Hostetter DR, Loeb CR, Simko J, Craik CS. Inhibition of Granzyme B by PI-9 protects prostate cancer cells from apoptosis. *The Prostate*. 2012;72:846-55.
197. Jiang X, Orr BA, Kranz DM, Shapiro DJ. Estrogen induction of the granzyme B inhibitor, proteinase inhibitor 9, protects cells against apoptosis mediated by cytotoxic T lymphocytes and natural killer cells. *Endocrinology*. 2006;147:1419-26.

198. Endt J, McCann FE, Almeida CR, Urlaub D, Leung R, Pende D, et al. Inhibitory receptor signals suppress ligation-induced recruitment of NKG2D to GM1-rich membrane domains at the human NK cell immune synapse. *J Immunol.* 2007;178:5606-11.
199. Chalupny NJ, Sutherland CL, Lawrence WA, Rein-Weston A, Cosman D. ULBP4 is a novel ligand for human NKG2D. *Biochemical and biophysical research communications.* 2003;305:129-35.
200. Bacon L, Eagle RA, Meyer M, Easom N, Young NT, Trowsdale J. Two human ULBP/RAET1 molecules with transmembrane regions are ligands for NKG2D. *J Immunol.* 2004;173:1078-84.
201. Malarkannan S. The balancing act: inhibitory Ly49 regulate NKG2D-mediated NK cell functions. *Seminars in immunology.* 2006;18:186-92.
202. Ashiru O, Boutet P, Fernandez-Messina L, Aguera-Gonzalez S, Skepper JN, Vales-Gomez M, et al. Natural killer cell cytotoxicity is suppressed by exposure to the human NKG2D ligand MICA*008 that is shed by tumor cells in exosomes. *Cancer research.* 2010;70:481-9.
203. Zheng X, Wang Y, Wei H, Sun R, Tian Z. LFA-1 and CD2 synergize for the Erk1/2 activation in the Natural Killer (NK) cell immunological synapse. *The Journal of biological chemistry.* 2009;284:21280-7.
204. Helander TS, Timonen T. Adhesion in NK cell function. *Current topics in microbiology and immunology.* 1998;230:89-99.
205. Gross CC, Brzustowski JA, Liu D, Long EO. Tethering of intercellular adhesion molecule on target cells is required for LFA-1-dependent NK cell adhesion and granule polarization. *J Immunol.* 2010;185:2918-26.
206. Becker JC, Dummer R, Hartmann AA, Burg G, Schmidt RE. Shedding of ICAM-1 from human melanoma cell lines induced by IFN-gamma and tumor necrosis factor-alpha. Functional consequences on cell-mediated cytotoxicity. *J Immunol.* 1991;147:4398-401.
207. Fiore E, Fusco C, Romero P, Stamenkovic I. Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell-mediated cytotoxicity. *Oncogene.* 2002;21:5213-23.
208. Sanchez-Rovira P, Jimenez E, Carracedo J, Barneto IC, Ramirez R, Aranda E. Serum levels of intercellular adhesion molecule 1 (ICAM-1) in patients with colorectal cancer: inhibitory effect on cytotoxicity. *Eur J Cancer.* 1998;34:394-8.

209. Ueda R, Kohanbash G, Sasaki K, Fujita M, Zhu X, Kastenhuber ER, et al. Dicer-regulated microRNAs 222 and 339 promote resistance of cancer cells to cytotoxic T-lymphocytes by down-regulation of ICAM-1. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106:10746-51.
210. Porgador A, Mandelboim O, Restifo NP, Strominger JL. Natural killer cell lines kill autologous beta2-microglobulin-deficient melanoma cells: implications for cancer immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94:13140-5.
211. Blattman JN, Greenberg PD. Cancer immunotherapy: a treatment for the masses. *Science*. 2004;305:200-5.
212. Siegler U, Kalberer CP, Nowbakht P, Sendelov S, Meyer-Monard S, Wodnar-Filipowicz A. Activated natural killer cells from patients with acute myeloid leukemia are cytotoxic against autologous leukemic blasts in NOD/SCID mice. *Leukemia*. 2005;19:2215-22.
213. Koepsell SA, Miller JS, McKenna DH, Jr. Natural killer cells: a review of manufacturing and clinical utility. *Transfusion*. 2013;53:404-10.
214. Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94:333-9.
215. Giebel S, Locatelli F, Lamparelli T, Velardi A, Davies S, Frumento G, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood*. 2003;102:814-9.
216. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. 2005;105:3051-7.
217. Zama L, Ponti C, Mirandola P, Gobbi G, Papa S, Galeotti L, et al. NK cells and cancer. *J Immunol*. 2007;178:4011-6.
218. Klingemann HG. Cellular therapy of cancer with natural killer cells-where do we stand? *Cytotherapy*. 2013.
219. Romagne F, Vivier E. Natural killer cell-based therapies. *F1000 medicine reports*. 2011;3:9.
220. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK cell-based immunotherapy for malignant diseases. *Cellular & molecular immunology*. 2013;10:230-52.

- 221. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nature reviews Immunology*. 2012;12:239-52.
- 222. Levy EM, Roberti MP, Mordoh J. Natural killer cells in human cancer: from biological functions to clinical applications. *Journal of biomedicine & biotechnology*. 2011;2011:676198.
- 223. Romagne F, Andre P, Spee P, Zahn S, Anfossi N, Gauthier L, et al. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. *Blood*. 2009;114:2667-77.
- 224. Decot V, Voillard L, Latger-Cannard V, Aissi-Rothe L, Perrier P, Stoltz JF, et al. Natural-killer cell amplification for adoptive leukemia relapse immunotherapy: comparison of three cytokines, IL-2, IL-15, or IL-7 and impact on NKG2D, KIR2DL1, and KIR2DL2 expression. *Experimental hematology*. 2010;38:351-62.
- 225. Spear P, Barber A, Rynda-Appl A, Sentman CL. NKG2D CAR T-cell therapy inhibits the growth of NKG2D ligand heterogeneous tumors. *Immunology and cell biology*. 2013;91:435-40.
- 226. Barber A, Meehan KR, Sentman CL. Treatment of multiple myeloma with adoptively transferred chimeric NKG2D receptor-expressing T cells. *Gene therapy*. 2011;18:509-16.

APPENDIX. SUPPLEMENTARY FIGURES FOR CHAPTER 5

Figure A-1. Histone deacetylase inhibitor sodium valproate upregulates mRNA and protein levels of other NKG2D ligands in neuroblastoma.

(A) Relative MICB, ULBP1 and ULBP2 mRNA levels in untreated (white) and 5mM treated NB-1691(black) four days after treatment. (B) Histograms of surface MICB, ULBP1 and ULBP2 on untreated (black) and sodium valproate treated (red) neuroblastoma cells. Isotype control was presented as shaded histograms. Data are representative of 3 experiments.

Figure A-2. Histone deacetylase inhibitor sodium valproate significantly upregulated ULBP2 mRNA and protein expression on group 2 leukemia and lymphoma cell lines.

(A) Relative ULBP2 mRNA levels in untreated (white) and sodium valproate treated (black) group 2 cancer cells. (B) Histograms of ULBP2 surface expression on untreated cells (black line) and sodium valproate treated cells (red line) 48 hours after treatment. Isotype control was presented as shaded histogram. Data are representative of 3 independent experiments.

Figure A-3. Bortezomib does not increase MICA mRNA.

Relative MICA mRNA levels in untreated (white) and bortezomib treated (black) group 2 cancer cells.

Figure A-4. Bortezomib does not downregulate HLA-ABC in patient cells. Histograms of HLA-ABC surface expression on untreated cells (black line) and bortezomib treated cells (red line) in the indicated patient samples. Isotype control was presented as shaded histogram.

Figure A-5. Histone deacetylase inhibitor treatment marginally upregulated MICB mRNA and surface protein in the group 2 leukemia and lymphoma cell lines. (A) Relative MICB mRNA levels in untreated (white) and sodium valproate treated (black) group 2 cancer cells. (B) Histograms of MICB surface expression on untreated cells (black line) and sodium valproate treated cells (red line) 48 hours after treatment. Isotype control was presented as shaded histogram. Data are representative of 3 independent experiments.

Figure A-6. Histone deacetylase inhibitor treatment marginally upregulated ULBP1 mRNA and surface protein in the group 2 leukemia and lymphoma cell lines.

(A) Relative ULBP1 mRNA levels in untreated (white) and sodium valproate treated (black) group 2 cancer cells. (B) Histograms of ULBP1 surface expression on untreated cells (black line) and sodium valproate treated cells (red line) 48 hours after treatment. Isotype control was presented as shaded histogram. Data are representative of 3 independent experiments.

Figure A-7. HLA inhibition does not further increase NK susceptibility of group 1 cells.

(A) Expression of inhibitory ligands HLA-ABC (black line) on the indicated group 1 cell lines. Isotype control was presented as shaded histogram. Data are representative of 2 independent experiments. (B) NK susceptibility of group 1 cell lines untreated (blue line), treated with anti- HLA-ABC blocking antibody (red line), treated with anti-NKG2D antibody (purple line), treated with anti-HLA blocking antibody+anti-NKG2D blocking antibody (green line) to NK cells at E:T ratios 5:1, 10:1, 20:1. Data are mean % lysis $\pm$ SEM from 3 independent experiments.

Figure A-8. HLA inhibition does not increase NK susceptibility of group 2 cancer cells.

(A) Expression of inhibitory ligands HLA-ABC (black line) on the indicated group 2 cell lines. Isotype control was presented as shaded histogram. Data are representative of 2 independent experiments. (B) NK susceptibility of group 2 cell lines untreated (blue line), treated with anti- HLA-ABC blocking antibody (red line), treated with anti-HLA blocking antibody+anti-NKG2D blocking antibody (green line) to NK cells at E:T ratios 5:1, 10:1, 20:1. Data are mean % lysis $\pm$ SEM from 3 independent experiments.

VITA

Neha Das Gupta was born in Lucknow, India in 1984. She obtained her Bachelor of Science degree from University of Mumbai, India with a major in Biotechnology in 2005. She enrolled at the Institute of Science of the University of Mumbai and completed her Master of Science degree with a major in Biotechnology in May 2007. She joined the Integrated Program in Biomedical Sciences at the University of Tennessee Health Science Center in August of 2007 to pursue her Ph.D. degree. She joined Prof. Wing Leung's laboratory at the St. Jude Children's Research Hospital in Memphis, Tennessee in August 2008 to conduct basic research in the field of natural killer cells and cancers. She is expected to obtain her Ph.D. degree by December 2013. After successfully defending her dissertation, she will begin her postdoctoral fellowship in the lab of Dr. Richard Gilbertson at St. Jude Children's Research Hospital.