NK CELL FUNCTION AND TUMOR RESISTANCE IN MICE TRANSGENIC FOR ANTIBODY TO NK INHIBITORY RECEPTORS

APPROVED BY SUPERVISORY COMMITTEE

_____
Michael Bennett, M.D.

_____
John Schatzle, Ph.D.

_____
Dorothy Yuan, Ph.D.

_____
Victor Garcia-Martinez, Ph.D.

_____
Charles Sentman, Ph.D.

To my parents

NK CELL FUNCTION AND TUMOR RESISTANCE IN MICE TRANSGENIC FOR ANTIBODY TO NK INHIBITORY RECEPTORS

by

Namita Anilkumar Gandhi

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

January, 2005

ACKNOWLEDGEMENTS

I have been blessed to be surrounded by people who have wanted me to achieve and be successful. Their support has elevated me and allowed me to persevere through this process of earning a PhD.

First, I would like to thank my mentors, Michael Bennett and John Schatzle. I have been fortunate to have the insight and expertise of two accomplished investigators. Because of their complementary scientific interests, I have expanded my knowledge of research in many ways. Mike's approachable style made me feel comfortable and at ease from the first moment I stepped into this office. He appreciates that we all come from different walks of life and have different ways to contribute. That is why I think he is able to recognize his students strengthens and brings out their best. Mike encourages new interpretations and insight into scientific questions and allows you to pursue your own ideas. Mike has always been motivating spirit around me, and his style of mentoring has allowed me to mature into independent researcher. I have been fortunate to have been trained in a lab that has produced so many outstanding scientists and it is a credit to Mike's love of science, mentoring capacity and personality. John's strength in mentoring comes from his love of science and teaching. Since he remembers what it is like to be a graduate student, he has been an invaluable emotional support for me especially during frustrating times of failed experiments. He has pushed me to pay special attention and explore area of science that I identified as my weaknesses in order to help me to be a better-rounded scientist.

My thesis committee is composed of a team of researchers with vast expertise in many dimensions of science and experience in training many graduate students. I want to

thank them for their support and direction. Dorothy Yuan has been always taken special interest in me and my work. She has always encouraged me and filled me with hope of accomplishment. I have enjoyed working with Victor Garcia who has great knowledge in many of areas of science. His fair objective criticism in evaluating my work has made me strive to do better and push harder for success and approval. It was very special for me to have Charles Sentman (from Dartmouth University), a former member of the Bennett lab, involved in awarding me this PhD. He has contributed scientifically to the area of research relating to my thesis project.

My first year of graduate school was difficult as my grandmother had passed away and I was adjusting to academic learning after working for several years. Joining the Bennett lab helped build my confidence to be able to meet the challenges of graduate school. Each member of the lab that I met had their own story of overcoming obstacles and as I saw them succeed, I became motivated. The environment of the lab has always been a great environment to learn, discuss science and ask simplistic questions without getting judged. Since the day I joined the lab, I have had guidance from people such as Tad George, Jennifer Klem, Maggie Morris and Gene Devora. In addition, Mesha Taylor and Maria Johansson were a vital part of elevating my learning. I realized a long time ago that I could not learn from just reading articles but I needed discussions and interactions with others for my intellectual growth. Maria was always open to discussing what ever got in my mind about NK cells and science and without realizing it, I was learning from an expert in the NK field. Mesha took special interest in me, training me on many technically aspects and going out of her way to make sure I stayed on a fruitful direction with my research project. Altogether,

Jennifer, Mesha and Maria and I had a wonderful time in lab as good friends and peers. I have encounters similar experiences with Leslie Greenlee, Beth Cameron and Veera Arora. They are great friends that have provided a great comfort to me in lab. They are the friendly faces that make my days in lab more enjoyable.

In addition to their administrative support, I want to thank Karen Kazemzadeh and Marilyn Gibson for their kindness and willingness to address all the needs that I have had in the past few years. Karen, especially, is special to me as has provided me great encouragement and perceptive. Thank you to Wayne for his help over these years. And I would like to thank Borna Mehrad for the scientific discussion in our lab meetings and help with my final defense presentation. Carlos Reyna, Silvio and Maria Pëna have not only helped with animal husbandry, they are my friends and encouragers. What magic Maria possesses to make my non-breeding mice to bred is still unclear to me, but I have appreciated all that they do.

I have developed wonderful friends at UT Southwestern, friends I hope to sustain forever. I would especially like to thank Renee Tall, Kathy Ivey, Vidya Subramanian, and Bo Purtic. Sometimes over wine and cheese, we would discuss our troubles in lab and through the process of these discussions, I think we all comforted each other as we understood the tough moments of graduate school. I could not have made it without you.

My best friends, Claudia Berdugo and Angela Chargois are my outlet and my sanctuary outside science and school. Their pride in my work and understanding of my challenges that come with graduate's school has been a great comfort to me. In addition,

Daljeet Singh's love and support has been invaluable to me during these years. His spirit and energy rejuvenated me and brought me back to a proper perspective.

I want to thank my brother, Mehul and my sister, Hetal for their love and encouragement over these years. After so many years of bickering as young siblings, now, I know Mehul as an invaluable friend, someone who will go out of his way to protect and support me. Hetal with her strong material qualities, conserved values and soft creative side, has always wished for my well-being.

As a daughter of immigrants from India, I have been inspired by the life my parents have built for us. They experienced hardship, arriving to this country with only $6. I was raised with extremely high standards for academic achievement as my father believes that he can give me all the material assets in the world but an education can never be taken away from me. Since my youth, he has pushed me to excel in academic learning, spending countless nights tutoring me. My parent's determination for success has brought me to heights I never thought I would achieve, in spirit and in academic learning. So I dedicate this thesis to you, Mom and Dad, for your patience with me. Thank you for sharing your love, values, spirit to endure with me. Those too are things no one can take away.

NK CELL FUNCTION AND TUMOR RESISTANCE IN MICE TRANSGENIC FOR ANTIBODY TO NK INHIBITORY RECEPTORS

Publication No._____

Namita A. Gandhi, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2005

Supervising Professors: Michael Bennett, M.D. and John D. Schatzle, Ph.D.

Tumor surveillance has been proposed as a means whereby the immune system monitors and eliminates transformed cells before their growth. Transformed cells that survive the immune response are escape variants selected by nature as they have developed mutations in immune recognition components. To boost immune response to these tumors, several types of immunotherapies are being studied but so far have had minimal success

when translated into patient studies. Among proposed immunotherapeutic approaches, monoclonal antibody treatments have shown the best efficacy in human clinical trials.

NK cells, cytolytic effector cells of the innate immune system, are implicated in tumor surveillance. Inhibitory Ly49 receptors determine the specificity of murine NK cells by recognizing of MHC class I molecules expressed on the target cell. This allows the transmission of inhibitory signals through intracellular signals to block NK cytotoxicity. Many tumors express sufficient levels of self MHC class I and are able to escape lysis by NK cells.

Our lab has been studying the inhibitory regulatory pathways in natural killer cells and has developed an approach for enhancing the ability of NK cells to kill tumor cells. We have focused on studying the inhibitory function of the murine Ly49 receptors and provided evidence that blocking of negative signals on two inhibitory receptors, Ly49C and I, with a monoclonal antibody (5E6), allow NK cells to kill syngeneic leukemia cells more efficiently providing an enhanced anti-tumor effect. To study further the effect of Ly49C/I receptor blockade and improve tumor rejection, we developed a transgenic model whereby the 5E6 Fab antibody fragments are constitutively secreted to allow the sustained blockade of the Ly49C/I receptor. These studies detail the generation of these Tg mice and their characterization in relation to NK and T cell receptor development, tolerance, autoimmunity and tumor surveillance. In addition, we demonstrated an effect of blocking inhibitory receptors on NK cells to delay tumor establishment in a nascent tumor model of murine chronic myelogenous leukemia.

TABLE OF CONTENTS

CHAPTER TWO: Materials and Methods

LIST OF PUBLICATIONS

Gandhi, NA, Wolf NC, Greenlee L, Ilaria RL Jr, Schatzle JD and Bennett M. Enhancing NK cells Anti-tumor Immunity in Chronic Myelogenous Leukemia. (Manuscript in preparation)

Gandhi, NA, Greenlee, L, Devora, G, Arora, V, Bennett, M. Tolerance of NK cells in Class I^{high} to Class I^{low} Bone Marrow Cell Chimeras: Potential Role of Hematopoietic Micro-environment (Manuscript in preparation)

LIST OF ABSTRACTS AND PRESENTATIONS

Gandhi, NA, Wolf NC, Greenlee L, Harper A, Ilaria RL Jr, Schatzle JD and Bennett M. Evaluating and Enhancing NK cells Anti-tumor Effect in Chronic Myelogenous Leukemia. Center of Immunology 2004 Scientific Retreat (May 19, 2004) Dallas, TX.

Gandhi, NA, Wolf NC, Greenlee L, Ilaria RL Jr, Schatzle JD and Bennett M. Evaluating and Enhancing NK cells Anti-tumor Effect in Chronic Myelogenous Leukemia. Laboratory of Molecular Pathology 2003 Scientific Retreat (Jan 22, 2004) Dallas, TX.

Gandhi, NA, Yuan D, Schatzle JD and Bennett M. Blocking Negative Signals on NK cells to Enhance Anti-tumor Effect: Development of 5E6 Antibody Transgenic. Immunology Graduate Program 5th Scientific Retreat (May 14, 2003) Dallas, TX.

Gandhi, NA, Yuan D, Schatzle JD and Bennett M. Blocking Negative Signals on NK cells to Enhance Anti-tumor Effect: Development of 5E6 Antibody Transgenic. 34th Annual UT Southwestern Sigma Xi Research Forum/Graduate Students' Organization Poster Session (Nov 8-14 2002) Dallas TX.

Gandhi, NA, Yuan D, Schatzle JD and Bennett M. Blocking Negative Signals on NK cells to Enhance Anti-tumor Effect: Development of 5E6 Antibody Transgenic. Society for Natural Immunity International NK cell Workshop (Oct 5-10 2002) San Jose, Puerto Rico.

Gandhi, NA, Schatzle JD and Bennett M. Blocking Negative Signals on NK cells to Enhance Anti-Tumor Effect. Laboratory of Molecular Pathology 2002 Scientific Retreat (Dec 12, 2002) Dallas, TX.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

-/-	Knockout
5-FU	5-flurouracil
ADCC	Antibody dependent cell mediated cytoxicity
ASGM1	Asialo-glycosphingolipid ganglio-N-tetrasylceramide
B6	C57BL/6
bg	Beige
BMC	Bone marrow cells
BMT	Bone marrow transplantation
BW/I	BW tumor cell transfected with Ly49I
cDNA	Complementary deoxyribonucleic acid
CML	Chronic myelogous leukemia
Con A	Concanavalin A
Cr	Chromium
D8	B6 mouse transgenic for H2 D^d
E:T	Effector to target ratio
$F(ab)'_2$	Bound antigen binding fragments of Ig
Fab	Antigen binding fragment of Ig
Fc	Fragment crystallizable of immunoglobulin molecule
FITC	Fluorescein-isothiocyanate
GFP	Green Fluorescent Protein (GFP)

GVHD	Graft versus host disease
GVL	Graft versus leukemia
H-2	Histocompatibility-2
HC	Heavy chain
HLA	Human leukocyte antigen
HR	Hybrid resistance
Ig	Immunoglobulin
IL	Interleukin
ip	Intraperitoneal
IS	Immuno- surveillance
IUdR	^{125}I-5-iodo-2'-deoxyuridine
iv	Intravenous
IVS	Intervening sequence
K_D	Dissociation constant
KIR	Killer cell immunoglobulin-like receptor
LAK	Lymphokine activated killer
LC	Light chain
mAb	Monoclonal antibody
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
mL	Milliliter
mRNA	Message ribonucleic acid

NK	Natural Killer
NKC	NK gene complex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RBCs	Red blood cells
RNK	Rat natural killer cell line
RPMI	Roswell Park Memorial Institute (medium)
RT-PCR	Reverse transcription-polymerase chain reaction
scFv	Single chain antibody fragment
SHP-1	(SH)-containing tyrosine phosphatase
TAP	Transporter associated with antigen presentation
Tg	Transgenic
WBCs	White blood cells
β_2M	Beta-2-microglobulin

CHAPTER ONE

Introduction

I. TUMOR SURVEILLANCE HYPOTHESIS

Tumors develop when normal cells accumulate genetic mutations in cellular growth or tumor suppressor genes that lead to uncontrolled proliferation. These genetic mutations can affect DNA integrity caused by environmental mutagens, genetic predisposition or transformation by viruses (1). To protect cells from the harmful effects of DNA mutational damage, intracellular processes have evolved to control and repair DNA damage. One way the harmful effects of DNA damage are prevented is by controlling cell cycle progression. A network of protein kinases regulate the cell cycle (2) by establishing a series of checkpoints (1). These checkpoints maintain genomic integrity and cell viability as they may arrest cell cycle progression in cases of extensive DNA damage. Defects in these pathways may result in genetic instability and ultimately result in tumorigenic processes (2).

Besides the molecular mechanism controlling tumor progression, a theory was set forth by Macfarlene Burnet. He believed that genetic changes to human cells probably occurred somewhat frequently and since these changes had harmful consequences, additional mechanisms must have evolved to remove these cells. Burnet refined a theory originally suggested by Paul Elrich (in 1909) and Lewis Thomas (in 1957) which implicated the immune system as one of these mechanisms. Burnet described “cancer immunosurveillance” (IS) as the ability of the immune system to identify and destroy developing tumors (3).

Burnet crafted this theory in great detail taking into account two concepts in immunology noted in the literature from 1950-1960's. First, tumor antigens were discovered by E.J. Foley. He observed that chemical induced tumors possessed antigenic properties (4). In addition, while studying hypersensitivity, Thomas discussed "homograft rejection" as a way the immune system could attack "self" molecules. Thomas went on to propose that this rejection was mediated by thymus-dependent processes and must be involved in natural defense against neoplasia. Together, these findings led Burnet to envision a method for the immune system to distinguish tumor cells from normal cells and induce an effective response that prevented tumor progression. He believed that IS involved a thymus-dependent mechanism of "homograft" rejection due to the existence of tumor antigens (5;6).

Burnet discussed four well-established observations regarding cancer and proposed IS as an explanation for these findings. The first observation noted that the incidence of cancer was higher in the elderly. It was thought that age was a factor that diminished the immune response. This notion was supported by a vaccine study in 1947 (5;6) which showed that older subjects had reduced immune responses following vaccine administration compared to children and young adult subjects. Burnet deduced that as people age, they mount less effective immune responses and therefore their ability to monitor developing tumors may also be impaired. This would provide an explanation for why there was a higher incidence of cancers in the elderly (5;6).

Secondly, it was observed that patients on immunosuppressive treatments after organ transplants (7) or with immunodeficiencies (8) had a higher incidence of tumor development. In this example, as well, the ability of the immune system to patrol and eliminate developing

tumors was deemed defective. Yet, if the immune system was restored, possibly IS could be effective in regressing tumor progression. Burnet pointed out one such example in which an immunosuppressed transplant patient developed carcinoma. Once the patient was removed of immunosuppressive therapy, the cancer regressed (5;6).

Burnet also proposed IS was the mechanism of spontaneous regression, when an established tumor suddenly disappears. Burnet believed that in these rare cases, the initial immune response was ineffective, allowing tumor growth. He predicted that if the immune response could become effective, by an unknown mechanism, the tumor mass could regress or be eliminated. In an examination of over 100 cases of spontaneous regressions, it was noted that the commonality among the majority of patients was that they were children or young adults. Presumably, they would have strong immune systems that could respond and "spontaneously" eliminate the tumor mass (5;6).

Finally, through studies analyzing post-operative tumor specimens, several researchers noted a correlation between patients with higher lymphoid infiltrates in the tumor mass and an increased rate of survival. Burnet reviews these studies to point out that lymphoid cells infiltrating tumor masses indicated that IS was occurring (5;6).

As a result of Burnet's claims, studies were launched to corroborate the hypothesis of IS and its mechanism of action. One such study supported predictions that IS was mediated by cellular components of the immune system. In this study, neuroblastoma cells were removed from human patients and treated *in vitro* with patient's lymphocytes or serum. It was noted that tumor growth was only inhibited by treatment with lymphocytes, not serum (9) therefore supporting IS required cellular components of the immune system.

To explore the validity of the hypothesis further, experiments were conducted by several groups using inbred strains of mice with immunodeficiencies. As in the human system, mice with immunodeficiencies were predicted to have a higher incidence of tumors. One study tested the effects of virus inducing transformation in thymectimized mice. Polyvoma virus caused tumors in C3H mice but C57BL/6 (B6) mice were resistant to transformation. F1 progeny of C3H x B6 mice had an intermediate phenotype and showed 50% incidence of tumor growth after viral infection. But when mice underwent thymectomy at early an age, F1 and B6 mice had significant increases in tumor growth (5;6).

In a tumor study conducted by O. Stutman, using athymic nude mice the findings were surprisingly opposite. When Stutman treated nude mice with 3-methylcholanthrene (MCA), a potent carcinogen, they did not have a higher incidence of tumor development or a shorter tumor latency compared to heterozygous nude controls (10). Stutman concluded that the absence of an intact thymus-dependent immune system did not affect the risk of solid tumor formation after MCA exposure. This study demonstrated that non-virus associated cancer (which are found more common in humans) are not more frequent in an immunodeficient model (11). Even with all the supporting evidence in favor of IS, the extensiveness of Stutman's studies and additional studies using nude mice as a model system (3), temporarily set aside the hypothesis of IS.

As better understanding of the immune system was established, studies in nude mice were re-evaluated. It was shown that nude mice were not completely devoid of cellular immunity and that it was therefore not an absolute model of immunodeficiency (12;13). In more recent years, the IS hypothesis has been reexamined. Now, there is sufficient

supportive evidence validating the existence of tumor surveillance. Studies that linked the immune response with the development of non-virus associated cancer were pivotal in causing the resurgence of the IS hypothesis.

These studies utilized genetically engineered mice to study immune components and molecules involved with immune effector function. First, Interferon-γ (IFNγ) was shown to play a role in promoting tumor destruction and elimination. Tumor cells were made insensitive to the effects of IFNγ by transfection of dominant negative IFNγ α chain receptor. When these IFNγ-insensitive tumors cells were implanted *in vivo*, they did not elicit a strong immune response (14). This prompted more extensive studies using mice unresponsive to the effect of IFNγ. IFNγ receptor α chain knockout (IFNγR-/-) mice and STAT1 knockout (STAT1-/-) mice were developed, since both molecules are required for IFNγ signaling. When these mice were exposed to the chemical carcinogen, MCA, the IFNγ unresponsive mice developed tumors earlier than controls. In addition, IFNγ unresponsive mice were crossed to p53 knockout mice (p53-/-). Since p53 is a tumor suppressor, its absence results in spontaneous tumor development. The double KO mice, (IFNγR-/- x p53-/-) and (STAT1-/- x p53-/-) mice, formed tumors more rapidly than controls. Thus, it was concluded that IFNγ plays a critical role in providing the host with a mechanism to eliminate tumors arising from chemical or genetic mutations (15). In addition, studies were conducted in RAG2 knockout mice (RAG-/-). These mice, having a defect in recombination-activating gene 2, do not produce B or T cells and exhibit tumor development more rapidly than wild type controls following MCA exposure. When RAG-/- mice were crossed to STAT1-/- mice, the effect on tumors after MCA exposure was additive. These double knockout mice developed

more spontaneous tumor growth than RAG-/- mice. This indicated that IFNγ did not only aid T and B cells to mount an immune response to spontaneous tumors, but also may act in a T/B cell independent manner (16).

Another series of studies that provided credibility to the IS hypothesis examined the role of perforin dependent cytotoxicity in the elimination of chemically induced and virus induced tumors. Perforin knockout mice (PKO) were challenged with established tumor lines, MCA exposure and Moloney murine sarcoma and leukemia virus (MoMSV). By assessing tumor development, it was demonstrated that perforin played a role in the elimination of a variety of established tumor lines and MCA induced tumors (17). Additional studies confirmed the finding that perforin was critical for IS of spontaneous developing tumors as PKO mice exhibited enhanced spontaneous tumor formation (18).

When tumors were induced in PKO and B6 mice with the virus, MoMSV, all mice developed tumors at a similar rate and kinetics. In B6 mice, tumor size was the largest between day 10 and12, and the tumor decreased in size by day 20. Interestingly, in PKO mice, the size of tumor was larger and the regression of tumors was slower than compared to B6 controls. This finding was interpreted to suggest that perforin-dependent effector cells may not be effective in the early innate response to viral induced tumors but may be more effective in adaptive phase of tumor rejection. Experiments where CD8+ cells were depleted confirmed that adaptive immune responses were responsible for the observed tumor regression (17). These results suggest that perforin-dependent cells of the innate and adaptive immune system are not equivalent in their response to viral induced tumors and may have differential response to tumors.

Taken together, these studies buttressed the concept of IS because they demonstrated that the immune system was patrolling and eliminating spontaneously occurring and virus-associated tumors. This research also indicated that IS entailed a complex response derived from the innate and adaptive immune system, involving multiple cell types and cytokines, a extension of Burnet's original hypothesis. T cells and natural killer (NK) cells have been implicated as main effector cell types in IS due to their known ability to secrete of IFNγ and lyse targets in a perforin-dependent manner.

II. NATURAL KILLER CELLS

A. Description

The discovery of NK cells forced researchers to revisit initial studies regarding the IS hypothesis. NK cells exhibited natural cytotoxicity or an innate ability to lyse tumor targets without prior sensitization (19). Since their discovery, the base of knowledge regarding NK function, regulation and specificity for targets has grown (which will be discussed in this section).

NK cells have the morphological appearance of large granular lymphocytes. NK cells were found to circulate in peripheral blood and reside in the spleen by assessing NK activity using functional lytic assays (19-23). However, NK activity is low in bone marrow (BM), where NK cells originate. And NK activity is absent in other lymphoid tissues such as the lymph nodes. NK cells are also found in the lung specifically within the interstitial compartment of the lung (19) shown by immunofluorescent staining (24). After NK cells

mature in the BM, they move into the periphery in response to cytokines or chemokines (19-23).

NK cells originate from a T/NK common progenitor cell. This developmental link accounts for many commonalities seen between NK cells and T cells in regards to their cytolytic mechanisms. NK cells and CTLs use exocytosis of granules containing apoptosis-inducing proteins and interactions between Fas-FasL and other death receptors to lyse target cells (25;26). Yet, NK cells differ from T cells in their regulation of cytotoxicity. NK cells, unlike T cells, are part of the innate immune system and do not express antigen specific TCR type receptors and do not require prior sensitization to function. The mechanism by which they initiate cytotoxicity is thought to be different than T cells. Recent visual microscopy data supports this prediction. By observing effector: target coupling events, it was noted that CTL are decisive in their action to lyse a target cell. NK cells are more tentative and appear to undergo a series of checkpoints before they kill (27). By studying cell surface receptors, it has been shown that T cells and NK cells are regulated by their receptors-ligand interactions (see NK regulation).

NK cells can be distinguished from T cells by surface expression markers. Murine NK cells are CD3 negative and express CD16 (FcRIII) and CD49a (detected by DX5 mAb) (28). NK cells in some strains are NK1.1 positive (19). Human NK cells are also CD3 negative and express CD16 and CD56 (19;29). A type of T cell, the NKT cell has aspects of both T and NK cell types. NKT cells are defined by their shared expression of NK1.1+CD3+. They are mostly CD8+ or CD4+ and have a limited TCR repertoire and exhibit specificity to glycolipid antigen (30).

B. NK Functions

1. Tumor Surveillance

NK cells were first identified for their ability to spontaneously lyse tumor targets. Specifically, lymphoid cells from many inbred strains of mice were found to have cytotoxicity against various syngeneic and allogeneic tumors. Effector cells responsible for this action were characterized by two investigators simultaneously. Kiessling and Herberman observed remarkably fast lysis of tumor targets, such as Moloney Leukemia cell (YAC-1), by splenocytes. Visual evaluation of this cell of interest revealed morphology similar to other lymphocytes. Depletion of B and T cells from splenocytes did not abrogate cytotoxicity; instead it was enhanced, possibly due to the enrichment of the responsible cell type. Studies in athymic and nude mice confirmed that natural cytotoxicity was mediated in a T- independent fashion. And it was noted that natural cytoxicity was not present until mice were of 3 weeks of age. Because of these findings, it was thought that the natural cytoxicity was attributed to an undefined immune cell, distinct from other known immune effectors (20;21;31;32). This was an important finding because natural cytoxicity had significant implications for controlling *in vivo* tumor growth and cancer (33). Kiessling described these cells as spontaneous or 'natural killer' cells (22;23).

To better understand the effectiveness of NK lysis of tumors *in vivo*, several studies utilized a mutant strain of mouse with an NK deficiency, the beige mouse. Mice homozygous for the beige (bg/bg) defect exhibited impairment of NK activity. *In vitro*, splenocytes from bg/bg mice displayed severely reduced cytotoxicity compared to

heterozygous beige (bg/+) mice (34). *In vivo*, when mice were challenged with sub-cutaneous injections of tumor cells derived from chemically and virally induced tumors, bg/bg mice developed tumors faster and died earlier compared to their bg/+ controls (34). Additionally, tumor growth in beige mice was investigated using an NK sensitive and insensitive tumor line, where sensitivity was determined by *in vitro* experiments. In this study, the NK sensitive tumors grew faster in bg/bg mice compared bg/+ controls while no difference in growth was observed using the NK insensitive line (35). In a study by Haliotis, bg/bg and bg/+ mice were challenged with several types of tumors such as spontaneous tumors, chemically, virally and irradiation induced tumors. NK defective bg/bg mice died with spontaneous tumors and tumors induced by the chemical carcinogen, benzo [alpha] pyrene (BP) earlier than normal mice or bg/+ mice, supporting the role of NK cells in IS. But when tumors were induced by other chemical carcinogens such as dimethylbenzanthracene (DMBA), irradiation or virally induced tumors, there was not a statistical difference between bg/bg and bg +/-mice. This suggested that NK cells may be effective in the surveillance of certain types of tumors (36).

2. Hybrid Resistance

The ability of NK cells to acutely reject MHC-mismatched bone marrow cells (BMC) was observed prior to the "discovery" of NK cells. It was generally assumed that total body irradiation resulted in total immunosuppression (37). Gustavo Cudkowicz made an observation that challenged this notion and the established laws of transplantation which were based on the idea that all parental histocompatibility antigens were expressed by F1

progeny and should have allowed for tolerance to parental strains grafts. Cudkowicz observed that irradiated F1 hybrid progeny mice could often reject BMC from either parent, implying that histocompatibility antigens were not co-inherited in F1 hybrids. This finding, termed "hybrid resistance" (HR), was very controversial at the time but a retrospective view of the literature revealed that other researchers observed similar findings (38).

Studies were initiated to understand the immune mechanism responsible for hybrid resistance. Rejection of parental BM grafts by F1 mice was thought to be due to the absence of hybrid or hematopoietic histocompatibility antigens (Hh). It was hypothesized that these Hh antigens required homozyosity and therefore were not co-dominantly expressed in F1 mice. Genetic analysis mapped Hh antigens to an H2 gene locus (38). In addition, Cudkowicz predicted that cellular immunity was involved in HR because anti-parental strain antibodies were not detected in F1 mice (38). But interestingly, parental BMC rejection by F1 mice was not affected by thymectomy (37;39).

Cudkowicz and Bennett defined several characteristics of the effector cells of HR. HR was not affected by varying doses of irradiation (37;39). Infant mice between 15-20 days old could not reject parental BMC, but at day 22 mice become resistant to parental BM grafts (37;39). It was determined that the effector cells had a radiosensitive hematopoietic precursor. HR was weakened in mice pretreated with sub-lethal dose of irradiation, to deplete hematopoietic precursors, 1-2 weeks prior to bone marrow transplantation (BMT) with parental BMC (38). In addition, Bennett showed that ^{89}Sr, a bone seeking isotope, was able to abrogate HR in a dose dependent manner (40). This suggested that a BM dependent cell was required for HR. The timing of rejection supported the prediction that HR was

performed by the innate immune system. At different time points after primary BMT, host splenocytes were transferred into syngeneic secondary recipients. By assessing engraftment in secondary recipients, it was determined that primary rejection began no sooner than 12 hours and was completed within 24-36 hours, too short a time to implicate adaptive immunity (40).

After the discovery of NK cells by Herberman and Kiessling, it became apparent that NK cells had many similarities to the effector cell involved in HR (41). The development of anti-NK antibodies and the use of the NK-defective beige mouse strain allowed for further studies that showed reduction or elimination of NK activity weakened or abrogated acute BM graft rejection. Therefore, it was established that NK cells were the main effector cell involved in HR (38).

3. Additional NK functions

After the discovery of NK cells by *in vitro* cytotoxicity assay results, their role in tumor clearance and rejection of MHC mismatched BMC became apparent. As more research regarding their function has been conducted, their role in immune activities has been expanded. It is thought that NK cells play a role during pregnancy since these cells have been found in high numbers at the maternal-fetal interface, specifically decidualized human uterus. Investigators predict that NK cells may participate in the allorecognition mechanisms during pregnancy (42). NK cells also play a role in regulating immune responses of other immune cell types through secretion of cytokines. Through this process, NK cells have been implicated in autoimmunity and tissue inflammation (43) by immune modulation of T cell or

B cell dependent processes (44). NK cells also play a role in eliminating virus-infected cells. NK cells respond to virus-induced interferon α/β (IFN-α and β) which allows them to participate in innate defense against viral infections. The role of NK cells in viral infections was supported by observations that patients with impaired NK cells were more susceptible to severe infectious diseases (45;46).

C. NK regulation

1. Missing Self Hypothesis

NK cells and T cells vary in their regulation and specificity for target cells. T cell activation relies on TCR recognition of antigenic peptides in the context of MHC class I molecules. Klas Kärre suggested that NK cells can recognize and lyse targets because they express reduced levels of 'self' MHC class I molecules. This meant that NK cells do not kill targets because they express foreign antigens but instead respond to the lack of 'self' MHC molecules. Using H2 class I-deficient tumor variants, the hypothesis was formally tested. Tumor challenges in B6 mice showed that H2 deficient tumor variants were rejected while parental H2 positive tumors were not. The nature of the rejection was consistent with known characteristics of NK cells as rejection did not require prior immunization and was independent of T cell activity. Experiments using NK depleting antibody treatment confirmed rejection of H2 deficient tumors was mediated by NK cells. In addition, restoring H2 expression on tumor variants by transfection re-established their resistance to NK mediated lysis (47).

The missing self hypothesis also provided an explanation for HR, as an alternative to the Hh hypothesis. F1 hybrids ($H2^{b/d}$) reject parental BM grafts ($H2^{b/b}$) because the graft fails to express one H2 allele of the host. This causes NK cells to eliminate the graft since it does not display all self MHC molecules of host (47).

The missing self hypothesis states that there is an inverse correlation between MHC class I levels and target lysis by NK cells. The significance of NK regulation in this manner suggests that NK cells and T cells may have complementary roles in the elimination of harmful cells. Adapting to immune selective pressures, transformed or virus infected cells can evade T cells by downregulating MHC class I molecules since T cell identification of targets requires MHC class I expression. In turn, these cells may become sensitive to NK cell mediated lysis.

2. NK Receptors

NK cell activity is controlled by a diverse set of cell surface receptors. The function of these receptors is contingent on environmental factors, such as the nature of the target and cytokines (48). Each cell can express multiple activating and inhibitory receptors and cytotoxicity depends on a balance of these signals (48-53). In most cases, it is thought that the inhibitory signal dominates to prevent autoreactive effects (51;53). Some of these receptors that will be discussed in this section are Ly49 in mice and KIRs in human, CD94, NKG2 family and 2B4.

a. Inhibitory MHC class I specific receptors:

The discovery of NK receptors specific for MHC class I molecules gave further credence to the missing self hypothesis as the mechanism by which NK cells identify their targets. Many of these receptors are encoded by a cluster of genes, called the NK gene complex (NKC), found on chromosome 6 in mice and chromosome 12 in humans. These genes are preferentially expressed in NK cells (48;54).

i. Inhibitory Ly49 and KIRs

By genomic sequencing of the B6 mouse strain, the Ly49 family was one of the first to be identified and has been found to contain at least 16 full-length genes and pseudogenes (*Ly49a* to *Ly49q*) (53). The Ly49 family of receptors is highly related and share greater than 80% amino acid identity. These receptors are C-type lectin transmembrane proteins found as disulfide linked homodimers. Diversity among the gene family is established by alternative splicing and allelic polymorphism (50;53). Analysis of other inbred strains of mice has revealed further variation among this receptor family (55;56).

Ly49 receptors are expressed randomly (57). In addition, NK cells can express multiple inhibitory receptors but "at least one" inhibitory receptor is present for each self MHC class I allele. And, NK cells can co-express inhibitory receptors for non-self MHC class I, the purpose of which is not well understood (58). Ly49 receptors are expressed on overlapping sub-populations of NK cells and with overlapping specificity, in which more than one receptor sees the same MHC ligand. Ly49A was one of the first described NK inhibitory receptors (48-53). It is expressed on 10-20% of NK cells in most inbred strains and binds the MHC class I molecules, $H2D^d$, D^k. The Ly49A ligand specificity and

inhibitory function was demonstrated in functional assays that showed Ly49A+ NK cells were unable to lyse $H2D^d$ or D^k+ target cells (59). Another inhibitory Ly49 receptor, Ly49G2, found on 50% of NK cells and binds $H2D^d$, $H2L^d$. In functional studies, inhibition of lysis of H2-D^{d+} targets was blocked by use of a monoclonal antibody (mAb) specific for Ly49G2, named 4D11 (60).

The 5E6 mAb stained a subset of NK cells, later determined to be Ly49C+ and Ly49I+ cells. Initial studies described the function and specificity of the 5E6+ subset in BM rejection. The 5E6 mAb was developed by immunizing 129/J mice ($H2^b$) with intrasplenic injections of purified B6 NK cells, for the purpose of identifying new NK receptors in the B6 strain of mice. Flow cytometry showed that the 5E6 mAb stained approximately 40-50% of B6 NK cells. Functional assays determined that the 5E6+ subset of NK cells was inhibited by $H2^b$, and this subset was involved in the rejection of $H2^d$ BM (*in vivo*) and lysis of $H2^d$ tumor targets (*in vitro*) (61;62). Later studies showed that the 5E6 mAb cross-reacted with two Ly49 receptors, Ly49C and Ly49I (63). Ly49C was shown to bind $H2^d$, $H2^k$, $H2^b$, $H2^s$ (64). And *in vitro* binding studies showed that Ly49I interacts with $H2^b$, $H2^k$, $H2^q$, $H2^r$ and $H2^v$ (65). Functional assays show strong inhibition of Ly49I by $H2K^b$ (64).

In humans, killer cell immunoglobulin-like receptors (KIRs) are encoded by the NKC. These receptors can be considered functionally similar to Ly49 receptors since both families have paired activating and inhibitory activities and interact with MHC class I molecules. But structurally, they are divergent as KIRs are part of the immunoglobulin (Ig) superfamily and found as monomers. Individual KIR receptors are named in accordance with the number of extracellular Ig domains they contain. For example, KIR3D has three Ig

domains in the extracellular region and binds HLA-A and HLA-B. KIR2D has two Ig domains in the extracellular region and sees HLA-C. Like Ly49 receptors, there is great diversity among KIR receptor expression due to alternative splicing and allelic polymorphism (50).

ii. Inhibitory Ly49 receptors on T cells

In recent years, several transgenic (Tg) mice have been developed to express Ly49 inhibitory receptors on all NK and T cells. In nature, inhibitory NK receptors have been found to be expressed on a small subset of T cells including NKT cells, CD8+ αβ T cells, CD4+ αβ T cells and γδ T cells (66). Ly49 expression on T cells may be increased after viral infection (67). In general, the function of Ly49 receptors on T cells is not well understood. To better elucidate the role of Ly49 on T cell function, Ly49 inhibitory receptor Tg mice have been studied.

In one set of studies by W. Held's group, the Ly49A transgene was regulated by MHC class I promoter and these mice showed MHC class I-specific inhibition of NK cells by preventing rejection of $H2^d$ BM grafts and inhibiting T cell proliferation (68). To further study Ly49+T cell responses, a Ly49A Tg line developed by C. Sentman expressing the receptor transgene using the CD2 promoter, was bred to αβ TCR Tg mice (69;70). In these mice, Ly49A+ T cells showed reduced responsiveness to target cells expressing $H2D^d$ (Ly49A ligand) unless Ly49A was blocked by mAb or an excess of antigenic peptide was provided. This indicated that Ly49 inhibitory function on T cells is similar to its function on NK cells, but the inhibitory signal can be overcome by a strong activating signal through the

TCR (71). These results led to a separate study by P. Duplay, which assessed what factors attributed to T cell activation in the presence of inhibitory Ly49 engagement using Ly49A and Ly49C transfected cells. Based on *in vitro* assays, it was predicted that strength of inhibition by NK inhibitory receptors correlated with surface receptor density and affinity for encountered ligand (72). This claim was supported by plasmon resonance analysis which shows that $H2D^d$ and Ly49A interactions are the strongest among the inhibitory Ly49-ligand combinations studied (73), with K_D values similar to TCR-MHC interactions (74).

To demonstrate the significance of Ly49 inhibitory receptor activity on T cells, Ly49A was compared to CTLA-4, known to downregulate T cell activity (75). CTLA-4 knockout (CTLA-4 -/-) mice develop lymphoproliferative disorder due to the lack of a negative regulator of T cell activity (76). When CTLA-4-/- mice, in D^d background, were bred to Ly49A Tg mice, Ly49A engagement on T cells rescued these mice from the lymphoproliferative phenotype found in the original CTLA-4-/- mice. This study suggested that Ly49A compensated for lack of inhibitory signals on T cells and prevented activation and expansion of the potentially harmful T cells.

Similar to the murine system, inhibitory KIRs may be negative regulators of certain T cells since their engagement has been shown to impair T cell activation by affecting cytoskeleton reorganization (77). To study human lymphocyte activation *in vivo*, Tg mice were created that overexpressed the KIR receptor, KIR2DL3 or CD158b, which recognizes HLA-Cw3. CD158b KIR expression was functional on NK cells in these mice since the Tg mice were unable to reject BM grafts expressing HLA-Cw3. In regards to T cell activity,

these findings mirrored results of the Ly49A Tg mice (70) where inhibitory receptor expression could modulate but not prevent T cell activation (50;66;77).

Ly49A may be a powerful regulator of T cell activity in the presence of its MHC class I ligand, but studies in Ly49I Tg mice suggest that not all Ly49 receptors have such an impact in T cell activation. Ly49I Tg mice (in FVB background) did not exhibit defects in T cell activation or proliferation. In these Tg mice, the Ly49I receptor, driven by a Thy-1 promoter, was functional since these mice were unable to reject of $H2^b$ BM (78;79). Yet, when T cell receptor repertoires and activation was studied, no significant changes were noted from controls. Even though Ly49 receptors have great genetic and structural similarities, this finding suggests that individual Ly49 receptors interact differently with molecules affecting T cell activity (80). The crystal structures of Ly49A and Ly49I were compared and revealed that spatial arrangement of the Ly49I dimer differed from the Ly49A dimer. The Ly49I dimer shows a more open spatial configuration. This further implies that there may be fundamental differences in the manner in which Ly49A and Ly49I interacts with their cognate ligands (81).

In mice and in humans, these inhibitory receptors are mostly found on CD8+ T cells and rarely found on CD4+ T cells. Inhibitory receptor expressing T cells do accumulate with age and infection. Initially, it was believed that these cells have a memory phenotype because Ly49+ T cells also expressed some markers that are associated with memory cells (66;82). More recent data suggests that these cells do not function as memory cells in response to infection (67). The earlier thought was that inhibitory receptors may function on memory T cells to help maintain a senescent state until a significant activating signal is

provided. Now the data is shifting to a notion that these cells are present in a transitional capacity until true memory cells develop (67).

iii. Influence of Ly49 receptors on Tolerance

Immunological tolerance is vital to prevent autoreactivity. The mechanisms involved in developing and maintaining NK tolerance are not well established. It is believed that inhibitory NK receptors play a crucial role in NK tolerance. Even though NK cell receptors are germline encoded, Ly49 and KIR receptors expression varies among different inbred strains and between individuals. There is evidence that NK receptor expression adapts to the environment according to MHC class I expression (83-85). β2m knockout mice (β2m-/-) have an increased surface expression of Ly49 on their NK cells compared to B6 controls (84;86). In more detailed studies, it was observed that Ly49-MHC ligand interactions influenced the Ly49 receptor repertoire in mice double Tg for Ly49A and Ly49C, developed by C. Sentman (69;70). Expression of other Ly49 receptors (Ly49G2, Ly49A and Ly49D) was evaluated in Ly49A, C and C/A Tg mice in the presence and absence of their cognate ligands. When the ligand for the Ly49 transgene was present (Ly49ATg in $H2^d$ background and Ly49C Tg in $H2^b$ background), other Ly49 receptor expression was down-modulated. When ligands were not present for the Tg Ly49A or C (Ly49C Tg in $H2^d$ background), receptor expression was comparable to controls. And when both transgenes were expressed in a $H2^{b/d}$ background, the expression of Ly49G2, Ly49A and Ly49D was further reduced (87). Ly49 receptor alteration may be a process required to maintain a balance between activating and inhibitory signals. MHC class I may help NK cells calibrate receptor

expression as a way to limit the generation of hypoactive NK cells with excess inhibitory receptors (88).

The influence of host MHC class I molecules on NK cell function is also demonstrated by the generation of BM chimeras. For example, by creating B6 to $\beta 2m^o$ radiation chimeras, it was apparent that B6 NK cells became tolerant to class I deficient $\beta 2m^o$ BMC (89). In addition, HR can be abrogated by transfer of BMC into irradiated mice ($H2^{b/k}$ into $H2^b$ mice). These chimeras accept $H2^b$ but reject a third-party $H2^d$ BMC (38). The D8 Tg mice express a transgene for MHC D^d in the B6 background (90;91). NK cells of these mice reject B6 but not D8 tumor cells or BMC grafts. However, when chimeras were made using BMC from B6 mouse into D8 host (B6>D8), these mice accepted B6 and D8 BM grafts (38). When spleen cells from these mice were sorted for D^d+ and D^d- cells and cultured *in vitro*, D^d+ cells could lyse D^d- but not D^d+ target cells. This suggested that D^d+ cells were not tolerant to D^{d-} targets unless they were in a mixed population (D^d+ and D^d- cells) (90;91). Additional studies have examined NK cell activity in fully MHC-mismatched chimeras. In experiments using BALB/c to B6 long term BMC chimeras, these mice accepted BALB/c and B6 BM grafts but rejected β2m-/- BM. As previously reported, NK tolerance was reversed when NK cells from chimeras were cultured in IL-2, but *in vivo* administration of high doses of IL-2 did not alter NK tolerance (92). A more striking result was observed when B6 to TAP-1º BMC chimeras were generated in our lab. In these chimeric mice, the Ly49 receptor repertoire was not altered on B6 NK cells but these chimeras were tolerant to TAP-1º and BALB/c (a third party) BM grafts. To study the effect of host environment on NK tolerance, secondary TAP-1º chimeras were made by transferring

B6 to TAP-1º chimeric BM into irradiated B6 or TAP-1º host (B6>TAP→B6 or B6>TAP→TAP). When challenged with TAP-1º BMC, these secondary chimeras rejected BM based on the previous host environment (unpublished data). NK cells from chimeric mice (such as B6 >TAP chimera) were tolerized to new host environment (such as TAP). All together these studies support the idea that the host MHC class I environment dictates NK tolerance.

In addition, NK receptors influence tolerance mechanisms of T cells. Ly49A Tg mice experience a severe inflammatory disorder in which the mice die within the first few weeks of age (69). By assessing TCR specificities, it was determined that certain Vβ T cell clones normally deleted in normal mice, were present in the Ly49A Tg mice. Proper T cell development relies on signaling between TCR and MHC molecules. This finding suggested that Ly49 receptors could participate in T cell development, maybe by attenuating TCR signaling threshold. Therefore, an alteration of Ly49 expression on T cells may affect T cell tolerance mechanisms (69).

iv. Inhibitory NKG2 Family and CD94

Similar to the Ly49 receptors, the NKG2 family of receptors is c-type lectin proteins. They require dimerization with CD94 for cell surface expression (29;50;53;88). CD94 is an invariant common subunit that has limited allelic polymorphism. NKG2/CD94 receptors were first identified in humans but are highly conserved in the murine system. These heterodimers bind non-classical MHC class I molecules, HLA-E or the HLA-E related molecule, Qa-1 in mice. These molecules are nonpolymorphic and present peptides from

classical MHC class I or HLA leader peptides (50;53;88). Therefore, CD94/NKG2 receptors allow NK cells to indirectly examine the state of MHC class I expression on target cells (53)

v. Inhibitory Receptor Signaling

Inhibitory receptor signaling has been more extensively studied with regards to KIRs. Since both mouse and human inhibitory receptors contain immuno-receptor tyrosine based inhibitory motifs (ITIMs) in their cytoplasmic domains, it is thought that their signaling mechanisms are similar (52). These ITIM motifs become phosphorylated once the receptor is engaged by its ligand. The phosphorylated ITIMs then recruit tyrosine phosphatases, (SH)-containing tyrosine phosphatase (SHP)-1 and SHP-2. Phosphatases are thought to de-phosphorylate substrates required for NK cell activation. Substrates for SHP-1 and SHP-2 are not well described, but may include molecules such as Syk, ZAP 70, phosolipase Cγ, SLP-76, vav and Rac (48;50;51;93). It is believed that the block in NK activation, initiated by inhibitory receptor engagement, occurs early in the signaling cascade since inositol-1,4,5-triphoshate production and calcium flux are not detected (51).

b. Non-MHC Specific Inhibitory Receptors: Example of 2B4

Over the past several years, there has been much debate regarding the function of the 2B4 (CD244) receptor. It differs from other inhibitory NK receptors because its signaling is not mediated by MHC class I and its does not contain intracellular ITIM motifs. Instead 2B4 interacts with CD48 ligand (94) and contains immuno-receptor tyrosine-based switch motifs, ITSMs, intracellular tyrosine phosphorylation sites. There are two alterative spliced isoforms

of murine 2B4. Each form was transfected into the rat NK cell line, RNK-16, and a series of functional lytic assays revealed that 2B4L and 2B4S had contrasting functions. 2B4S was thought to be activating and contains one ITSM and 2B4L was thought to be inhibitory containing 4 ITSMs (similar to human form of 2B4) (95). 2B4L is predominantly expressed on B6 NK cells (96). Originally, functional studies using anti-2B4 mAb, showed that the lytic activity and IFNγ secretion was enhanced. This data was interpreted identifying 2B4 as an activating receptor (97;98). Recently, the role of murine 2B4 was investigated using 2B4 knockout (2B4-/-) by *in vivo* experiments. These studies showed that 2B4-CD48 interactions inhibited lysis and IFNγ production by NK cells (98). This was confirmed by *in vitro* studies testing lytic activity of B6 or 2B4-/- NK cells on CD48(+/-) variants of the P815 tumor cell line (96).

3. NK Activating Receptors

NK activation allows for the release of cytokines to modulate immune responses and the initiation of cytotoxic mechanisms (19). It is believed that NK target lysis is determined by ligand cross linking of activating receptors and the lack (or reduction) of inhibitory receptor engagement. The majority of information regarding NK receptors relates to inhibitory receptors and ligands. But, recently, there has been progress to elucidate the role of NK activating receptors and their ligands in BM rejection, clearance of transformed cells and viral-infected cells.

Activating receptors, such as Ly49D and Ly49H, are similar to the inhibitory Ly49 receptors except they lack ITIMs for cytoplasmic signaling. Instead, these receptors contain

a positively charged amino acid residue in the transmembrane region allowing association with the DNAX-activating protein of 12 kDa (DAP12) molecule. DAP12 is an adaptor molecule that transduces the activating signals through cytoplasmic immuno-receptor tyrosine-based activation motifs (ITAMs) (53;99). NKG2D associates with DAP10 adaptor molecule which transmits an activating signal through a YxxM cytoplasmic motif (100). It differs from other NKG2 family members because it does not require CD94 for expression but is actually expressed as a homodimer (101)

a. Activating MHC class I specific receptors:

As previously discussed, the missing self hypothesis provided explanation for how HR occurred. It also helped explain why D8 mice (B6 mice Tg for D^d) can reject B6 ($H2^b$) BM, as the B6 graft fails to express an H2-D^d allele of the host. But, the missing self hypothesis did not explain why B6 mice rejected D8 BMC (102-104). The expectation was that B6 mice would accept D8 BMC because $H2^{b/d}$ F1 hybrid BMC are accepted by the B6 parental host (105) . The rejection of D8 BM by B6 mice was reversed in the presence of an anti-Ly49A/D antibody (4E5 mAb) (106) and *in vitro*, anti-Ly49D mAb blocked lysis of $H2^d$ Con A lymphoblasts by B6 NK cells. These results implicated Ly49D+ cells as the effectors of rejection of D^d+ cells. To properly test lytic ability of Ly49D+ cells, B6 NK cells were selected that did not express inhibitory receptors, Ly49A, C, G2 and I. A small subset of NK cells, Ly49D+ and Ly49A/C/G2/I- was isolated by flow cytometric sorting. These Ly49D+ cells, not affected by inhibitory Ly49 receptor signaling, were able to lyse D8 target cells.

Thus, it was concluded that this small subset of NK cells was responsible for B6 host rejection of D8 BM (107).

Ly49D has also been found to recognize a molecule termed Hm1-C4 on Chinese hamster ovary (CHO) cells. In fact, CHO cell lysis could be inhibited by blocking the function of Ly49D on B6 NK cells with the 4E4 mAb. Interestingly, Hm1-C4 structurally resembles classical MHC class I molecules (108). Hm1-C4 is an example of how activating NK receptors detect MHC class I "like" molecules.

b. Activating receptors recognizing MHC-like molecules:

Other activating receptors have also been found to recognize the MHC class I like molecules. Ly49H binds to m157, a viral glycoprotein of mouse cytomegalovirus (MCMV) (109). This finding followed another pivotal discovery made by studying genetic resistance to MCMV. B6 mice are resistance to MCMV *(Cmv1^r)* but other inbred strains, such as BALB/c and DBA/2 are susceptible to MCMV *(Cmv1^s)* infection. MCMV susceptibility was genetically mapped to the *cmv1* locus found on murine chromosome 6 at the NKC (110;111). The relationship between the NKC and MCMV resistance was revealed in studies by S. Vidal, using the recombinant inbred strain BXD-8. BXD-8 mice derived from B6 (*Cmv1^r*) and DBÃ2 (*Cmv1^s*), are highly susceptible to MCMV infection but contain a B6 haplotype at *Cmv1*. Analysis of expression patterns of the NKC indicated a deletion of Ly49H in BXD-8 mice. As this was the only alteration among the two strains, it was believed to account for MCMV resistance. This conclusion was supported by additional studies that showed blocking Ly49H or depleting Ly49H+ cells in B6 mice induced susceptibility to MCMV

infection (112-114). Moreover, Ly49H- susceptible strains could be made resistant with Tg expression of Ly49H (115).

L. Lanier initiated experiments to identify the ligand for Ly49H. Since it was shown that Ly49H conferred MCMV resistance and that other Ly49 receptors bound MHC class I molecules, it was speculated that Ly49H probably recognized a 'self' MHC class I molecule presenting a viral peptide or 'self' MHC molecule altered by viral infection. To test this hypothesis, an innovative experimental design was implemented. T cell hybridoma cells were transfected with Ly49H and DAP12, required for activation signaling. NFAT-green fluorescent protein (GFP) was used as a reporter for cell activation, therefore cross-linking Ly49H with mAb allowed the cells to fluoresce. To determine the interaction between Ly49H and MHC, CMV uninfected and infected β2M-/- fibroblasts were used to test the Ly49H reporter system. Most Ly49 receptors bind fully assembled MHC class I molecules but Ly49H did not need MHC expression to distinguish infected and uninfected cells. This supported a previous finding which showed that regardless of MHC expression, NK cells could respond to MCMV infected cells. Therefore, a Ly49H-MHC direct interaction did not mediate lysis of infected cells. Investigators considered that Ly49H was reacting to viral encoded proteins and exposed Ly49H reporter cells to a panel of MCMV deletion mutants. By this approach, m157 was identified as the ligand for Ly49H. By structural analysis, m157 was shown to be a MHC-like protein (109).

Another activating receptor that has been found to bind MHC-like molecules is the NKG2D activating receptor. The molecule, human MHC class I chain related protein A (MICA), was known to be an antigen for human γδ T cells and it was commonly found on

epithelial tumors cells. T cells clones were able to lyse MICA transfected cells unless a soluble form of MICA protein was used to block T cell binding to cell bound MICA. These observations implied that MICA was involved in T cell activation. Investigators were interested in determining whether MICA interacted with TCR or other surface receptors on the effector cells. Several primary cell types and cell lines were evaluated for binding ability to MICA. It was assumed that cell types that bound soluble MICA expressed a MICA specific receptor. In attempts to identify the MICA specific receptor, expression patterns were compared between cell lines staining positive with soluble MICA protein and cells that did not bind MICA protein. DNA subtraction-hybridization was used for this analysis and revealed NKG2D as a candidate receptor. Immunoprecipitation of MICA transfected cells with NKG2D mAb confirmed the MICA-NKG2D association. Functionally, it was shown that NKG2D engagement by MICA receptor mAb could induce redirected lysis of Fc receptor (FcR)-bearing P815 cells. The summary of these findings stated that activating function of NKG2D is dependent on engagement by MICA (116).

MICA homologs have not been found in the murine system. But to identify the murine ligands for NKG2D, florescent-labeled tetramers of the extracellular domain of NKG2D were used on a panel of primary cells and tumor lines. Several cell lines stained for NKG2D binding including the J774 macrophage cell line. A cDNA expression library of J774 was used to identify potential NKG2D ligands. Positive clones were able to trigger NK cytotoxicity and IFNγ release. Upon further analysis, two molecules, H60 and retinoic acid early transcript (Rae1) were identified. These molecules are distant relatives of MHC class I (101). When H60 or Rae1 were expressed on NK resistant tumor cells by transfection, these

cells become sensitive to NK killing in a NKG2D dependent fashion (101;117). Rae I is upregulated on many tumor cells and is not expressed on normal cells. H60 expression may be induced by cellular stress since H60 mRNA has been detected Con A+ LPS activated lymphoblasts but not in unstimulated splenocytes (100).

Intact or normal MHC class I expression acts as an indicator of the health of cells which prevents NK activation by engagement of NK inhibitory receptors. Additional MHC-like molecules, such as MICA, H60, Rae1 and m157, appear to be expressed only when the cell has been altered or undergone stress (101;116). Cellular stress can be induced by tumorigenic processes or viral infection. The study of NK activation ligands has provided insight into the positive signaling mechanisms used by NK cells to identify harmful cell types. These recent findings further implicate the role of NK cells in tumor surveillance.

Additional activating receptors have been implicated in tumor clearance but the identification of their ligands is still under investigation. The natural cytotoxicity receptors, NKp46, NKp44 and NKp30, were discovered on human NK cells to respond in a non-MHC specific fashion (118). New insights to these receptors may be critical in understanding other NK activation mechanisms.

III. IMMUNOSELECTION OF TUMOR VARIANTS

A. Tumor Editing

Recently, investigators reviewing the progress of cancer immunobiology research have concluded that the term “IS” is insufficient to describe the exact interactions between the immune system and cancerous cells (3;119;120). These interactions are more complex than

first recognized. IS was thought to only exist during the early stages of cancer but now it is believed that the immune response persists even as the tumor progresses. Not only does the immune system protect the host from harmful transformed cells, but it is thought that the immune response actually sculpts or shapes the immunogenicity of tumors (119;120). This concept came from observations that tumor cells grown in immunocompromised hosts are easily rejected when transplanted into immunocompetent hosts. Tumors induced by MCA in RAG-/- mice are cleared when transplanted into wild type controls. Similar results were observed when tumors were induced in PKO mice and transferred into controls (16;121). This implies that tumors adapt to the immune environment. They become more immunogenic in an immunodeficient host and more resistant in the presence of a fit immune system (120). Therefore, the preferred term to describe the interactions of the immune system and tumor cells is "tumor immunoediting." This term not only describes the efforts of the immune system to monitor and eliminate transformed cells, but it includes how the immune system influences tumors in evolving immune escape variants. Tumors undergoing immunoediting are subject to mutagenesis permitted by the genetic instability within the tumor cells and selection by the immune response. The tumor cells that survive an immune response are naturally selected as they likely developed alterations or deletions in molecules important for immune regulation or recognition (120;122;123). These tumor variants can go on to evade immune responses and progress into cancerous masses.

The interactions between the host response and cancer cells can be categorized into three stages: elimination, equilibrium and escape (119;120). Elimination is the stage of the immune response proposed by the IS hypothesis. Evidence of its existence is the relative

infrequent incidences of cancer in immunocompetent individuals. The idea of tumor equilibrium can be described as a balance reached between the host immune response and tumor cell variant growth that has survived the elimination phase. At this point, the tumor may be contained but may not be not fully eradicated. Similarly, patients in cancer remission may have a balance between the immune response and the cancer, following anti-cancer treatments. Whether the tumor persists depends on new variants that arise that are resistant to the host immune response (119;120).

B. Tumor escape

Examples of how the shift from equilibrium to escape can occur are demonstrated in some transplant recipients. Two kidney transplant patients, receiving allografts from the same donor, developed metastatic melanoma 1-2 years post transplant. After reviewing the donor's medical history, it was discovered that the donor had undergone treatment for melanoma several years prior but was considered tumor free at the time of his death. It is thought that since the recipients were on immunosuppressive therapies to prevent allograft rejection, the tumor could escape their limited immune response and progress (119). Additional examples such as these have also been reported in the literature in liver transplant patients (124;125).

Tumor escape variants breach host immune defenses. There are several identified mechanisms and probably many other unidentified mechanisms by which tumors escape immune detection (120;126). One way tumor cells avoid immune recognition is by causing immunosuppression. The T cell subpopulation defined by the markers CD4 and CD25

regulates or suppresses the immune response. Evidence has shown these cells maintain T cell homoeostasis and prevent autoreactivity. T regulatory cells secrete immunosuppressive cytokines such as TGFβ and IL-10. It is believed that tumor cells stimulate T regulatory cells as many cancer patients appear to have a higher number of T regulatory cells in peripheral blood than healthy persons (120). Depleting or blocking T regulatory cell function, *in vivo*, can improve tumor surveillance (126).

Tumors can secrete immunosuppressive soluble factors to limit the immune response. Some examples of these cytokines and soluble factors include IL-6, IL-10, TGFβ, vascular endothelial growth factor (VEGF) and prostaglandins. IL-6 inhibits T cell differentiation and stimulates IL-4 to drive a TH_2 response. The TH_1 response is more effective against tumors shown by studies that associated TH_1 reduction with increased cancer risk (127;128). IL-6 also disrupts IFNγ signaling by upregulating IFNγ signaling inhibitors. IL-10 inhibits expression of co-stimulatory molecules and inhibits TH_1 responses. In experimental systems, many transformed cell lines have been shown to secrete IL-10. In patients with B cell lymphomas, high IL-10 serum concentration is predictive of a poor clinical outcome. TGFβ levels are also found to be higher in cancer patients than healthy cohorts. Serum TGFβ levels correlate with the levels of tumor present in patients with colorectal cancer. In lung, breast, prostate and gastric cancers, TGFβ levels are also predictive of a poor clinical outcome. TGFβ suppresses the immune response by limiting T cell proliferation and it affects the lytic ability of T and NK cells by inhibiting expression of perforin (129).

VEGF is known to induce the development of new blood vessels or angiogenesis. It can also suppress the immune response by affecting T cell development (130) and inhibiting

dendritic cell (DC) differentiation and maturation (131). Prostaglandins participate in many physiological processes and are widely expressed in many tissues. DCs and macrophages can make prostaglandins, specifically E2 (PGE_2). This factor can act as an immunosuppressive agent by inhibiting receptors on T cells involved in response to lipopolysaccharide (LPS), IL-2 and IL-12 (132). It also affects NK activity by suppressing NK cell synthesis of IFNγ (133). Prostaglandin synthesis requires catalysis by the enzyme, Cyclooxygenase-2 (Cox-2). Recently, inhibitors of Cox-2 enzyme have been shown to reduce tumor growth and suppress metastases and are now being developed as a cancer drug therapy (134).

Tumor variants can also disrupt signaling pathways required by the immune system. IFNγ is needed by the immune system for effective elimination of primary tumors. As previously discussed, when tumors become insensitive to the effects of IFNγ, they become more resistant to the immune response (14-16). This can occur when tumors develop mutations in key intracellular signaling molecules such as JAK and STAT proteins (135). In addition, the death receptor signaling pathway is vital for tumor clearance. Receptors required for initiating apoptosis are FAS, TNF, and TRAIL. Interactions with their ligands leads to the activation of caspases and cleavage of specific cellular substrates (136). Mutations or deletions in the genes encoding these signaling proteins can affect death receptor killing mechanisms and favor tumor escape and tumor survival. In several types of cancers, it has been seen that tumors downregulate Fas or express FasL to evade immune mediated apoptosis (123;137;138). Tumors can also become resistant to death receptor

signaling by expressing anti-apoptotic molecules or inhibitors of the death pathway that halt the intracellular signaling pathways leading to apoptosis (120;123).

Through acquiring mutations in co-stimulatory molecules, some tumors can evade the immune response. Tumors that lack expression of B7-1 and B7-2, co-stimulatory molecules, can not only prevent T cell activation but also induced anergy in T cells that recognize tumor antigens (123).

The most common defect seen in human tumor cells is an alteration of MHC expression (123;139). As previously discussed, T cells are very effective in eliminating harmful cells but require antigen processing and presentation by MHC molecules. Viruses and transformed cells have evolved ways to disrupt the biochemical pathways allowing proper antigen presentation (120). Epstein-Barr virus (EBV), Herpes simplex virus and cytomegalovirus (CMV) express proteins that upset intracellular processes of proteasomal degradation and peptide translocation, which inhibits MHC expression of antigenic peptides. Many laboratory cell lines and tumors found in patients have deletion mutations in genes required for antigen processing and presentation such as β2m, TAP1 and 2, low molecular weight protein (LMP) subunits (120;123). In addition, tumor cells can have deletions that result in the loss of MHC class I expression.

Tumor cells lacking MHC class I expression should become very sensitive to NK mediated lysis. Though this is well-established *in vitro*, it is puzzling why a high percent of cancer patients have expansion of MHC negative tumors. It is expected that these tumors would be eliminated by NK cells. *In vivo*, NK cells may have a part in elimination of some MHC deficient variants but the surviving cells may have additional mutations that evade the

NK cell response. As MHC class I expression inhibits NK activation, the lack of MHC class I should enable NK cell activation in the presence of an activation signal. In addition, ligands for activating receptors can be downregulated. Some stress-induced activating ligands such as MICA have been identified. But, tumor cells can downregulate activating ligands to avoid NK activation as seen in colorectal cancer patients. In addition, soluble MIC proteins are found in serum of these patients. Soluble MIC can bind to NKG2D to internalize these receptors, preventing NK lysis of tumor cells (140). In addition, more evidence suggests that NK cells interact with other immune cells such as DCs to achieve optimal expansion and activation (141). If these cells are not available or have been disabled by the tumor cells, NK cells may be less effective (123). Tumor variants surviving a NK mediated response may have modulated levels of MHC class I. Tumors may have a selective loss of MHC in which a reduction of MHC expression evades T cell responses but a preservation of sufficient expression engages NK inhibitory receptors. In these cases, tumors could escape immune surveillance (139).

Recently, it has been discovered that tumor cells engage NK inhibitory receptors by expressing HLA-G, a non-classical MHC class I antigen. HLA-G is the ligand for inhibitory NKG2A/CD94 receptors. HLA-G was originally identified in first trimester placental blood vessels and found to protect fetal tissue from NK cell attack. Studies evaluating NK resistant cell lines and ex vivo melanoma biopsy samples, have revealed the expression of HLA-G on transformed cells (142). Leukemia (143) and renal cell carcinomas (144) are some of the human cancers that have been shown to express HLA-G. HLA-G can be expressed in a

membrane bound form or a soluble form (145). The soluble form of HLA-G can be involved in tumor evasion as it can bind and inactivate to NK cells without tumor cell contact.

IV. IMMUNOTHERAPIES

Presently, the course of treatment for most cancer patients with solid tumors is surgery, to remove solid tumor mass, followed by chemotherapy and radiation. Patients with hematopoietic malignancies rely mainly on chemotherapy and radiation (though some receive BMTs). Chemotherapy drugs inhibit cell growth and division by affecting important cellular components such as DNA or the cytoskeleton or they can act as anti-metabolites. The nature of the drugs, to kill all dividing cells, does not exclude normal dividing cells of the immune system, the digestive tract (mouth, stomach, intestines, esophagus), reproductive system (sexual organs), and hair follicles (146). These broad and systemic targets of chemotherapy cause damage to many normal tissues resulting in clinical adverse effects.

Although toxic, chemotherapy, presently, appears to be the most potent weapon against cancer. Due to the great progress in understanding the immune response to cancer, more specific targets for cancer therapies are being identified. Pre-clinical investigations of immune modulating therapies have been promising. Yet, translating these approaches into the human system has been challenging.

A. Cytokine therapies

In 1893, William Coley was one of the first to modulate the immune response using bacterial toxins. This stimulated the immune system in a nonspecific manner resulting in an

enhancement of anti-tumor effects (147). Bacterial adjuvants have been shown to increase the production of pro-inflammatory cytokines and drive TH_1 responses (148). The goal of cytokine therapies is to restore or enhance the immune status that has been diminished by tumor immunosuppression (149). A number of cytokines have been observed to have anti-tumor effects. Potent immunomodulators, IFNα and IL-2 appear to be the most consistent in their effects as demonstrated in animal and clinical studies (150).

IFNα is the first cytokine to be produced by recombinant technology. It has been used for treatment of infectious diseases, solid and hematologic malignant diseases. Results from clinical trials indicate that IFNα treatment increases overall survival of melanoma, multiple myeloma and chronic myeloid leukemia (CML) patients (151;152).

IFNα demonstrates a reduction in cancer cell growth by a mechanism not fully understood. Immunologically, IFNα is thought to enhance CTL and natural killer cell activity and upregulate tumor antigens and MHC class I and class II antigens (152). Similar to other proteins used for therapy, IFNα is administered intramuscularly, subcutaneously or intravenously and not orally to avoid proteolytic degradation. It has a serum half life of several hours and is not measurable in serum after 24 hours. Therefore to improve efficacy, high doses of IFNα are given but high doses are not tolerated by approximately 40% of patients (151;152). Recently, due to the process of pegylation, combining a polyethylene glycol moiety to a biologic protein, the half life of IFNα has been greatly increased. Clinical trials tested its improved efficacy are ongoing (153).

IL-2 is a T cell growth factor and activates T cells and NK cells. In a number of murine tumor models, IL-2 has been shown to be effective in enhancing anti-tumor activity

(154). The use of IL-2 to improve tumor clearance in patients has been dampened by modest response rates and problems with toxicity. Only high-doses of IL-2 appear to exhibit a benefit to patients with metastatic melanoma and renal cell carcinoma (155;156). However, high dose IL-2 causes acute toxicity that is reversed with reduction or cessation of therapy. But more promising, low dose IL-2 in combination with chemotherapies, or other cytokine treatments such as IFNα or TNF appear to have additive anti-tumor benefits (157-159).

Data from animal studies indicate that IL-2 has contrasting roles. IL-2 knockout mice develop lymphoproliferative disorders and therefore it is believed that IL-2 is needed to promote activation induced cell death and limit T cell expansion (160). This finding may help explain the disappointing response rates seen in patients. Yet, a more effective use of IL-2 may be *ex vivo*. IL-2 shows promise as a means of generating effectors cells *in vitro* for adoptive transfer. Lymphokine-activated killer (LAK) cells are IL-2 activated splenocytes or peripheral blood cells and the adoptive transfer of these cells appears to produce higher response rates than with interleukin-2 administration alone (161) (see cellular immunotherapies).

B. Vaccines

Immunomodulatory agents have shown promising results in animal models, but human clinical trials have exhibited limited success. A possible explanation for this is that patients with malignant disease have suppressed immune responses or have become tolerant to tumor antigens. To elicit a stronger antigen specific response, vaccination has been investigated. The goal of vaccination is to manipulate the immune system and prevent disease by priming

the immune system to specific antigens. Therefore, upon re-exposure to antigens, a rapid and robust memory response can be initiated. Commonly, vaccines have been used to provide the host with protection upon repeated exposures to an infectious agent. But, the same concept could be utilized to strengthen the immune response to tumor antigens. Vaccines are being developed that use allogeneic or autologous tumor antigens in the form of proteins, peptides or naked DNA. In addition, tumor antigen pulsed DCs are being evaluated for their effectiveness in priming the immune response to tumors. Using the vaccine approach, there is a potential to develop a more refined and powerful secondary response mediated by T cells that will be directed towards the tumor (120). The limitation to this approach is that the tumor antigen must be defined. Though many common tumor antigens have been identified, the expression of tumors antigens may vary among patients which would negate the effectiveness of vaccination (120).

The success of a vaccine has been measured by the induction of an immune response. Studies using protein or peptide based vaccines noted activation and expansion of tumor-reactive $CD8^+$ T cells in a majority of melanoma or colon carcinoma and chronic lymphocytic leukemia patients. Recent reports evaluating clinical trials results of cancer vaccines have not been able to correlate the increase in immune cell activation with an improved clinical course for patients. So far, the improvement in clinical response has been modest, with only 20% of patients benefiting from tumor vaccination. Even the use of different vaccine designs has not noted better clinical response (162). The transient nature of responses seen in CLL vaccine studies implies that the anti-tumor responses exhibited are short-lived (129). Investigators have been trying to determine how anti-tumor specific

immune responses can be improved in order to see an effect clinically. It is believed that combining cancer vaccines with other interventions may be more effective in achieving good therapeutic response (162).

C. Cellular immunotherapies

1. Bone marrow transplantation

BMTs are effective treatment for several hematological malignancies and immunodeficiencies with the goal of reconstituting a healthy immune system.

In the 1950's, lethally irradiated mice were rescued with BMTs from donor of the same strain (163). But in humans, the first BMTs were performed before the development of conditioning regimens, knowledge of HLA matching and prevention of graft-versus host disease (GVHD) (when donor immune cells attack host tissue) and therefore were less successful than BMT conducted in more recent years. As the field of BMT has advanced, the success of the procedure has dramatically improved. Transplantation conditioning has commonly involved myeloablative therapy which consists of high dose chemotherapy and total body irradiation. This therapy destroys most rapidly dividing cells and non-proliferating lymphocytes and progenitor cells, as well as malignant cells and many hematopoietic cells. This creates "hematopoietic space" which allows engraftment. The harsh nature of myeloablative therapy has been associated with many side effects and transplant related death. Recently, mini-transplantations have been performed that use a less-intensive conditioning regimen of irradiation but requires more immunosuppression with this procedure (164;165).

The source of donor BM cells used in BMT can be autologous (isolated from recipient) or allogeneic (related or unrelated HLA matched, or partially HLA matched donor). The success of the transplant can depend on where the donor cells originated. Use of autologous BM cells has less risk of transplant related mortality but patients have higher rates of relapse due to residual tumor burden. Allogeneic BMT are limited by genetic factors in which only 25% of patients have sibling HLA-matched donors available (165;166). While risks involved in HLA matched cases is relatively low, risk of HLA mismatched transplant is much greater due to GVHD. GVHD is caused by donor T cells that recognize the recipient cells as foreign due to differences in MHC class I and class II antigens. GVHD is particularly severe in immunosuppressed recipients. The clinical signs of GVHD include skin rash and jaundice due to liver damage and diarrhea due to colon damage. Anemia, weight loss and immunosuppression also occur (164).

To prevent GVHD, T cells can be depleted from the BM graft. Though this minimizes the effects of GVHD, it results in reduced engraftment (164) and increased relapses (167). In T cell depleted BMT, engraftment can be improved by administration of higher numbers of hematopoietic stem cells demonstrated by experimental data (167). T cell depletion inhibits remission in patients because the GVHD reaction provides donor T cells that can eliminate residual tumor in the host. This is called graft versus leukemia (GVL) effect. The higher incidence of GVHD in allogeneic BMT is associated with lower relapses due to GVL. In an ideal BMT, the negative effects of GVHD are minimized while benefits of GVL are maximized. In recent studies, approximately 50% patients are receiving deliberately MHC mismatched BM, in which one or more MHC alleles are different (168).

These transplants appear to be successful because they allow a GVL effect and improve BM graft survival.

As more MHC mismatched BMT are conducted, questions regarding the effects of NK cell alloreactivity in human BMT have been raised. NK cells are a relevant cell type to study as they mediate acute BM rejection in inbred mice (38;38;41) and since NK cells are the first lymphocyte population to recover after BMT (169). Because NK cell reactivity is governed by inhibitory receptors, in humans, the expression of KIR and KIR ligands are important to evaluate in patients receiving BMTs. The expression of HLA alleles dictates KIR specificity. For example, individuals homozygous for group 2 HLA-C alleles will possess NK cells with KIR specific for group 2 HLA-C alleles. In an autologous BMT, NK cells are tolerant to host tissues since inhibitory KIR receptors of donor NK cells are engaged by KIR specific ligands found in the host. But, NK alloreactivity is predicted in the case of allogeneic HLA mismatched BMT where donor NK cells do not have KIR specific for the host MHC. This can be defined as "NK mismatched". In a "NK matched" scenario, donor NK cells express KIRs that can recognize HLA class I of host (167).

Interestingly, studies of "NK mismatched" BMT show favorable effects in inducing remission of myeloid leukemia patients. Evidence supports the notion that NK cells do not mediate GVHD but can provide GVL effects. Data from retrospective studies show a difference in relapse rates depending on the method of T cell depletion that was used to remove T cells from BM grafts. One protocol for T cell depletion utilized anti-CD2 mAb which is thought to have broad specificity and deplete T cells and NK cells. Whereas another protocol used other T cell specific mAbs for depletion (without anti-CD2 mAb) and

therefore this method is thought to have a more narrow specificity and retain the presence of NK cells in the BM graft. When T cell depleted BMT were conducted using narrow specificity T cell depleting mAbs, the rate of relapse in patients was comparable to patients that did not have T cell depleted grafts. But, broad T cell specific mAbs for T cell depletion was associated with higher relapse rates. This suggested that NK cells in BM graft were beneficial for improved BMT results, probably due to their ability to facilitate GVL (170).

Other studies have shown the benefit of NK cells in BMT, by studying related and unrelated allogeneic BMT. In one study, investigators predicted that unrelated donor BMTs should display more GVL compared to related donor BMT. Related donors possess more "NK matched" cells with the recipient compared to unrelated donors which will have more "NK mismatched" cells. This study looked at patients with hematopoietic malignancies receiving unrelated allogeneic BMT in which one MHC allele was mismatched compared to related allogeneic BMT from MHC identical donors. All BM grafts were T cell depleted using anti-TCR mAb or anti-CD3 mAb, which should preserve NK cell content of the BMT. The rate of relapse at two years was higher in patients receiving related matched BMT (42%) compared to unrelated mismatched BMT (26%) (170;171). The conclusions from this study were further supported by investigations conducted by L. Ruggeri and colleagues who evaluated the effect of KIR mismatch on transplantation outcome. Prior to BMT, several leukemia patients were evaluated and predicted to have KIR epitope mismatch. By *in vitro* functional assays, donor derived NK clones were tested for alloreactivity against recipient T cell lymphoblasts. These assays revealed a potential for donor alloreactivity but no patients developed GVHD and donor NK alloreactive clones were not found in the patients four

months post transplant. In addition, it was determined that donor derived NK clones were able to lyse recipient myeloid leukemia cells, but not lymphoid leukemia cells. These results correlated with clinical outcomes in which no relapses were reported for the myeloid leukemia patients receiving unrelated mismatched BMT (167;170;172-174).

In addition, NK cells have been associated with the prevention of GVHD. In murine studies, investigators wanted to determine if infusion of NK cells would prevent GVHD. Mice were conditioned with irradiation and adoptive transfer of NK cells prior to the allogeneic BMT. NK cell infusion protected mice from GVHD even with administration of alloreactive T cells in increasing doses (up to 10^7 T cells). Infusion of non-alloreactive NK cells did not provide protection from GVHD (173;175). As an explanation for these findings, it was hypothesized that NK cells may be affecting alloreactive T cell activation by eliminating the APCs that present to alloreactive T cells. This idea was supported by evidence from additional murine studies by L. Ruggeri (173). $H2^{b/d}$ chimeras ($H2^{b/d}$ BMC into $H2^{b}$ irradiated host) were developed so that chimeric mice would have $H2^{b/d}$ APCs. As a control, $H2^{b}$ BMCs were put into an $H2^{b}$ host and these mice have APCs that of the $H2^{b}$ MHC haplotype. Both chimeras were challenged with $H2^{d}$ BMC. $H2^{b/d}$ chimeric mice developed GVHD while $H2^{b}$ mice did not. These results suggest that $H2^{b/d}$ APCs resistant to NK lysis can prime T cells for GVH reactions. Specifically, $H2^{b/d}$ APCs were resistant to lysis by donor ($H2^{d}$) NK cells (since NK inhibitory receptors were engaged). Therefore APCs survived and were available to prime donor ($H2^{d}$) T cells causing GVHD. In the control experiment, $H2^{b}$ APCs are not protected by NK inhibitory receptor engagement and were lysed by donor ($H2^{d}$) NK cells, therefore T cells were not primed by $H2^{b}$ APCs (173).

2. Adoptive transfer and Purging Approaches

The induction of GVL by NK cells is not surprising as data from numerous murine studies have demonstrated the anti-tumor effects of NK cells. In trying to exploit the anti-tumor property of NK cells, S. A. Rosenberg was one of the first to use adoptive transfers of IL-2 activated splenocytes (which include non-immunized T cells and NK cells) in mice with pulmonary metastases. IL-2 administration plus adoptive transfer of LAKs were effective in reducing the size and the number of tumor nodules in these mice (176). In addition, other studies showed that LAK cultures derived from peripheral blood of CML patients were functional and able to lyse NK sensitive targets *in vitro* (177;178). Taken together, these studies provided a rationale for clinical trials. The initial reports of using IL-2 and LAKs in patients with malignancies were encouraging but with further investigations, results were inconclusive regarding the effect on clinical outcome. Patients treated with IL-2 had 10-fold increases in circulating NK cells and these NK cells had improved lytic activity against NK resistant targets. But there were minimal differences between study patients and controls in regards to survival or relapse (179). Rosenberg was involved with many of the clinical trials testing the efficacy of IL-2 and LAKs treatment for cancer. One study enrolled 25 patients with various metastatic malignancies. Patients were treated with 1.8 to 18.4 x 10^{10} autologous LAK cells and up to 90 doses of interleukin-2. Eleven of the 25 patients experienced regression of cancer by 50% of volume (commonly considered a complete response) and complete tumor regression was seen in one patient with metastatic melanoma which was sustained for 10 months after therapy. Partial responses (defined as by less than

50% reduction in tumor burden) were seen in nine patients with pulmonary or hepatic metastases from melanoma, colon cancer, renal-cell cancer and lung adenocarcinoma (180). Another study reported more modest results, with 8 patients having complete responses and 15 patients exhibiting partial responses of 106 metastatic cancer patients receiving LAK cells plus interleukin-2. (181). In a phase II trial with 35 metastatic renal cancer patients, an overall response rate to IL-2 and LAKs treatment was 16%. Two patients displayed complete responses and remained disease free for 12 and 9 months. Three partial responders experienced greater than 50% reduction of all measurable tumor, but one of these patient relapsed in 4 months (182). Different cancers may have different responses to IL-2 and LAK treatment. Clinical trials enrolling patients with melanoma (183), renal cell cancer, colon cancer (184) and Hodgkin's disease (185) only reported modest to low clinical responses. In addition, studies testing LAK administration with continuous infusion of IL-2 also only displayed minimal clinical efficacy (186;187). Some investigators have been optimistic by reporting that results from IL-2 plus LAK trials display a trend toward increased survival (188), while others concluded that the lack of significant clinical responses and high toxicity of the treatment is not justified (189). Treatment toxicity was displayed in a majority of patients in these reported studies but only persisted while on IL-2 treatment.

The use of tumor-infiltrating lymphocytes (TIL) instead of LAKs appears to display a more potent response and appears to be less dependent on IL-2 stimulation. In a murine pulmonary metastasis model, TILs were more effective in eliminating tumor burden than IL-2 LAKs (190). And when human TIL were tested *in vitro*, similar findings were reported (191). A prospective pilot study evaluated the safety of using TIL as treatment from patients

with malignant gliomas. The results indicated that TIL plus IL-2 treatment was tolerated by patients and some efficacy of treatment was also demonstrated (192). But as a treatment option, using TIL is limited to patients with solid tumors. In addition, other studies have showed that TIL have functional defects caused by immunosuppression in the tumor microenvironment (193). Ex vivo experimental data has reported promising findings on the use of TIL as an immunotherapy, but more clinical trials are need to further assess its efficacy.

Ex vivo purging is another cellular immune therapy that has shown great promise. Allogeneic NK cells (194;195), LAKs and now even an NK cell line (NK-92) (196) have been studied for the ability to eliminate transformed cells from stem cell preparations prior to BMT. Purging can be utilized in autologous BMT cases, where recipient BM grafts are thought to contain residual tumor burden following standard cancer therapies (197). Clinical trials have been testing other non-cellular approaches to purging that use chemotherapeutic drugs, monoclonal antibodies and complement, and CD34+ cell selection to isolate healthy cells from the BM preparation. Phase I trials have established the feasibility of purging procedures, but more advanced trials are needed to confirm if purging provides an improved clinical outcome (198).

D. Monoclonal Antibody Therapies

1. Introduction

The majority of the immunotherapeutic approaches have displayed encouraging results in animal models. Unfortunately, this has not translated into demonstrated safety and efficacy

in clinical trials. Presently, the use of mAbs as a treatment for cancer is an exception. Antibody therapies have advanced from exhibiting impressive results in animal models to earning FDA approval status.

Antibody therapies have been designed to act directly or indirectly on the immune system to facilitate the elimination of tumors. By binding tumor antigens on cancerous cells, antibodies initiate Fc-mediated lysis by antibody dependent cellular cytotoxicity (ADCC) or by complement fixation. In addition, mAb can be used to modulate the immune response by interfering with receptor-ligand binding that affect tumor clearance mechanisms (199-201).

The current FDA approved mAb used in patients provide passive immunity to specific tumor antigens. Passive immunity has been used successful for decades with treatments such as Rhogam (mAb to blood RhD antigen), tetanus immune globulin, and rabies immune globulin (202) For cancer treatment, Rituximab (anti-CD20) is one of the first mAbs to be successfully used in non-Hodgkin's lymphoma patients. CD20 is present on 90% of malignant B-cell lymphomas. Rituximab has displayed impressive results in clinical trials with a 46-48% overall response in two Phase II studies and when combined with chemotherapy, 95% of patients responded to treatment with 55% of patients experiencing a complete response (199;200). Alemtuzumab (anti-CD52, Campath-1) is another approved mAb therapy that targets a glycopeptide expressed on B and T cells. It is effective in patients with chronic lymphocytic leukemia or non-Hodgkin's lymphoma. Phase II trials of 50 non-Hodgkin's lymphoma patients demonstrated a 20% overall response. Herceptin or Trastuzumab binds HER2/neu tumor antigen found on breast cancer cells and has been seen to improve one year survival by 16% (200).

These treatments appear to be effective in reducing tumor burden and are well tolerated by patients. The use of immunoglobulin (Ig) that binds cancer cells with limited effect on normal tissue is thought to account for the low level of side effects. Monoclonal antibody therapies are targeted and extremely specific since the antibody only binds to a very precise epitope compared to other immunotherapy approaches that may affect multiple systems and regulatory pathways not yet appreciated.

2. Antibody Engineering

Among the Ig isotypes, IgG has been used commonly in the development of cancer therapies. IgG is a very stable 150 kDa protein. IgG can be broken into three subunits by enzymatic digestion. Papain digestion breaks down the protein into two antigen binding fragments (Fab) and one non-antigen binding (Fc) subunit. Pepsin digestion separates the Fc portion but retains the disulfide bond (hinge) between Fabs to form $F(ab)'_2$ (110kDa). The genes encoding Ig consist of a variable and a constant region of the light chain (LC) and the heavy chain (HC). The epitope binding region of Ig contain three hypervariable or complementarity-determining regions (CDRs) spaced between four conserved framework regions (200;203).

Antibodies are conventionally produced *in vitro* by hybridomas, cells derived from fusion of antibody producing cells and a transformed myeloma cell. These cells grow in culture and secrete 10-100μg/ml of antibody. For large scale mAb production, this method can become expensive and laborious due to the cost of culturing media and purification. Instead, more advanced equipment can be used for large scale production such as hollow-

fiber cell culture bioreactors. The hybridoma cells are grown in extra-capillary spaces of hollow fibers where media can circulate. Cells can stay alive and produce mAb for months. In addition, due to techniques in DNA cloning, recombinant mAbs can be developed in bacteria, yeast, plants, insect and mammalian cells by expression of mAb genes . Mammalian cells are ideal for making functional whole mAb since they contain all the cellular machinery required for correct Ig assembly, post-translational modification and secretion. Growing mAb in bacteria, called phage display, is limited because the cells do not have the necessary machinery to assemble and glycosylate the whole mAb. Yeast cells also do not have all the required machinery to make functional mAb as whole mAb made in yeast appear to have defects in complement mediated lysis. But, bacterial cells and yeast cells are acceptable for the production of recombinant mAb fragments which do not require specialized protein modifications (204).

Previously, whole Ig, $F(ab)'_2$ and Fabs were the only tools for antibody-based therapies. Through advances in antibody engineering, smaller recombinant mAb derivatives have been developed with effective antigen binding. These additional antibody based fragments can be constructed using genes encoding variable and constant domains of specific mAbs. These recombinant molecules range from peptides of one CDR to multivalent molecules of antigen binding subunits. The single chain Fv (scFv) molecule is composed of variable heavy chain and variable light chain fragments connected by a flexible peptide linker. It is a small molecule of 25 kDa and can be manipulated and dimerized or made into a multivalent molecule (200;203;204). Valency of antigen binding subunits affects avidity and the strength of antigen binding. Therefore, dimers of scFv exhibit higher valency and

improved tumor targeting (205). Antibody engineering allows investigators to customize mAb design to exploit the unique features of Ig.

Because scFv and other antibody fragments do not contain the Fc portion, their kinetics and effects *in vivo* are altered. The Fc portion of Ig molecules is recognized by specialized receptors on immune cells. As previously mentioned, binding of the Fc portion to Fc receptors (FcR) allows activation of ADCC and the complement pathway. Therefore without the Fc portion of Ig, cells bound to mAb fragments are not depleted *in vivo*. In addition, these molecules have reduced serum half-life (206). Yet, these small antibody derivatives lacking Fc regions have been found to penetrate tissue more effectively than whole mAb. The pharmokinetics of radiolabelled IgG, F(ab)'$_2$, Fab and scFv specific for the same tumor antigen were tested *in vivo*. At various time points after systemic administration of the reagents, tumors were removed and radioactivity of the tissue was measured. The study showed that the majority of IgG was found concentrated in regions adjacent to blood vessels while scFv was found distributed throughout the tumor mass. F(ab)'$_2$ and Fab appeared to have intermediate tumor penetration based on their size (207). Small antibody fragments provide a greater advantage in designing mAb therapies for the elimination of solid tumors. In fact, intact IgG diffusion into solid tumor masses is limited to 100μm in 1 hour (207). ScFv can reach their maximum tumor penetration in 0.5 hours but for whole IgG to reach that same level of penetration takes 48-96 hours (207). Therefore, whole antibodies have limited effectiveness in patients with solid tumors. But, a barrier for the usefulness of small mAb fragments as cancer treatment is in finding optimal dose to maintain antibody levels at an effective threshold due to their short half-life (208).

It was thought that prolonging K_{off} rates by increasing mAb affinity for a tumor antigen would improve tumor retention of therapeutic Ig. Mutants of HER2/neu antibody gene were made using site directed mutagenesis. Antibody mutants were tested for improved binding to tumor antigens *in vitro*. Results of these experiments correlated increase affinity to longer K_{off} rates. But *in vivo*, high affinity variants displayed reduced tumor retention compared to parental antibody. It was speculated that higher antigen affinity may limit the ability of mAb to diffuse through the tumor mass (205;209).

Modification of antibody structure has provided interesting options to improve cancer therapy. Bispecific antibodies have been developed that contain two antigen binding domains. Bispecific antibodies were first utilized *in vitro* to target tumor antigens and activating receptors on effector cells to show redirected cellular cytotoxicity. Bispecific antibodies facilitate the formation of cell couples between effector and target cells when in close proximity (210). L.M. Weiner has been furthering the development of these molecules by initiating studies with dual specificity Ig for HER2/*neu* and human FcR. In clinical trials, the antibody treatment presented some clinical responses but antibody was not well tolerated because it engaged FcR on circulating leukocytes resulting in massive cytokine release (200;208). The use of bispecific antibodies is still promising, but more attention should be placed on the selection of targets in order to maximize tumor clearance and limit toxicity of treatment.

Antibodies can be conjugated or bound to radioisotopes or linked to a toxins. Radio-immunoconjugates bind a specific target and deliver radiation to facilitate tumor clearance. Presently, radio-conjugated anti-CD20 antibody, called Zevalin, has been approved by the

FDA for treatment of non-Hodgkin's lymphoma. In randomized clinical trials, Zevalin was well tolerated and had significantly improved response rates compared to Rituximab (211). Immunotoxins are mAbs linked to toxin from bacteria (diphtheria toxin or pseudomonas exotoxin) or plant (ricin or gelonin). Upon antigen binding, immunotoxins are thought be internalized by tumor cell and cause inhibition of cellular protein synthesis pathways leading to apoptosis (201;211;212). Mylotarg is an anti-CD33 immunotoxin approved by the FDA for treatment of acute myelogenous leukemia (212).

The use of mouse derived mAb in humans was found to initiate human anti-murine antibody response against the administered antibodies. In addition, murine mAb were less effective in ADCC in humans and have a shorter half life compared with human antibodies. These problems were circumvented by "humanizing" mouse derived mAb. The human hybridomas were developed by immortalizing antibody secreting cells with EBV infection but following this transformation the cells had reduced fusion rates and poor cell stability. Interestingly, several years ago, mice were genetically manipulated to produce a broad range of antigen-specific human Ig molecules. This was accomplished by first developing Ig knockout mice where host antibody genes were silenced by deleting J_H (joining region) for HC and C_κ for LC by homologous recombination. Secondly, transgenic mice were created using yeast artificial chromosome containing human IgG genes and regulatory elements. Human Ig transgenic mice were crossed onto Ig knockout mice (Figure 1.1). These mice would make human antibody upon immunization (204;213;214). The development of these mice was an important step in the production of human mAb for therapies.

Presently, better human fusion partners, myeloma cell lines, have been developed. In addition, using DNA cloning techniques, humanized mAb can be genetically manipulated to contain only murine Fab with human Fc region. The newer humanized mAb retains only a minimal part of the murine Ig. The murine CDRs of antigen binding subunits can be isolated and reassembled with human Ig subunits including human framework regions (215).

3. Immunomodulatory antibodies

Presently, the success of mAb therapies has required the identification of tumor antigens. Since the knowledge of tumor antigens is limited, additional targets for mAb therapy for tumor elimination are needed. The use of mAb to modulate the immune response is currently being investigated. MAbs are being used to interfere with receptor-ligand binding interactions that affect tumor clearance mechanisms. As a potential target, negative regulatory pathways of the immune system are being studied. Negative regulators are required to help maintain tolerance to self and prevent autoimmunity. But for the purposes of anti-tumor immunity, negative regulation of the immune system may limit the effectiveness of immune cells.

T cells are negatively regulated by CTLA-4, which binds to B7-1 and B7-2. Experiments by J.P. Allison showed that blocking CTLA-4 with mAb enhances T cell proliferation. This finding led to the possibility that CTLA-4 blockade could enhance anti-tumor immunity by T cells. To test this hypothesis, mice were injected with B7 negative tumors and treated (ip) with anti-CTLA-4 or control antibodies. Mice treated with anti-CTLA-4 completely rejected tumors compared to control antibody groups (216;217).

These studies were extended and showed similar results against colorectal carcinoma, renal carcinoma, lymphoma prostate, and fibrocarcoma cell lines. CTLA-4 blockade showed increase tumor rejection in established tumors (218) and improved T cell response to parasitic infection (219). CTLA-4 blockade has been combined with potential vaccine therapies. GM-CSF tumor cell vaccines demonstrated improved cross-priming of host APC, but by itself, was not effective in the rejection of established tumors. When combined with anti-CTLA-4 mAb treatments, clearance of poorly immunogenic tumors was greatly enhanced (218). The consequence of abrogating negative regulatory pathways of T cells can be autoimmunity. The initial tumor challenges testing anti-CTLA-4 mAb in animal models did not display autoimmune symptoms. But when CTLA-4 blockade was tested in murine models of encephalomyelitis, autoimmune disease was exacerbated (220;221).

But the strong anti-tumor effects of the receptor blockade rationalized the creation of a humanized form of anti-CTLA-4 mAb and experiments in monkeys with high doses of humanized anti-CTLA-4 did not show increased autoimmunity. In addition, Phase I clinical trials in prostate and melanoma patients also showed no signs of autoimmunity or severe reactions to treatment. Patients were evaluated for levels of prostate specific antigen which correlated to disease. In this study, anti-CTLA-4 treatment appeared safe and showed reduced levels of tumor antigen (222). In another study, 9 previously immunized patients with metastatic melanoma and ovarian carcinoma also displayed no symptoms of autoimmunity, though transient production of auto-antibodies was reported. Five patients exhibited anti-tumor effects suggested by an increase of levels of tumor necrosis factor (223). Additional clinical trials with melanoma patients tested the effects of anti-CTLA-4 mAb with

peptide vaccination for two melanoma associated antigens. Of the 14 patients, only 2 displayed a complete response and one patient had a partial response. But 9 patients suffered high grade toxicity with symptoms of diarrhea, pulmonary infiltrates, dermatitis and colitis (224). Further investigation into anti-CTLA-4 treatment is required to determine if the benefits of treatment justify the potential autoimmunity and toxicity causes by treatment.

NK cells are negatively regulated by inhibitory receptors. In fact, inhibitory receptor engagement limits NK cytotoxicity as a mechanism to maintain tolerance. But, the function of inhibitory receptors also provides an explanation for why NK-based adoptive immunotherapies are only moderately effective. The presence of certain MHC class I ligands can lead to inhibition of over 50% of the NK cells. Yet, when inhibitory receptor engagement by MHC class I ligands is blocked, NK cells display increased cytotoxicity to syngeneic targets. This has been exhibited with the use of the 5E6 mAb that binds Ly49C and Ly49I receptors (Figure 1.2). This was first demonstrated using NK cells sorted from (NZBxB6) F1 splenocytes. The 5E6+NK1.1+ subset of NK cells lysed BALB/c but not B6 con A blasts. The resistance to lysis of B6 con A blasts was reversed when NK cells were treated with 5E6 mAb. These initial studies also established that 5E6+ NK cells were strongly inhibited by the H2-K^b MHC haplotype as B10.D2 5E6+ NK cells were unable to lyse B6 (H2-K^b+) con A blasts but this inhibition was voided by 5E6 mAb treatment (64). Studying the potential for increased cytotoxicity by NK cells lead to the idea that blockade of NK inhibitory receptors may allow NK cells to provide more effective anti-tumor immunity.

The administration of the whole 5E6 mAb depletes NK cells that express Ly49 C/I receptors. This was first recognized because splenocytes isolated from a mouse treated with

5E6 antibody showed a 50% reduction of NK lytic activity (61). But, 5E6 F(ab')$_2$ antibody fragments block Ly49C/I receptor function without depleting NK cells. Throughout these studies described below, where not specified, 5E6 F(ab)'$_2$ fragments were used.

In vitro, the engagement of inhibitory receptors of NK cells results in reduced cytotoxicity. This is demonstrated in Figure 1.3, where IL-15 activated Ly49C transgenic LAKs were used as effectors against MHC class I low target, YAC and YAC cells transfected with H2-K^b (YAC-K^b) targets. Without 5E6 antibody treatment, YAC-K^b tumors were resistant to Ly49C LAKs mediated lysis presumably because Ly49C receptors were engaged by H2-K^b molecules. But these effectors were functional as they lysed parental YAC targets very well. When the Ly49C receptor was blocked by treatment of 5E6 mAb, YAC-K^b lysis was restored to that of parental YAC targets.

In addition, 5E6+ NK cells have exhibited low cytotoxicity against two H2^b tumor cell lines, EL-4 and C1498. But when these 5E6+ NK cells were treated with 5E6 mAb, tumor lysis was significantly enhanced. Also reported, NK receptor blockade impeded growth of these tumor cells *in vitro*. IL-2 activated NK cells from SCID mice were pre-incubated with 5E6 mAb or control normal mouse serum Ig or 4D11 (anti-Ly29G2) for 2-3 hours. NK cells were then cultured with EL-4 and C1498 or allogeneic P815 (H2^d) tumor cells at different effector: target ratios for 48 hours. Viable tumor growth was quantitated by colony formation assay where cells from co-cultures were transferred to methyl cellulose media. Colony growth was enumerated after 6-7 days. Growth of EL-4 and C1498 was significantly reduced following exposure to 5E6 mAb treated NK cells. Inhibition of tumor growth was also reported by *in vivo* studies. Mice were given a lethal tumor dose of C1498

leukemia cells. Mice receiving a biweekly injection of 5E6 F(ab')$_2$ exhibited a fifty percent increase in survival compared to control mice (225).

The effectiveness of NK cells after inhibitory receptor blockade was also demonstrated in ex vivo purging experiments. To mimic a purging scenario, BMC were spiked with a small amount of leukemia cells. As effectors for purging, B6 SCID NK cells were pretreated with 5E6 F(ab)'$_2$ or 4D11 F(ab)'$_2$ or no antibody. BMC preparations were mixed with NK cells at a 1:1 ratio (representing a NK: tumor ratio of 100:1) for 24 hours. Results determined by colony forming assays showed growth of C1498 cells was significantly decreased in both antibody treated groups. When cells from co-cultures were injected into lethally irradiated mice, cells purged with 5E6 treated NK cells survived significantly longer than cells purged with 4D11 treated NK cells. This suggests, for optimal results, that the MHC haplotype of the tumor should be considered as it determines which inhibitory Ly49 specific antibody treatment is needed (195).

These studies were extended to show that purging with allogeneic NK cells is more effective than purging with syngeneic NK cells. Data from "NK matched" and "NK mismatched" BMT, discussed earlier, corroborate this finding (170;226). But in addition, allogeneic NK cells treated with inhibitory receptor blocking antibodies demonstrated greater elimination of tumors compared to antibody treated syngeneic NK cells. Specifically, B10 ($H2^b$) and B10.D2 ($H2^d$) ALAK cultures were treated with 5E6 or 4D11 mAb and incubated with C1498 ($H2^b$) or P815 ($H2^d$) tumor cells. 4D11 treated B10 NK cell were more effective than 4D11 treated B10.D2 NK cells in purging P815, whereas, 5E6 treated B10.D2 NK cells were more effective than 5E6 treated B10 NK cells in purging C1498 tumors (194). These

experiments indicate that blocking interactions of inhibitory receptors and their specific MHC class I ligand can improve tumor clearance efforts by NK cells.

These findings validate an interesting approach for enhancement of NK cell function. Tumor cells escaping NK lysis by selective expression of MHC class I may become susceptible to lysis with blocking antibody treatment. With further research, blockade of inhibitory signals may be a viable approach to be translated into humans. KIR receptors blockade may provide a very specific and useful treatment for tumor cells that are resistant to NK mediated lysis.

V. OBJECTIVES

Project Goal

Development of a model to further study the enhancement of the anti-tumor property of NK cells by blocking inhibitory Ly49 receptors in vivo.

A. Development of 5E6 Transgenic Mice

A limitation to these previous studies of blocking negative signals on NK cells has been the reliance on F(ab)'$_2$ antibody fragments for blocking Ly49C/I receptors. The i*n vitro* production and reduced *in vivo* serum half-life of this molecule has hindered further progress of these studies. In addition, we wanted to study the effects of a sustained Ly49C/I receptor blockade on autoimmunity and tumor surveillance efforts by NK cells which required a continuous source of blocking mAb. To address these issues, we developed a transgenic mouse that secretes the monovalent 5E6 Fab antibody fragments into circulation. Chapter 3 describes the development and characterization of these 5E6Tg mice. We confirmed the presence of the transgene in several founder lines and the presence of RNA transcript for the transgene in the founder line used in these studies. We showed that the transgene was functional as it could reverse inhibition of NK cells caused by Ly49I receptor engagement.

B. Evaluation of NK and T cell receptor expression in transgenic mice

Ly49 receptor expression on NK cells adapts to the MHC environment in which they develop (84;86;87;227;228). In addition, it has been reported that T cell expression of Ly49s affects T cell proliferation and TCR mediated response to antigen in some Ly49 transgenic mice (84;229;230). It has been suggested that blocking Ly49-MHC interactions could alter activation thresholds of T cells. For these reasons, we wanted to determine if 5E6Tg mice

have altered NK and T cell receptor expression. Using flow cytometry, cells from various lymphoid tissues were assessed. In Chapter 3, data from these staining experiments is presented.

C. Functional Characterization of 5E6 Transgenic Mice

Blocking negative regulation of immune cells can result in autoimmunity as seen in CTLA-4 receptor blockade studies (220;223;224). We wanted to determine if sustained NK receptor blockade in 5E6Tg mice would result in a loss of tolerance. We performed syngeneic BMT to assess if 5E6Tg mice would reject syngeneic BM grafts. In addition, we assessed the effect of the transgene on rejection of tumor cells. *In vitro* ^{51}Cr release assay showed that activated splenocytes from transgenic did not have altered cytotolytic activity compared to B6 controls. But, *in vivo* tumor challenges with a syngeneic B16 melanoma cell line resulted in enhanced tumor rejection by transgenic mice.

D. Assessment of the role of NK cells in tumor surveillance in a nascent tumor model

Presently, studies investigating the effects of blocking negative signals on NK cells for enhanced tumor elimination, have utilized tumor models that are not predictive of physiological development of cancer. Therefore, we wanted to study effects of inhibitory NK receptor blockade in a nascent tumor model. We chose to study CML as a well established murine model is available and is comparable to disease in humans. In Chapter 4, characteristics of murine CML are presented. In addition, we studied the susceptibility of

NK lysis of tumors at different stages of murine CML. And finally, we demonstrate that onset of CML can be delayed in 5E6Tg mice.

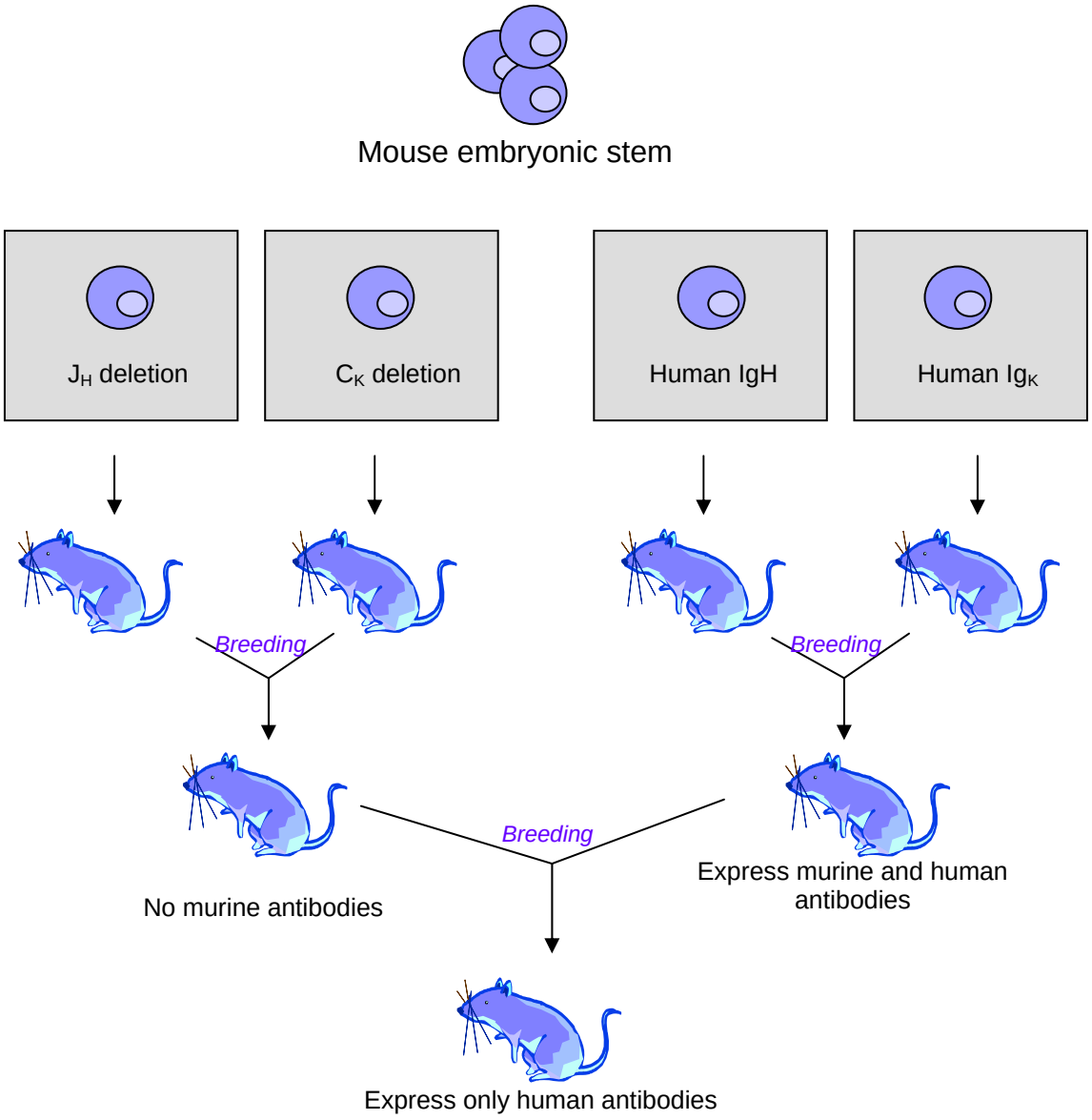
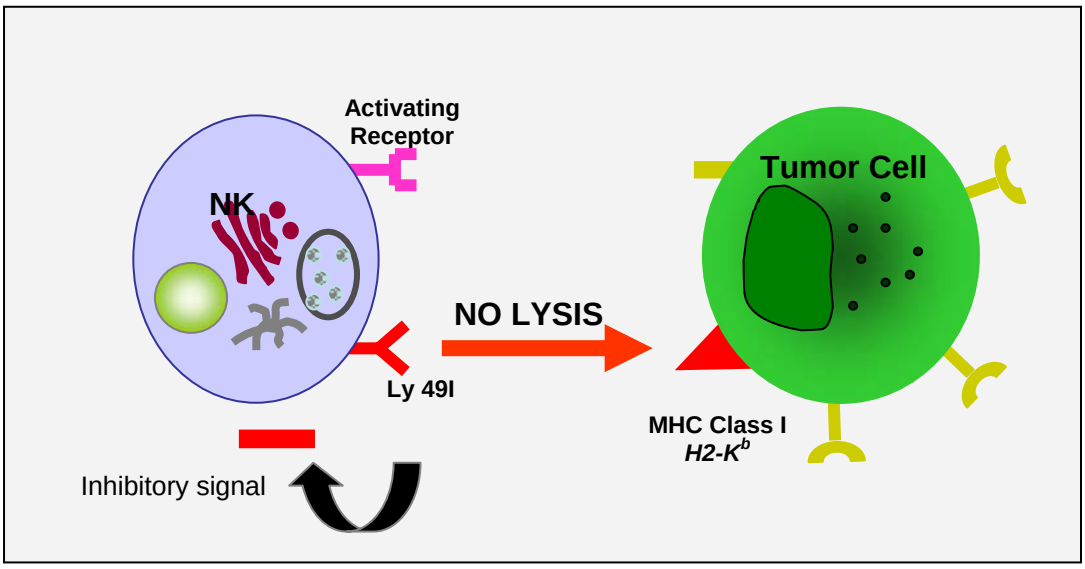


Figure 1.1: Schematic representation of the development of transgenic mice able to produce human antibodies in the absence of murine antibodies. Adapted from *M. Little. Immunology Today. 21.8 (2000) 364-70.*

A



B

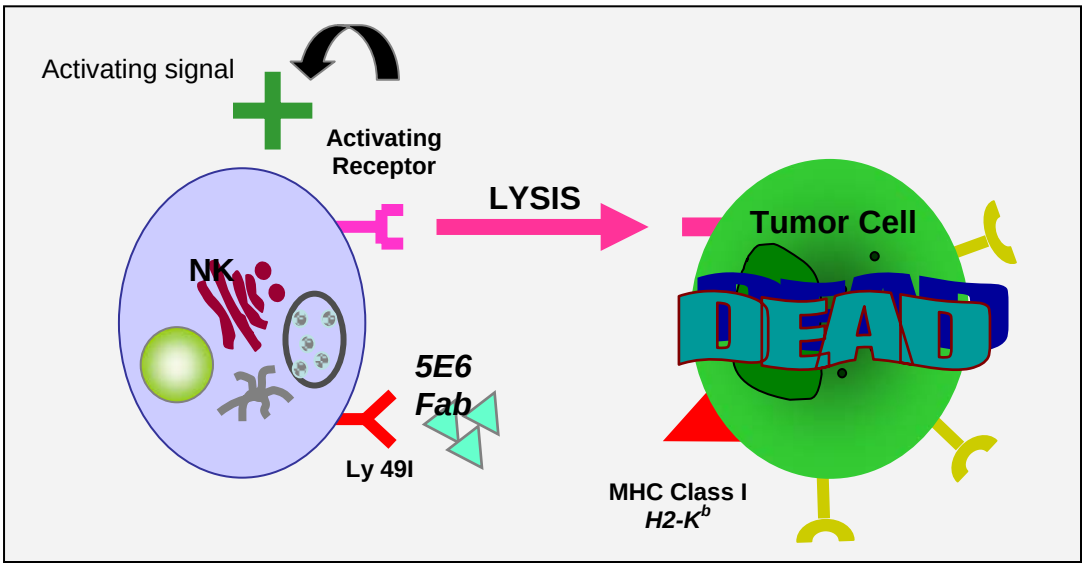


Figure 1.2: Depiction of blocking negative signals on NK cells to enhance anti-tumor immunity. A NK cell in contact with a tumor cell, (A) can be inhibited from lysis by engagement of inhibitory receptor Ly49I by H2-K^b on tumor cell, but (B) when Ly49I receptor is bound by 5E6 Fab, inhibitory receptor interaction with H2-K^b is blocked and therefore NK cell lysis is driven by activating receptor engagement.

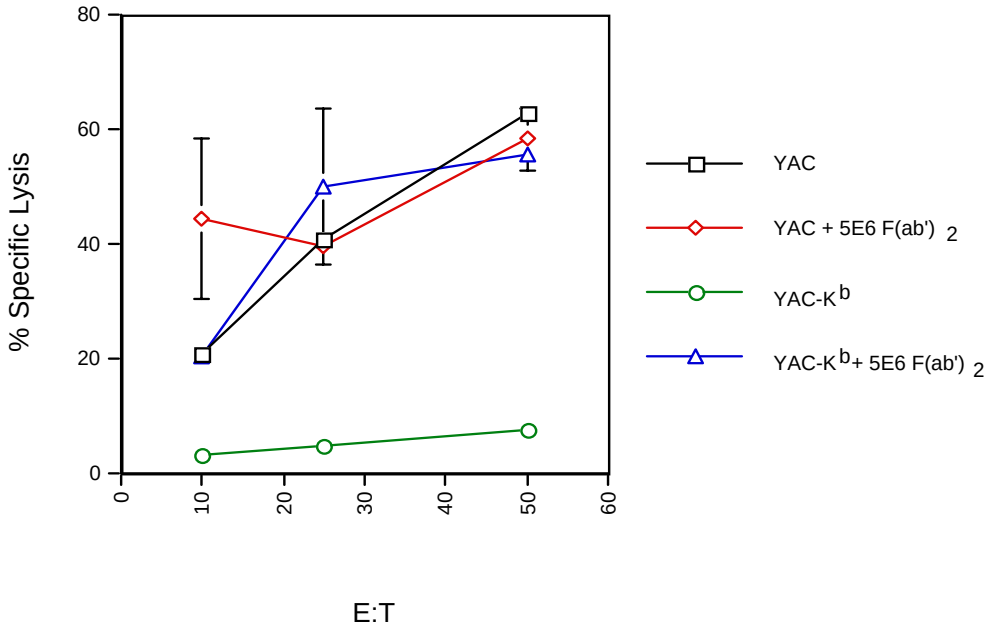


Figure 1.3: 5E6 antibody treatment can reverse inhibition of lysis of Ly49C+ LAK cells by H2-K^b . Splenocytes from Ly49C Tg mice were cultures in IL-15 for 5 days and used as effectors in 4 hour ^{51}Cr release assay. YAC and YAC H2-K^b were used as targets with or without 5E6 F(ab)'$_2$ at 10μg/ml. Graph shows percentage of specific lysis. Error bars indicate standard deviations.

CHAPTER TWO

Materials and Methods

I. DEVELOPMENT OF RECOMBINANT 5E6 ANTIBODY AND TRANSGENIC MICE

A. Cloning of the 5E6 antibody genes

Cloning of the 5E6 antibody genes was conducted for the purpose of developing a non-depleting recombinant antibody. As mentioned in Chapter 1, 5E6 antibody has been adequately tested *in vitro* and *in vivo* for its ability to block the inhibitory action of Ly49C/I (194;195;231) cDNA was made using oligo dT primers from RNA isolated from 5E6 hybridoma cells. cDNA was amplified using degenerate primers for murine heavy chain (HC) and light chain (LC) specific primers (232). The primers used were the following: EcoR1/ Leader murine Light Kappa Chain (5'end): GGG AAT TCA TGG GCA TTC AAG AAT GGA AGA ATC ATC AT; HindIII/murine Light Kappa Chain constant region (3'end): GGA AGC TTA CTG GAT GGT GGG AAG ATG GA; EcoR1/Heavy chain FR-1 (5'end): GGG AAT TCG CAG GTC GAC AAG CTG CAG CGA GTC T; HindIII/Mouse Heavy chain construct (3' end): GGA AGC TTA TCC TCC ACA CAC AGG AGA GCC AGT GGA TAG A. The PCR product was sub-cloned into pGEM T-easy vector (Promega) by rapid ligation. Sequence analysis showed that conceptual translation of each gene fragment, (HC and LC) had high homology to other mAb sequences but diverged at the N-terminal CDR regions.

B. Development of Fc-mutant 5E6 antibody

The 5E6 mAb developed by immunizing 129/J mice ($H2^b$) with intrasplenic injections of B6 LAKs (61), has an isotype of mIg_{2a} and has been found to deplete the Ly49C/I subset of NK cells. All studies to demonstrate blocking negative signals to enhance anti-tumor effects of NK cells have utilized antibody fragments of 5E6, but antibody fragments have a reduced half-life (233). The goal of developing a recombinant antibody to study blocking negative signals required a mAb with a prolonged half life which does not deplete NK cells. One approach used to develop this recombinant whole antibody involved with making alterations to Fc region of existing 5E6 mAb. It was reported that human and mouse IgG1 isotype do not bind human FcγR (234). In order to alter the 5E6 HC constant region from IgG2a to IgG1, a muIgG1 vector was utilized (235),(a gift from E. Sally Ward from UT Southwestern) to fuse the VDJ region of 5E6 HC to the muIgG1 constant region (Hinge-CH2-CH3). Primers were designed and used to amplify the IgG1 coding region using PCR creating a fragment with HindIII 5' and EcoRI 3' restriction sites to facilitate cloning in frame with existing 5E6 HC cDNA fragments. The HindIII digested IgG1 and 5E6 HC fragments were ligated to maintain the open reading frame (ORF). This ligated product was cloned into the pGEM T-vector and the sequence of the construct was confirmed. The Sal/EcoR1 digested 5E6H/IgG1 fragment and 5E6LC fragment were cloned into Sal/EcoR1 digested eukaryotic expression vector (pcDNA (-) and pZeoSV2 (+)) resulting in 5E6HC pcDNA (-) and 5E6LC pZeoSV2 (+) (vectors from Invitrogen). Further alteration was needed to assure that the antibody did not deplete. Site directed mutagenesis was used to convert asparagine to alanine at residue 297 thereby removing the antibody N-carbohydrate attachment site (236). This resulted in converting mIgG1 mAb into a non-depleting antibody (Figure 2.1). For the

production of 5E6 Fc Mutant antibody, double transfections with mutated 5E6HC pcDNA (-) and 5E6LC pZeoSV2 (+) were conducted by electroporation of SP2/0 cells. Stable transfectants were expanded and tested for antibody production. For more efficient *in vitro* expression of 5E6 Fc Mutant antibody, HC and LC cDNAs were cloned into a single vector, pBUDCE4 (Invitrogen), containing 2 promoters (pCMV for LC and pEF-1α for HC). The ~500bp 5E6HC insert was isolated by Kpn I-Not I (Invitrogen) digestion of pcDNA vector and ligated with Kpn-Not digested pBUD vector using T4 ligase at 18º C overnight. The ligation reaction was transformed into DH5α competent cells (Invitrogen) and plated on LB/Amp agar plates and incubated overnight at 37ºC. Bacterial colonies were expanded and mini-prepared (Qiagen). Restriction digestions of minipreps were conducted to identify recombinants. Bacterial clones containing HC cDNA were made into large preparations and used in cloning of LC cDNA. For LC cloning, no compatible restriction sites were available in LC cDNA, therefore restrictions sites Sal I and Sca I were added to LC cDNA by amplifying pZeoSV2 (+) 5E6L plasmid using the following primers: 5E6LSAL (5' END) GTC GAC GTG GGA AGA TGG AGT CAG ACA CAC, 5E6LSCA (3' END) AGT ACT GGT GGT GGC GTC TCA GGA CCT. The PCR product was sub-cloned into pGEM T vector for manipulation. Following DNA large preparation, LC cDNA insert was prepared by Sal I and Sca I digestion and ligated with Sal-Sca digested pBUD containing HC cDNA vector. Cloning was confirmed by sequence analysis. The final pBUD vector construct containing LC and HC cDNA inserts was sequenced. T7 primers were used to confirm LC cloning into multiple cloning site A and EF-1α primers were used to confirm HC cloning into multiple cloning site B. Overall, this recombinant antibody was created and tested to show

binding to Ly49C/I on splenic NK cells, but was not used in further experiments due to problems with its *in vitro* production.

C. Transgenic construct

To develop a transgenic construct for *in vivo* expression of 5E6 antibody fragments, pIRES (Clontech) was chosen due to its strong CMV promoter, presence of multiple cloning sites and inclusion of an internal ribosomal entry site (IRES) sequence. Successful transgenic mice, described in the literature, that secrete soluble molecules or antibodies were referenced when vector and promoter choices were considered. Several antibody transgenic mice have been developed to study questions in immunology, such as autoimmunity and anti-tumor effects (Table 1). For example, the ability of B cells and T cells to become tolerant to autoantibody was studied in the transgenic mice that expressed an antibody for a small nuclear ribonucleoprotein particles (237;238) and transgenic mice making antibody against mouse erythrocytes provided a model to study autoimmune hemolytic anemia (239). The IL-7Tg, mouse in which transgene expression was driven by the MHC class II promoter, was designed to study the effects of IL-7 overproduction on T cell development (240). The Hepatitis B virus transgenic mice secrete Hepatitis B surface antigen by hepatocytes, driven by an albumin promoter, to provide a model for chronic, immune mediated hepatitis (241). In the CTLA4Ig Tg mice, a rat insulin promoter regulated expression of the fusion protein which allowed enhanced allograft acceptance (242). In addition, CTLA4-Hγ1Tg mice which used a mouse Ig promoter for cDNA regulation allowed the study of the effect of blocking CD28/B7 interactions (243-247). Transgenic mice making IgM to ganglioside GD2, sialic

acid-containing glycosphingolipids, demonstrated anti-tumor responses to EL-4 and B16 tumor cells (248). Transgenic mice that secreted anti-CD4 (GK1.5) antibody using rat insulin promoter and CMV promoter provided a novel CD4-deficient model to assess the role of CD4 cells in co-stimulation of CTL activation (249;250). In addition, transgenic mice that produced anti-NK1.1 depleting antibody (by Ig promoter) were developed to study NK-B cells interactions (251). In most of these examples of antibody transgenic mice, HC and LC cDNAs were cloned into two separate vectors. Either, two transgenic mice were created and then crossed (as depicted in Figure 1.1) or both vectors were microinjected into embryos and founder lines were selected if they incorporated both cDNAs. In addition, using a B cell specific promoter is limiting because it requires B cell development for transgene expression. Since, we considered assessing the effects of 5E6 mAb transgene expression on NK development, we required a promoter that was active earlier in mouse development. Moreover, instead of developing 2 transgenic constructs for each antibody gene, we decided to use a bicistronic vector that contained an IRES sequence. Using PCR, restriction sites (Xho I and Mlu I), stop codon and 6x HIS tag was added to HC (excluding Fc region) cDNA. The primers used for this reactions were as follows: XHO1-HC (5'end): CTC GAG CTT ACA ATG AAA TGC AGC; HC-STOP-MLU (3' end): ACG CGT TTA ATG GTG ATG GTG ATG GTG CGA TGG GGC TGT TTT GGC. PCR amplification of plasmid containing HC was conducted and PCR product was sub-cloned into the pGEM T-Easy Vector. Recombinants were identified by restriction digestion. Meanwhile, LC cDNA insert (product of Sal-Not digest of 5E6LpzeoSV2) and linearized pIRES (digested with Sal-Not) were ligated. After transformation into competent cells, several recombinants were identified by

restriction digestion. LC–pIRES DNA was prepared by Qiagen Maxi prep and used for HC cloning. HC cDNA was digested with Xho I and Mlu I and ligated with Xho-Mlu digested LC-pIRES vector. External and internal primers were designed to confirm cloning success by sequencing the entire construct. The primers used were as follows: T7; T3; HC-5E6-SQ: GTC TCT GCA GCC AAA ACA GCC; LC-5E6-SQ: ATC AGC CCG TTT GAT TTC GAC; 5E6-IRES: CAGGTG CCT CTG CGG CCA AAA (Figure 2.2).

D. Testing transgenic construct *in vitro*

The DNA construct was evaluated for its ability to make functional protein. IL-6/SP20 cells (gift from W. Lai at UT Southwestern, IL-6 was transfected into SP2/0 to enhance antibody secretion; SP2/0 myeloma do not secrete Ig unless fused with B cell or transfected with Ig vectors) were transfected with 10 µg DNA by electroporation at 960µF and 300 mV. Cells were rested on ice for 15 minutes before being incubated at 37º C for 2 days without selection media. After two days, cells were plated in 0.4mg/ml Zeocin (Invitrogen) selection media in 96 well flat bottom plates and surviving clones were expanding in 24 well plate. After several days, supernatants were harvested, pooled together and concentrated using a Centricon filtration device (Millipore). Concentrated supernatants were used to stain Ly49I transfected cells (BW/I) followed by secondary stain with anti-mouse IgG H+L antibody (Jackson) to detect bound antibody. Cells treated with concentrated supernatants from transfected cells showed a shift in mean fluorescence intensity compared to untransfected media control (Figure 2.3). From this experiment, it was concluded that the DNA construct was able to encode functional antibody.

E. Preparation for transgenic injections

The transgenic construct was digested to remove extraneous vector sequences which may have interfered with incorporation into the mouse genome, specifically the Neomycin and Ampicillin resistance genes. The original 6088 bp vector after cloning included 421 bp HC sequence and 737 bp light chain sequence was digested with Sfi I (New England Biolabs) and Bgl II (Invitrogen) to result in a 4271 bp DNA fragment. After digestion was complete, DNA was eluted on a 0.8% agarose gel at 48 volts to achieve optimal separation of transgenic DNA fragment from vector sequence (Figure 2.4). The 4271 bp band was gel extracted and eluted in 10 mM Tris (pH 7.3) 0.1mM EDTA (as instructed by Center for Immunology Transgenic Core Facility).

F. Generation of scFv construct

ScFv molecule is a fusion protein of variable HC (V_H) and variable LC (V_L) regions of whole antibody connected by a flexible linker. A schematic diagram of the scFv compared to Fab antibody fragment is shown in Figure 2.5. To create 5E6 scFV, 5E6 mAb HC and LC sequence were assessed to determine the CDR and framework sequences. The location of required sequences for scFv was determined by evaluating conserved antibody sequences found in the Kabat reference (252) and confirmed by E. Sally Ward from UT Southwestern. The DNA construct used to generate 5E6 scFv antibody fragment is depicted in Figure 2.6. PCR primers were used to modify V_L cDNA, which included framework 1-4 regions, to add restriction sites required for cloning and to add the linker sequence. The following primers were used to amplify V_L DNA: 5E6scFv EcoR1-V_L (5' end): GAA TTC CTC GAG AAG

ATG GAG TCA GAC ACA CTC CTG CTA TGG; 5E6scFvLinkV_L (3'end): GC GGC CGC AGA GCC GCC GCC GCC ACC AGC GCC GCC ACC GCC CGA GCC ACC GCC TCC CCG TTT GAT TTC CAG CTT GGT GCC. To prepare V_H sequences for construction of scFv molecules HC vector (5E6H pcDNA) was amplified using the following primers: 5E6scFvLinkV_H (5' end)GCG GCC GCT GAG GTT CAA CTA CAG CAG TCT GGG GCA GAG CTT GTG AA CCA; 5E6scFv Sal-V_H (3' end) GTC GAC GGA TCC TTA CTT ATC GTC GTC ATC CTT GTA CTA CAG ATC CTC TTC AGA GAT GAG TTT CTG CTC TGC AGA GAC AGT GAC CAG AGT CCC TTG GCC. This PCR product contained sequences encoding epitope tags (myc and flag) added to the 3' end of the V_H sequence. PCR products of both reactions were sub-cloned into a pGEM vector. The V_L (5E6L pZeoSV2) vector was digested with Xho I and Not I. The V_H vector was digested with Not and BamHI. Triple ligation reactions were performed with XhoI-BamHI digested pcDNA and digested V_L and V_H cDNA segments. scFv was produced as an alternative approach to study blocking negative signals but not used in *in vivo* experiments.

G. *In Vitro* testing of scFv DNA construct

293T cells were transiently transfected using non-liposomal transduction (Qiagen Effectene reagent) with 2μg of the 5E6 scFv DNA construct. Culture supernatant from transfected cells was concentrated using a centrifuge filter device (Centricon by Millipore) and used to stain BW/I cells. Bound antibody was detected by anti-flag antibody and by anti-mouse IgG H+L specific antibody (Figure 2.7). In contrast to approach depicted in Figure 2.3, to test scFv DNA construct, 293T was transiently transfected, instead of developing stable transfectants,

for faster results. Because of this approach, only a subset of 293T was expected to make scFv after transfection resulting in low concentration of scFv protein in culture supernatant. The staining profile depicted in Figure 2.7does not represent an entire shift in MFI but instead shows only a small subset of BW/I cells binding to anti-mouse Ig secondary.

II. MOLECULAR TECHNIQUES

A. Cloning

When compatible restriction sites were not available on cloning vectors, special PCR primers were designed to add restriction sites 5' or 3' of insert cDNA. Primer sequence typically included 15-18 nucleotides of cDNA sequence in addition to restriction site and/or epitope tag sequence as needed. PCR amplification was conducted on dilutions of plasmid containing the cDNA of interest (1-10 ng). Standard PCR conditions were used (see PCR methods) with annealing temperatures chosen depending on specific primers. PCR products of expected size were extracted from the gel under UV light and purified using a gel extraction kit from QIAGEN. DNA was eluted in sterile H_20 and stored at -20º until used. PCR products were sub-cloned into pGEM T-easy vector (Promega) by rapid ligation at RT as described by Promega T-Easy kit. If compatible restrictions sites were available, most commonly directional cloning was conducted in which two different restrictions sites were used to extract insert DNA and to linearized recipient vector. This approach prevented false positive results in which the vector ligated onto itself. Cloning procedures were conducted as described in (253).

B. Isolation of genomic DNA

Genomic DNA was isolated from mice tail clips to assess 5E6 mAb transgene incorporation. A small clip of tail was collected and digested in a buffer 100mM Tris (pH 8), 5mM EDTA, 0.2% SDS, 200mM NaCl and 350µg Proteinase K at 55ºC for several hours or overnight. Digested tails were vigorously vortexed and centrifuged at full speed (14000 rpm) at 4ºC for 10 minutes. Supernatants were removed and DNA was precipitated by isopropanol. Samples were centrifuged at full speed 4ºC for 10 minutes again. The DNA pellet was dried on the bench top and re-suspended in 100-200µl of sterile H_2O. DNA samples were then utilized as template in PCR reactions.

C. Polymerase chain reaction

Primers for cloning are described in Development of Recombinant 5E6 antibody and Transgenic Mice portion. For genotyping of transgenic animals, primers were selected that would amplify vector unique sequences found in the CMV promoter region and HC region. Candidate primers were identified by putting the pIRES vector sequence into Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3). Of the several primers suggested, primers that amplified the promoter region and giving 500bp PCR product were chosen. Primers were tested on plasmid DNA to ensure a single product was amplified. The following primers were chosen for genotyping: 5E6Tg CMV-HC #1 (5' end): CAC CAA AAT CAA CGG GAC TT, 5E6Tg CMV-HC #2 (3' end) CCC AGC TGC ATT TCA TTG TA. For screening transgenic mice, transgenic vector plasmid was mixed with genomic B6 mouse DNA as a positive control until actual PCR positive mice were identified. B6

genomic DNA and reactions without template DNA were used as negative controls. Standard PCR reactions were conducted using hot-start Taq polymerase (Invitrogen or Sigma) (started at 94ºC for 3 minutes). The PCR cycles ran for 30-35 cycles: denaturation at 94ºC for 30 seconds, annealing at 42-55ºC (varies with primers) for 30 seconds and elongation at 72ºC for 90 seconds. A final elongation step at 72ºC was continued for an additional 5 minutes after which the reaction was completed and held at 4ºC.

D. RT-PCR

RNA was extracted from mouse animal tissue samples. Tissue samples were harvested, weighed and placed in PBS. Single cell suspensions were made by placing the tissue between two frosted microscope slides and gently rubbing slides together. Cells were centrifuged at 2500 rpm for 5 minutes. Cell pellets were resuspended at 60 mg/ml in RLT buffer (Qiagen) containing β-mercaptoethanol and kept at -20ºC until RNA preparations were conducted. RNA preparation was done using Qiagen RNAeasy kit, per manufacturer's instructions. RNA eluted in RNAase-free water was stored at -20ºC. cDNA was made by incubating 10µl of RNA with 1µl Oligo (dt) and 2µl of 10mM dNTP (Invitrogen) for 5 minutes at 65ºC. A mixture of the following reagents was made: 2µl 10x PCR buffer and 2µl 25mM $MgCl_2$ (both from Promega), 1µl Random primers, 1µl MMLV Reverse transcriptase (RT) and 1µl RNAaseOUT (Invitrogen). 7µl of reagent mixture was added to each sample and incubated at 37ºC for 2-3 hours. Samples were incubated at 65ºC for 10 minutes to inactivate the reaction and stored at -20ºC. cDNA preparations were done with and without RT as a control. The following HPRT primers (a gift from I. Stroynowski lab) were used to

assess quality of the cDNA preparation: Forward HPRT primers (7/8th Exon): GAT ACA GGC CAG ACT TTG TTG; Reverse HPRT primers (9th Exon) GGT AGG CTG GCC TAT AGG CT. Using HPRT primers, the samples lacking RT did not amplify a product. For assessment of Tg RNA in tissues, primers were designed to distinguish between template DNA and spliced RNA transcript. The forward primer corresponds to the CMV promoter region and the reverse primer corresponds to the HC region. This PCR product extended across an intervening sequence contained in the transgenic vector. The IVS sequence (132 bp) was spliced out of the final transcript. Therefore, amplification of the unspliced product results in a PCR product of 474bp and the spliced product results in a PCR product of 342bp. A tissue sample resulting in both products would indicate positive expression of transgene. cDNA was amplified for 35 cycles using methods described in PCR section. The following primers were used in RT-PCR: IVS-HC #7: CTT GCA GAA GTT GGT CGT GA; IVS-HC #8: TGT AAC CAT TCG CAG GAT CA.

III. CELL LINES

Tumor cell lines were maintained in culture at 37ºC with 5-10% CO_2 in the air. The following cell lines were grown in RPMI media supplemented (RPMI complete) with 1mM sodium pyruvate, 1mN non-essential amino-acids, penicillin/streptomycin, 25mM HEPES and 10% fetal calf serum (Atlanta scientific): YAC-1 ($H2^a$), YAC-Kb ($H2K^b$), EL-4, BW, BW/I and C1498. The following cell lines were grown in DMEM media supplemented (DMEM complete) with 1mM sodium pyruvate, 1mN non-essential amino-acids, penicillin/streptomycin and 10% fetal calf serum (Atlanta Scientific): B16, 293T, SP2/O and

IL-6/SP2/O. All other tissue culture additives were obtained from Invitrogen. For *in vivo* tumor studies, tumors were expanded and frozen in 90% FCS, 10% DMSO at -80ºC. Prior to each experiment, a new vial was thawed and only maintained in culture for 4-7 days prior to use.

IV. REAGENTS

A. Antibody Purification

Hybridoma cells were grown in complete DMEM media at 37ºC until cells reached a very high cell density. Culture supernatants were separated from cells and concentrated by ammonium sulfate precipitation. Following overnight dialysis in PBS buffer, culture supernatant was filtered through a 0.45 μm filter and prepared for protein purification by HPLC technique (Amersham) using Hi Trap Protein G column (Amersham) and reagents recommended by the manufacturer. Antibody concentration and purity of samples were verified by Coomassie stain (Invitrogen) of non-reducing 10% Tris SDS-PAGE gel (BioRad).

B. Antibody Digestion

For generation of bivalent $F(ab')_2$ antibody fragments, whole antibody samples in 100mM sodium citrate buffer were mixed with 0.2mg/ml pepsin (Sigma) at pH 3.5 and incubated at 37°C for optimal time of incubation (determined by pilot digestion). The reaction was neutralized with 1/10 volume of 3M Tris. Effectiveness of pepsin treatment was confirmed by non-reducing SDS-PAGE gel and Coomassie stain. For production of monovalent Fab

antibody fragments, whole antibody samples in 100mM sodium citrate buffer were mixed with activated papain enzyme (Sigma) and incubated at 37°C for optimal time of incubation (determined by pilot digestion). The papain slurry (at 1/10 volume) was activated by a 30 minute incubation at RT in 50 mM sodium citrate 20 mM cysteine 2 mM EDTA buffer.

V. FLOW CYTOMETRY

A. Preparation of peripheral blood

Peripheral blood was drawn from tail veins and placed in Alsiever's solution (ICN). Cells were centrifuged at 3000 rpm in a microcentrifuge (Sorvall MC12) for 2 minutes and resuspended in PBS. Red blood cells were lysed using warm NH_4Cl-Tris buffer (4g NH_4Cl, 1.58g Tris-HCl in 500 mL dI H_2O, pH 7.2) for 5 minutes. Cells were centrifuged as above and supernatants were aspirated. The remaining cell pellet was used in a cell staining protocol.

B. Preparation of splenocytes and thymocytes

Tissues were harvested and placed into DMEM complete media. Single cell suspensions were made by placing tissue between two frosted microscope slides and gently rubbing slides together. Slides were rinsed with complete DMEM media and cells were passed through a 18g needle or 1 ml pipet-tip to remove cell clusters. Cells were passed through a nylon mesh filter and centrifuged at 3000 rpm. RBCs were lysed with NH_4Cl-Tris buffer. Cells were centrifuged and supernatants were aspirated.

C. Cell Staining Protocol

Cells were treated with culture supernatant from the 2.4G2 hybridoma to block Fc receptors and placed at 4° C for 20-30 minutes. Cells were washed with PBS + 2% FCS buffer by centrifuging at 3000 rpm and supernatants were aspirated. Monoclonal antibodies were added to cell pellets in 100µl volume and incubated for 20-30 minutes at 4° C in the dark. Excess antibody was removed by washing in PBS plus 2% FCS buffer and cells were prepared for flow cytometry collection after filtration through nylon mesh. Flow cytometry was performed using FACscan or FACs Caliber instruments (Becton Dickinson).

VI. *IN VITRO* ASSAYS

A. Chromium release assay

Cytotoxicity assays were performed as previously described (254). Target cells were radiolabeled with 200 µCi Na $^{51}CrO_4$ (^{51}Cr) for one hour at 37ºC and washed to remove excess label. Effectors were plated at varying ratios to target cells and incubated for up to 4 hours. 100µl of the supernatant was removed and the amount of ^{51}Cr released from lysed cells was determined by liquid scintillation counting. The percent specific lysis was calculated by the following formula: [(^{51}Cr cpm experimental release -spontaneous release)/(maximum release-spontaneous release)] x 100. Triplicate samples were used. If antibody treatment was required, antibody was pre-incubated with effectors for 30 minutes prior to plating with targets.

B. Cellular competition assay

This assay was developed to detect the presence of 5E6 Fab antibody in the serum of 5E6Tg+ mice. Ly49I transfected cells (BWI) were treated with serum from transgenic mice and with a set amount of conjugated 5E6 whole antibody as a positive control. Although the binding avidity of whole antibody to Ly49I is greater than Fab, it was predicted that if Tg serum contained 5E6 Fab molecules, a reduction in mean fluorescence intensity (MFI) would be observed. 5E6 FITC (BD Pharmingen) was titered from 100ng to 1ng on 50,000 BWI cells . 5E6 FITC concentration of 12.5 ng was chosen because it appeared to be a sufficient amount of antibody for staining with little excess, so that if a competing antibody was provided, a change in FITC staining could be detected. Controls for the experiment were performed to establish background MFI levels and maximum MFI by 5E6 FITC staining. Background MFI was established by treating BWI cells with B6 serum. Maximum MFI was established by treating cells with B6 serum and 12.5 ng 5E6 FITC antibody. A standard was established by treating cells with B6 serum, 1000ng or 100ng or 10ng 5E6 Fab plus 12.5 ng 5E6 FITC. Ten-fold dilutions were performed for each sample. To determine percent competition by Tg serum, background was subtracted from each value and then this equation was applied to normalize values between experiments: [1-(sample MFI/maximum MFI)]x 100. The assay was validated by use of several non-transgenic serum samples in which no decrease in MFI was seen.

VII. GENERATION OF EFFECTORS

A. LAKS

Splenocytes from B6, B6.Ly49C Tg and BALB/c were cultured in complete DMEM, 2.25 x 10^{-5}M 2-ME, 1μg/ml indomethacin and 50-150ng/ml mouse recombinant IL-15(R&D) or 500units/ml of IL-2 (Chiron). Cells were plated at 3 x10^6 cells/ml in 24-well flat bottom plate for 5 days at 37°C in 10% CO_2

B. *In vivo* activated effectors

For effectors, mice were treated with 200-300 μg of polyinosinc-polycytidylic acid (poly IC) (ip) (Sigma or Calbiochem). Spleens were harvested the following day. Lymphocytes, suspended in 10 ml were layered above 10 ml of ficoll (Amersham Biosciences) and spun at 9000 rpm high speed for 20 minutes (Beckman). Buffy coat was removed and washed twice in complete DMEM.

C. Negative selection of NK cells

Occasionally, experiments required LAK cultures enriched for NK cells. This was done by removal of CD3+ cells from splenocytes by antibody depletion. Cells were suspended in PBS+ 2%FCS at 100 x 10^6/ml. CD3-biotin (BD Pharmingen) antibody were added to cells and incubated for 20 minutes at 4°C. Cells were washed of excess antibody and then incubated with magnetic strepavidin microbeads (BD or Miltenyi Biotec) for 15 minutes at 4°C. Cells were passed through a MACS CS magnetic separation column from Miltenyi Biotec. Cells that flowed through the column (which were depleted for CD3+ cells) were collected and put into culture for development of LAK cells, as previously described in LAKs section.

D. Positive Selection of NK cells

The surface marker DX5 was used to positively enrich for NK cells. Splenocytes were suspended in PBS+ 2%FCS at 200 x 10^6/ml. DX5-biotin or NK1.1-biotin (BD Pharmingen) antibody were added to cells and incubated for 20 minutes at 4°C. Cells were washed of excess antibody and then incubated with magnetic strepavidin microbeads (BD) for 15 minutes at 4°C. Cells were passed through Miltenyi MACS VS or LS separation column (according to manufacturer instructions). The column flow-through contains $DX5^-$ cells, which were discarded. The column was removed from magnet and cells were eluted by plunger action.

VIII. ANIMAL EXPERIMENTS

A. Bone Marrow Transplantation

Transplantation experiments were conducted as described (255-258). Bone marrow cells (BMC) were harvested, under sterile conditions, from femur, tibia, hip and spine bones of donor mice. Bones were crushed by mortar/pestle and collected in serum-free media. Cells were aspirated and filtered through sterile mesh and washed in PBS before resuspension to an appropriate concentration for transplant. Cells were injected intravenously (iv) through tail veins of lethally irradiated recipient mice. Proliferation of transplanted cells was determined by uptake of [^{125}I]UDR , a DNA specific precursor and thymidine analog, in the spleen 5 days post-transplant. Percent splenic uptake was determined by comparison to [^{125}I]UDR injected, noted as standard, (Splenic cpm value /standard cpm value) x 100. Parametric and non-parametric statistical analyses were used to determine differences between groups.

B. BCR/ABL Tumor Model

Procedures were conducted as described (259) with modifications (Figure 2.8). Briefly, donor mice were treated (ip) 4-5 days prior to BMT with 5 mg of 5-fluorouracil (Sigma). Approximately 1 to 1.5 donor mice were required for 1 recipient mouse. Donor mice were sacrificed and BMC were collected as described in BMT methods. Live lymphocytes from BMC were separated by ficoll gradient centrifugation and then stimulated with cytokines (at final concentration of IL-6, 10 ng/ml, SCF, 70 ng/ml and IL-3, 6 ng/ml (BioSource)) in 10 ml complete DMEM overnight at 37°C 10% CO_2 in the air. The cells were transduced with retroviral supernant in the presence of polybrene (to enhance virus uptake into cells) by centrifugation at 2500 rpm (in Sorvall RT6000) for 90 minutes to enhance transduction efficiency. Transduction was repeated with fresh viral supernatant the following day. Lethally irradiated recipient mice were infused with 0.2-0.5 $x10^6$ retrovirally transduced cells. Low transduction efficiency (less than 1%) was sufficient for mice to develop CML-like disease. Mice were monitored for GFP expression of peripheral blood leukocytes to assess disease progression, since the vectors used contained the GFP reporter gene (Figure 2.9).

C. Generation of Retrovirus Stocks for Transduction of Injected Cells

Procedures were conducted as described (259) with modifications. Retroviral vectors were purified by buoyant density centrifugation in CsCl or by Qiagen DNA large preparation. 293T cells were transiently transfected with 2 μg of retroviral GFP-containing vector (control

vector, MIGR1 or p210/GFP, shown in Figure 2.9) and 2 μg of MCV-Ecopack, an ecotropic single-genome packaging construct using the Qiagen Effectene transfection reagent, following manufacturer instructions. The culture supernatant was harvested 2 days after transfection and frozen at -80°C. Transduction efficiency was assessed by flow cytometry for presence of GFP expression compared to untransfected control cells. GFP expression was used to correlate with viral titer.

D. Antibody treatment of mice

For T cell depletion, 200μg of anti-CD4 mAb (GK1.5) and anti-CD8 mAb (2.43) were administered (ip) 2 days prior to BMT. Control experiments showed 95% reduction of CD3+ cells in peripheral blood. For NK depletion, 25μl of rabbit anti-asialo GM1 serum (Wako Chemicals) was administered (ip) one day prior to BMT or 200μg of anti-NK1.1 mAb (PK136) was administered 5 days prior to BMT.

IX. STATISTICS

Microsoft Excel was used to calculate means, standard errors and student T-tests. Values were considered statistical significant if $p<0.05$.

Table 2.1: Examples of soluble protein transgenic mice

Transgenic Protein	Purpose/Finding	Promoter	Reference
Antibody to small nuclear ribonucleoprotein particles	Studied peripheral B cell and T cells tolerance to an autoantibody	Mouse Ig promoter	(238;260)
Antibody to mouse erythrocytes	Provided a model for autoimmune hemolytic anemia	Mouse Ig promoter	(261)
IL-7 cytokine	Evaluated the effects of IL-7 overproduction on T cell development	MHC class II promoter	(240)
Hepatitis B surface antigen	Provided a model for chronic, immune mediated hepatitis	Albumin promoter	(241)
CTLA4Ig fusion protein	Demonstrated enhanced allograft acceptance	Rat insulin promoter	(242)
CTLA4-Hγ1	Provided a model for the study the effect of blocking CD28/B7 interaction	Mouse Ig promoter	(243-247)
Antibody to ganglioside GD2 sialic acid-containing glycosphingolipids	Demonstrated anti-tumor responses to EL-4 and B16 tumor cells	Mouse Ig promoter	(248).
Anti-CD4 (GK1.5) antibody	Provided a novel CD4-deficient model to assess the role of CD4 cells in co-stimulation of CTL activation	Rat insulin promoter and CMV promoter	(249;250).
Anti-NK1.1 (PK136) antibody	Developed to study NK-B cells interactions	Mouse Ig promoter	(251)

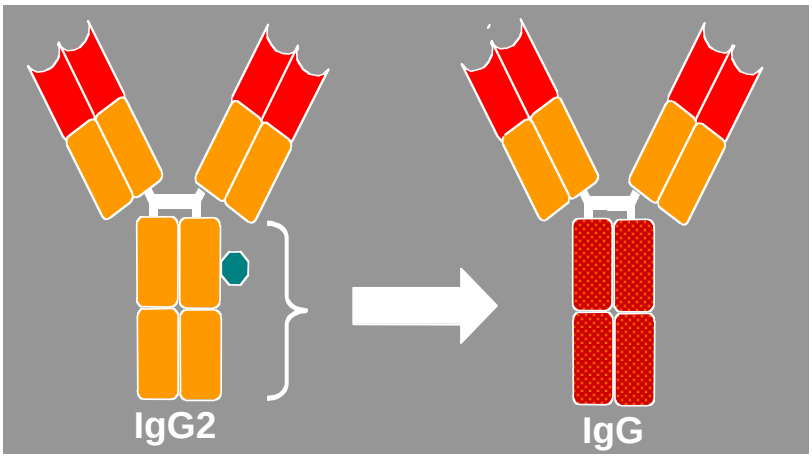


Figure 2.1: 5E6 antibody modifications to create non-depleting mAb. 5E6 HC constant region IgG2a was altered to IgG1 by molecular cloning methods. This was followed by site directed mutagenesis which destroyed the N-carbohydrate attachment site by converting asparagine to alanine at residue 297. The resulting recombinant antibody has been shown to bind but not deplete Ly49C/I, in vivo.

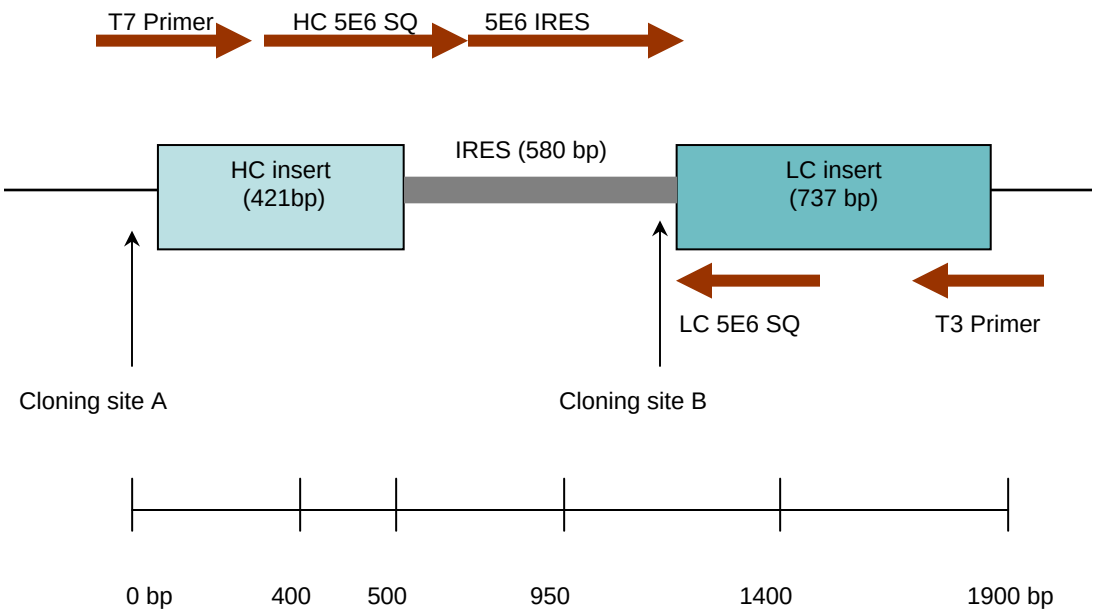


Figure 2.2: 5E6Tg DNA construct indicating primers used in sequencing. To confirm accurate DNA cloning, a variety of primers were designed to allow sequencing to span genes inserted in both cloning sites.

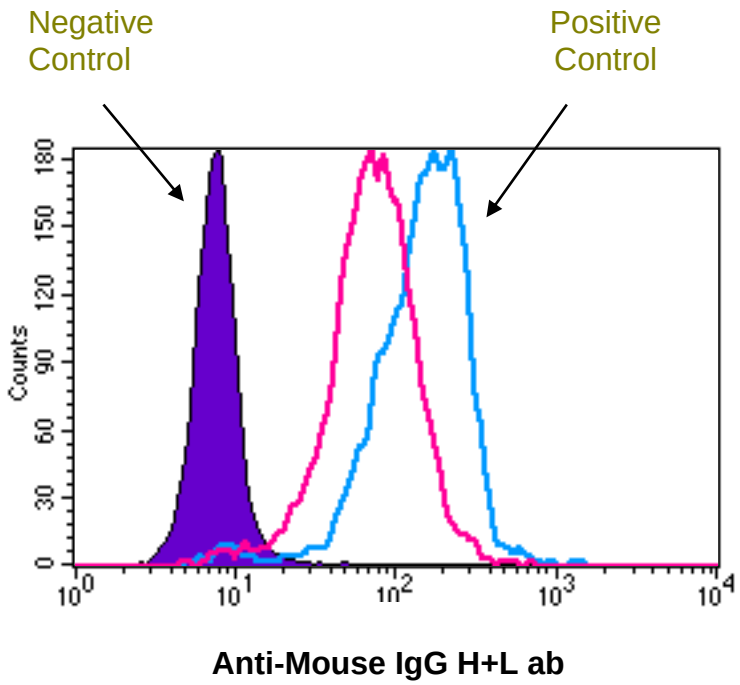


Figure 2.3: 5E6Tg DNA construct makes functional antibody that binds Ly49I. Ly49I transfected cells were treated with culture supernatant from control 5E6 Tg non-transfected cells (purple), 5E6 Tg transfected cells (pink), control supernatant spiked with 5E6 F(ab)'$_2$ (blue) and bound antibody was detected by secondary anti-mouse IgG H+L Ab.

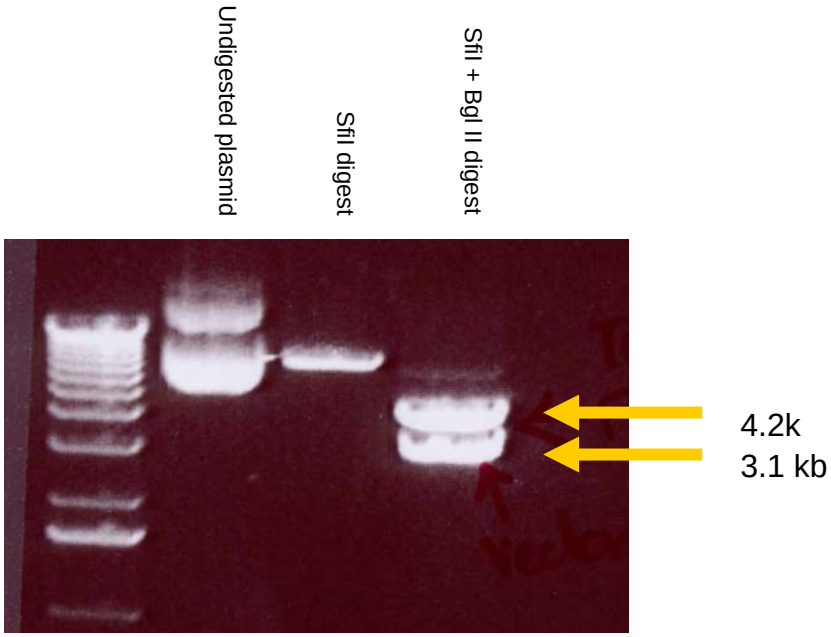


Figure 2.4: In preparation of transgenic infections extraneous vector sequences were removed using Sfi I I and Bgl II restriction digestion. 4.2 Kb corresponds to Tg fragment that was purified and used in transgenic injections. 3.1 kb band corresponds to remaining extraneous vector DNA.

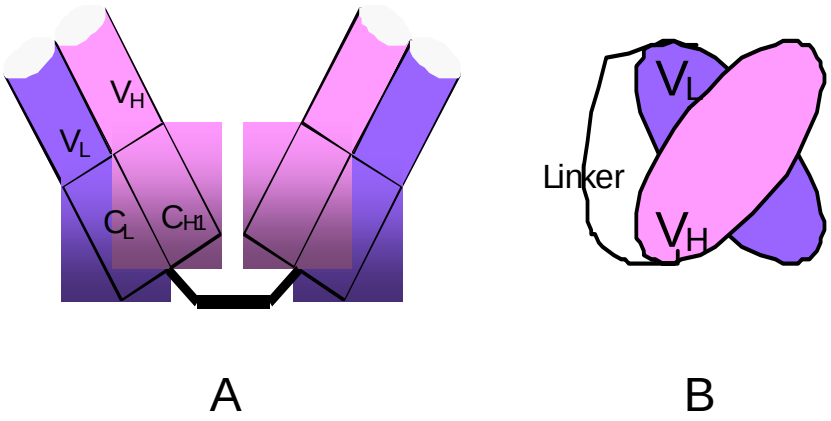


Figure 2.5: Schematic of (a) $F(ab')_2$ antibody fragment structure compared to (b) scFv structure.

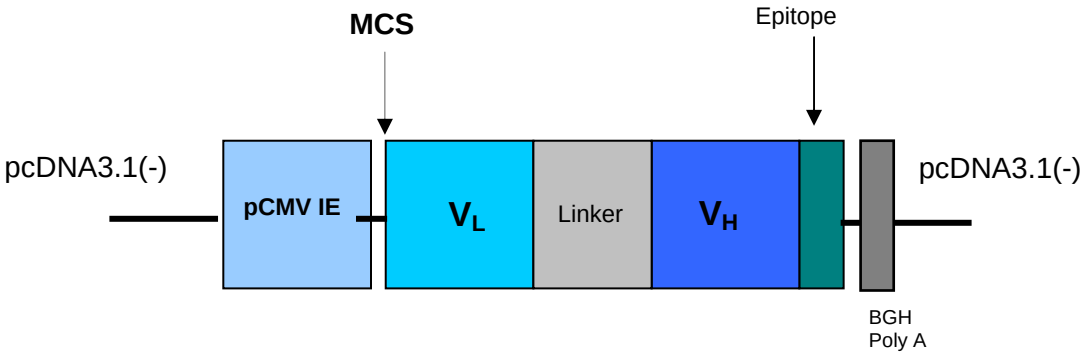


Figure 2.6: DNA construct used to generate 5E6 scFv antibody fragment. Gene segment of V_L cDNA , framework 1-4 regions, and V_H, framework 1-4, were cloned into pcDNA3.1(-) vector (Invitrogen). 15 amino acid flexible linker sequence was added to 3' end of V_L sequence by PCR. Epitope tags (myc and flag) sequences were added to 3' end of V_H sequence by PCR. Resulting 5E6 scFv vector is 6.2kb size.

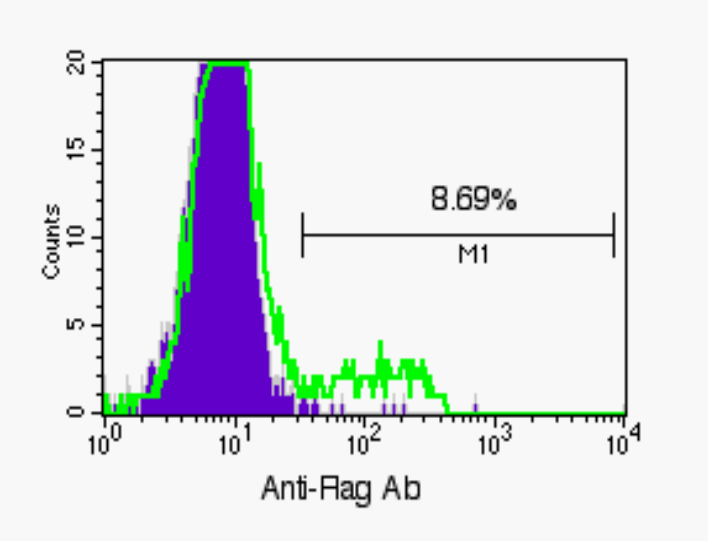


Figure 2.7: 5E6 scFv DNA construct makes functional protein. Ly49I transfected cells were treated with culture supernatant from non-transfected cells (filled purple) and 5E6 Tg transfected cells (green). Bound antibody was detected by secondary anti-flag Ab and anti-mouse IgG H+L ab (data not shown).

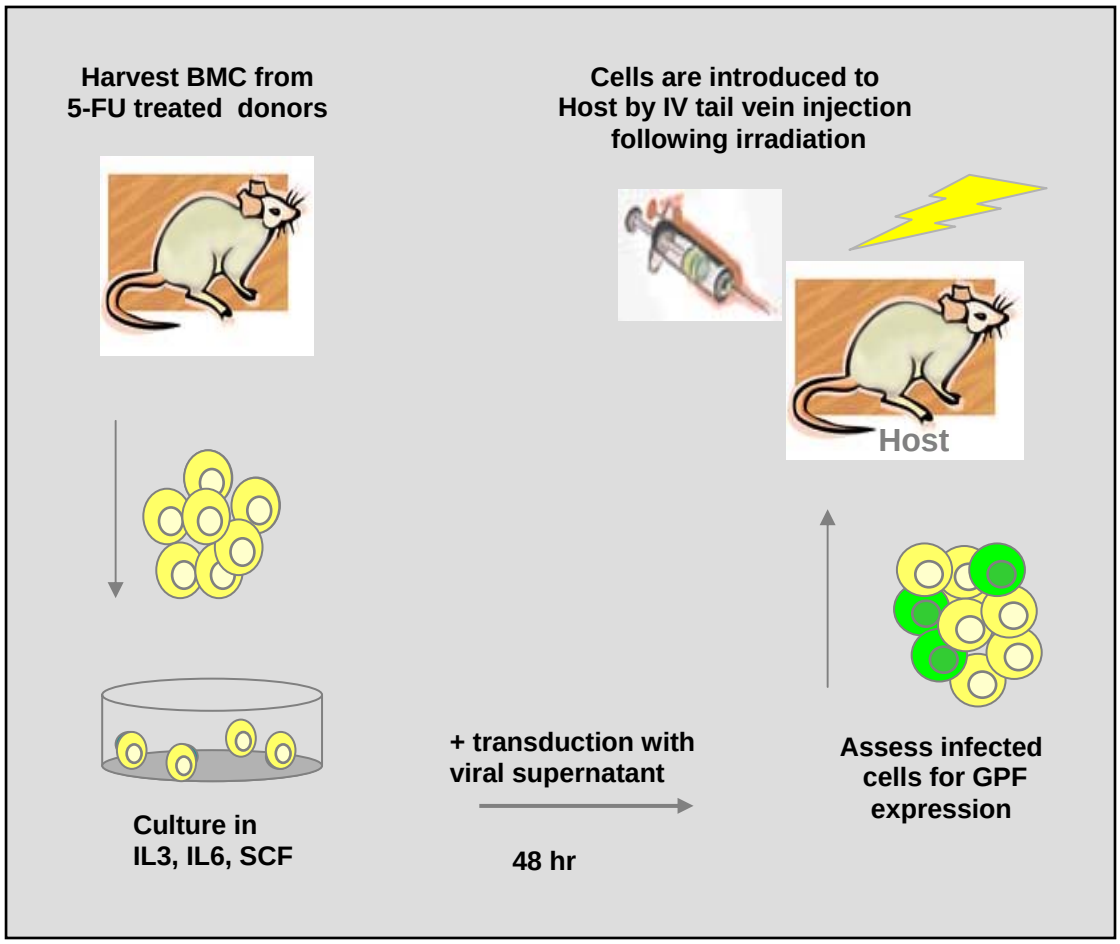


Figure 2.8: A schematic depiction of BMT used to establish murine CML. 5-FU treated BMC from donor mice are stimulated with cytokines prior to retrovirally transduction with p210/GFP. Transduced BMC are infused into lethally irradiated syngeneic hosts.

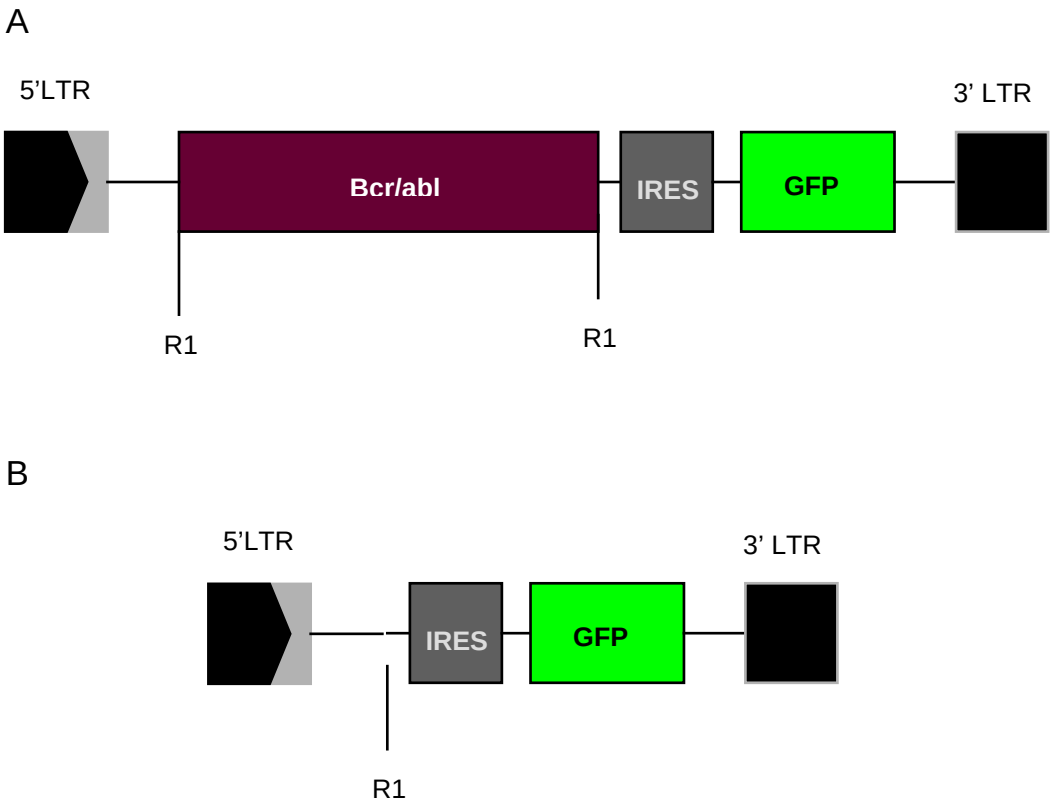


Figure 2.9:. The retroviral constructs used to transduce (A) BCR/ABL/GFP and (B) GFP- only genes. These constructs and retroviral packaging vector were used to transiently transfected 293Twhich produced retroviral supernatant. Retroviral supernatant was then used to transduce BMC for induction of CML or GFP control mice.

CHAPTER THREE

Development and Characterization of 5E6 Transgenic Mice

I. Introduction

As introduced in Chapter 1, NK cell regulation is mediated by inhibitory receptors such as Ly49s. When these receptors are engaged by MHC class I ligands, NK cytotoxicity is halted. Also discussed in Chapter 1, there is evidence that NK cells are involved in tumor surveillance. For example, beige mice, which have impaired T and NK function, are more susceptible to spontaneous and chemical induced tumor formation (36). NK cells are very effective in the lysis of MHC class I deficient targets as predicted by the missing self hypothesis (47). But, when MHC class I+ tumor cells engage inhibitory Ly49 receptors on NK cells, this results in a poor response by NK cells to potentially dangerous cells. Our lab has shown that NK cell responses to MHC class I expressing tumor targets can be improved by blocking inhibitory receptors with the 5E6 mAb (194;195;225). Specifically, an NK resistant tumor can become sensitive to NK lysis in the presence of 5E6 blocking mAb (Figure 1.3). In addition, administration of 5E6 mAb to mice receiving lethal tumor dose of C1498 leukemia cells resulted in significantly improved survival compared to controls (225). These studies utilized F(ab)'$_2$ antibody fragments in order to modulate NK response without depleting the 5E6+ subset of NK cells. But, efforts to fabricate a sufficient supply of F(ab)'$_2$ for *in vivo* studies has limited our progress. Therefore, we wanted to improve our model system to study the effects of blocking NK negative signals on tumor clearance. We developed transgenic mice that constitutively make non-depleting 5E6 mAb *in vivo*. This

model also allowed us to study if a sustained blocking of the Ly49-MHC interaction affects NK development and tolerance mechanisms. As discussed in Chapter 2, by using molecular techniques, a DNA construct was designed to express non-depleting recombinant 5E6 Fab mAb gene from CMV_{IE} promoter in transgenic mice.

II. Results

A. Generation of transgenic mice

1. Identification of transgenic lines

As a result of the transgenic injections, 22 pups of B6 background were produced. To determine which mice incorporated transgenic DNA, PCR of DNA extracted from tail clips was utilized. We designed primers that amplified unique sequences of the transgenic DNA construct, specifically the CMV_{IE} promoter region and the HC region. Figure 3.1 represents the PCR screen of all 22 mice. DNA from a B6 mouse was used as a negative control while transgenic plasmid mixed with B6 DNA provided a positive control. Approximately the same amount of DNA was used in all PCR reactions. However, determination of copy number was not assessed as PCR was not quantitative. In Figure 3.1, lanes without labels represent PCR (–) mice and mice labeled by a number were PCR(+) and backcrossed a single time to a B6 breeding pair. The F1 progenies of PCR (+) founders were assessed to determine if the transgene was incorporated germline. Most of these crosses resulted in progeny (described as F1 progeny) that did have germline transmission of the transgene as determined by PCR analysis (data not shown). Figure 3.2 displays PCR results from one such founder line labeled 2888 and its F1 progeny compared to transgenic DNA plasmid, as positive control.

2. Expression of the 5E6 transgene was predicted by increased anti-IgG staining of NK cells

Of the founder lines identified as positive for transgene germline incorporation, we needed to determine which founder lines would be studied. Initially, we considered choosing founder lines based on serum antibody levels detected by ELISA. We attempted ELISA using serum samples from several transgenic mice by epitope tag (His) detection and binding to soluble Ly49C and Ly49I. These methods were unsuccessful in accurately detecting serum transgenic mAb. Because we could not detect Tg mAb in serum, we considered the possibility that Tg mAb may be bound to Ly49C/I receptors on NK cells.

It is well established that endogenous antibody is found in serum and bound to B cells. But, endogenous antibody is less commonly bound to NK and T cells. However, the Tg mAb would be expected to bind Ly49C/I on NK cells and on those Ly49C/I+ T cells. Therefore, we predicted that these cells may be bound by mouse Ig in transgenic mice. To investigate this possibility, splenocytes from F1 Tg mice were stained with anti-mouse Ig and analyzed by flow cytometry. We predicted that if transgenic mice were making 5E6 antibody, this could be detected on the surface of NK cells and not on NK cells of wild type mice. Spleens were harvested from PCR (+) mice and B6 mice. After RBC lysis, cells were stained with rat anti-mouse Ig HC+LC specific antibody (Jackson). Excess antibody was washed off, and cells were stained with CD3, NK1.1 and B220 conjugated mAb. We expected a high percent of splenocytes would be double positive for anti-mouse IgG and B220, representing the B cells expressing surface IgM (HC). But we did not expect many B220- cells to bind the anti-mouse Ig secondary antibody unless they were also bound by the 5E6Tg antibody. In Figure 3.3E, a depiction of the staining scheme is shown. Of the nine

founder lines tested, only two founder lines (2888 and 2899) demonstrated an increase of anti-mouse Ig levels on NK cells above background seen in wild type controls. Results of staining for one such founder (2888) are represented in Figure 3.3. Figure 3.3A shows the negative control where 5.77% of total splenocytes are NK1.1+, of which very few cells are positive for anti-mouse IgG secondary antibody (0.70%), as expected (gate depicting 5.77% includes 0.70%). This background level could have been due to secondary antibody binding to Fc receptors on NK cells. As a positive control, B6 splenocytes were spiked with 250 ng of 5E6 F(ab)'$_2$ antibody prior to staining. Figure 3.3B shows approximately half of the NK1.1+ cells (2.5 % of 5.87% NK1.1+ cells) were stained with anti-mouse IgG antibody indicating that 5E6 mAb fragments were bound to the cell surface thus confirming the validity of the assay. Since Ly49C/I are expressed on approximately 40-50% of NK cells, the results of the positive control were within the expected range. When splenocytes from PCR (+) mice were stained, anti-mouse IgG staining was higher than background. Figure 3.3C and D show the staining results of 2 mice of the 2888 founder line. NK1.1 staining was 5.60% and 8.79% of splenocytes and of that 22% (1.25/5.60) and 19% (1.67/8.79) of NK cells also stained with anti-mouse IgG. These results suggested that this founder line may have produced just enough Tg antibody to decorate Ly49C/I+ NK cells in situ, even though the titer may have been extremely low.

3. Expression of transgenic mRNA detected in the 2888 founder line

While the results above were suggestive of Tg expression, we wanted to directly show that the 2888 founder was expressing Tg mRNA. Therefore, we performed RT-PCR in an

attempt to detect expression of transgenic mRNA. Since we used a CMV_{IE} promoter, we predicted that transgene DNA would be expressed in many tissues. In order to detect Tg mRNA, we harvested organs from various tissues of a homozygous mouse (determined by breeding results) of the 2888 founder line. RNA was extracted from spleen, thymus, lymph node, liver, kidney, muscle, heart, pancreas, gut, brain and skin. As a positive control, 293T cells were transiently transfected with transgenic DNA plasmid and used for RNA isolation. RNA was made into cDNA using oligo (dT) and random primers by reverse transcriptase reaction. As described in Chapter 2, the transgenic vector contained an intervening sequence (IVS) which is spliced out of the final transcript. We designed primers that spanned the IVS sequence to detect cDNA spliced product. Sequencing of PCR products from cDNA of transfected cells confirmed the larger product of 472bp contained the IVS sequence and the smaller product of 340bp did not contain this sequence, representing spliced mRNA. HPRT primers were used on each tissue sample to confirm the integrity of our cDNA preparations. Samples prepared lacking RT did not amplify a HPRT product indicating that RT was needed to obtain cDNA product. Transgenic mRNA was consistently detected in liver tissue of transgenic mice (Figure 3.4). Transgenic cDNA was also detected in the thymus and gut but these results were not easily reproducible as they required higher amplification, suggesting these tissues may have lower levels of transcript. Through RT-PCR, we were able to show that transgenic DNA was being transcribed at low but detectable levels in liver. However, tissue expression was not as broad as we expected in using the CMV promoter. Other reports of transgenes regulated by the CMV_{IE} promoter have demonstrated mRNA in a variety of tissues with the strongest expression in pancreas (249;262;263).

B. Assessment of protein expression in transgenic mice

1. Serum of Tg mice does not contain detectable free 5E6 mAb

Attempts were made to corroborate RT-PCR results with assessment of protein expression by measuring antibody titers in transgenic mice serum. We predicted that Tg mAb would be found in the serum since other soluble protein transgenic models showed detectable protein titers in serum by immunohistochemistry. For example, CTLA-4Hγ Tg mice measured 10-50μg/ml of CTLA-4Hγ in the serum (264) and 10-40ng/ml of Hepatitis B surface antigen (HBsAg) was detected in the serum of HBsAg Tg mice (241).

An ELISA using recombinant Ly49C or Ly49I soluble protein was developed to detect direct binding of Tg antibody from serum samples (in collaboration with D. Margulies, NIH). A standard curve was established by treating plate-bound antigen with a range of 500-10 ng of 5E6 Fab molecules. Bound antibody fragments were detected by alkaline phosphatase conjugated rabbit-anti mouse IgG H+L secondary antibody (Jackson). However, after several attempts, positive results of ELISA for samples from serum of transgenic mice were not reproducible.

Next, we tried using competitive binding assays to detect mAb in serum of transgenic mice. We hypothesized that if transgenic serum contained 5E6 Fab, it could compete for binding to Ly49I in transfected cells with a labeled 5E6 mAb. We used Ly49I transfected cells (BWI) cells to test mAb binding and florescence (FITC) conjugated 5E6 whole antibody, as the labeled mAb. The design of the experiment required 5E6 Fab molecules to compete directly with conjugated whole 5E6 mAb for binding to BW/I cells (further

described in Chapter 2). As data from another experiment indicated, 5E6 whole mAb binds better to BW/I cells than 5E6 Fab (Figure 3.5). This figure shows that when antibody of the same concentration (250µg) was used, intensity of binding determined by mean fluorescence intensity (MFI) varied in relation to antibody structure. Fabs exhibited lower MFI compared to $F(ab)'_2$ and whole mAb. Even with this information, we expected that if Tg serum contained a sufficient concentration of 5E6 Fab molecules, these antibody molecules would compete with 5E6 conjugated whole antibody causing a reduction in MFI as assessed by flow cytometry. Control non-transgenic serum samples were tested and showed no competition with conjugated 5E6 mAb for binding. For a positive control, B6 serum was spiked with 1000, 100, and 10 ng of 5E6 Fab and mixed with 5E6 conjugated antibody and applied to BW/I cells to develop a standard (Figure 3.6B). This exhibited a concentration dependent decrease in MFI suggesting that Fabs could compete with labeled whole 5E6 mAb for binding to Ly49I on BW/I cells. When transgenic serum was used the percent of inhibition varied between transgenic mice samples, where some mouse samples appeared to exhibit competitive binding while other samples appeared similar to negative controls (Figure 3.6). Specifically, 2 samples from transgenic mice showed competition at levels that would predict there was 100 ng of antibody in the serum. When samples were pooled, the level of competition was not statistically significant ($p=0.09$) compared to controls. After repeating experiment, we concluded that serum levels of the Tg mAb were either below detectable limits of these assays or that no functional protein was produced.

The reason we may not have been able to detect free mAb in serum of transgenic mice may have to do with the structure of the Tg mAb. Studies comparing recombinants of

Ig molecules, such as scFv, Fab, F(ab)'$_2$ and IgG, demonstrate that smaller mAb molecules have faster plasma and whole body clearance (265) but are more effective in penetrating tumor masses (207). 5E6Tg protein is a Fab and is a smaller molecule than used in other soluble protein transgenic systems (CTLA-4Hγ Tg, HBsAg Tg) in which free antibody in the serum was detected (241;264).

2. Presence of the 5E6 transgene can reverse Ly49I inhibition to reject B6 BMC

Since transgenic protein expression was not detected by ELISA or competitive binding assays, we investigated functional assays to detect Tg expression. When various Ly49 transgenic mice were produced, the function of transgenic Ly49 was tested by BMT. Inhibitory Ly49 expression inhibited rejection of BMC containing cognate ligands. For example, Ly49A Tg mice were unable to reject $H2^d$ BMC, (68;70), and KIR2DL3 Tg mice could not reject BMC expressing HLA-Cw3 (77). In addition, our lab reported that Ly49I expressed in $H2^q$ FVB mice (FVB.Ly49I transgenic mice) inhibited rejection of B6 BMC since B6 ($H2^b$) BMC engage Ly49I inhibitory receptor signaling(79;80). However, wild type FVB mice can reject B6 BMC very well (78). The inhibition of rejection by FVB.Ly49I (FVB.I) mice can be reversed by the administration of Ly49I blocking antibody (78;79). Therefore, we thought that if 5E6Tg mAb is bound to cells expressing Ly49C or Ly49I, it would block inhibition caused by Ly49I-$H2^b$ interactions during B6 BMC challenge thereby reversing phenotype of the mice.

In attempts to test this, we crossed 5E6Tg mice to FVB.I mice. F1 progeny were tested for presence of transgene by PCR. If 5E6Tg mice were producing 5E6 Fab, even at

undetectable levels but were bound to Ly49I on NK cells, we would predict that (5E6Tg x FVB.I) F1 mice would now reject B6 BMC. One limitation to this experiment was that many T cells in the heterozygous FVB.I transgenic mice express Ly49I (Figure 3.7) (78) in contrast to a much smaller percent of Ly49I on T cells in B6 mice. These T cells may also bind the 5E6Tg antibody thereby absorbing 5E6Tg Fabs. This could limit the effect of blocking negative signals on NK cells. In order to avoid this, we conducted the BMT assay after T cell depletion.

Figure 3.8 shows the results of BMT in which B6 BM was injected into (5E6Tg x FVB.I) F1, (B6 x FVB.I) F1 and B6 mice. B6 mice served as a positive control of engraftment. Groups of mice were treated with antibodies to deplete NK cells (Figure 3.8C), or T cells (Figure 3.8B) or received no treatment (Figure 3.8A). Results in figure 3.8A of both FVB.I F1 mice challenges with B6 BMC supported previous observations that FVB.I and FVB.I F1 mice are unable to reject B6 BMC due to the inhibition caused by Ly49I receptor engagement by $H2^b$ of B6 BMC. (5E6Tg x FVB.I) F1 did not reject B6 BMC, which shows that the presence of the 5E6Tg did not affect inhibition by Ly49I. These results could have been due to the high numbers of Ly49I+ T cells in FVB.I transgenic model competing for small pools of circulating antibody. Therefore, the following experiment was conducted.

In Figure 3.8B, the BMTs were repeated after all groups of mice were depleted of T cells. Again, the B6 group served as a positive control for engraftment which was not altered by T cell depletion. In addition, (FVB.I x B6) F1 mice were unable to reject B6 BMC even after T cell depletion. But interestingly, when T cell depleted (5E6Tg x FVB.I) F1 mice

were challenged with B6 BMC, they were now able to reject B6 BMC with strong statistical significance ($p<0.005$). This suggests that transgenic mice produce enough 5E6 Fab Tg antibodies to reverse inhibition by Ly49I following T cell depletion. We feel that since (FVB.I x 5E6Tg) F1 mice have Ly49I on a majority of their T cells, the depletion of these cells allowed NK cells expressing Ly49I access to the 5E6 Fab Tg antibody. T cell depletion was necessary to detect the effect of 5E6 Fab transgene since antibody titers are undetectable by other means. Finally, BMT assays were repeated after all groups underwent NK depletion (Figure 3.8C). When NK cells were not present, mice in all groups, B6, (FVB.I x B6) F1 and (FVB.I x 5E6Tg) F1 showed engraftment of B6 BMC. This supports previously reported claims that acute rejection of BM without immunization is mediated by NK cells (266;267). Because these experiments were conducted with a single founder line of transgenic mice, we can not rule out that the observed results were caused by the integration of the transgene into a region of the mouse genome that alters NK cell function.

Next, we wanted to determine if the presence of the 5E6 transgene affected the ability of FVB mice to reject B6 BMC. In addition, we wanted to assess if T cell depletion affected FVB rejection of B6 BMC. As previous mentioned, FVB ($H2^q$) mice which do not express Ly49I on NK cells can reject allogeneic B6 BMC. In addition, (FVB x B6) F1 mice reject B6 BMC by HR (38). Therefore, we predicted that (FVB x 5E6Tg) F1 mice would reject B6 BMC by the same HR mechanism. Shown in Figure 3.9, additional B6 BMTs were conducted with (5E6Tg x FVB) F1 and (B6 x FVB) F1 mice in which each group of mice was treated with antibodies to deplete NK cells, T cells or received no treatment. As compared to B6 mice, these mice rejected B6 BMC following T cell depletion or no

treatment, but without NK cells these mice could not reject B6 BMC. All FVB F1 mice rejected B6 BMC, regardless of the presence of the 5E6Tg. Since these mice do not express Ly49I on a majority of their NK cells, they are able to reject B6 BMC as seen in the previous scenario in Figure 3.8. In addition, this experiment shows that T cell depletion of (5E6Tg x FVB) F1 and (B6 x FVB) F1 mice did not affect their ability to reject B6 BMC. And finally, Figure 3.9C shows that (5E6Tg x FVB) F1 and (B6 x FVB) F1 rejection of B6 BMC is NK cell mediated, because without NK cells, these mice were unable to reject B6 BMC.

From the results of these experiments, we concluded that Ly49I expression in FVB.I mice inhibits rejection of B6 BMC. But, the presence of the 5E6 transgene allows reversal of inhibition caused by Ly49I in FVB.I mice. T cell depletion is required to show this effect, perhaps due to a limited level of 5E6 Fab circulating antibody. The rejection of B6 BMC by (FVB.I x 5E6 Tg) F1 mice is NK dependent, and the 5E6 transgene does not affect NK mediated rejection by FVB F1 mice of B6 BMC. Yet, we cannot rule out that the transgene integration affected NK cell function.

C. Phenotypic and functional characterization of NK and T cells of transgenic mice

The BMT assays results, discussed above, functionally demonstrate the presence of 5E6Tg mAb and the ability to block Ly49I-MHC interactions in transgenic mice. We wanted to evaluate if blocking Ly49C/I interaction with MHC class I during NK and T cell development would alter the receptor repertoire of these cells. As observed in class I deficient mice (β2M-/- or TAP-1º) and Ly49 (Ly49A, C and G2) transgenic mice, Ly49 receptor expression on NK cells adapts to the MHC environment in which they develop

(84;86;87;227;268). In addition, inhibitory Ly49 receptors can be important on T cells since splenic CD3+ cell expression of Ly49 increases to 30-40% in B6 mice after certain infections (269;270). In addition, these inhibitory receptors appear to maintain their inhibitory function in T cells. TCR-mediated activation can be impeded by Ly49 interactions with MHC class I (84;229;230) although this was not observed in the FVB.Ly49I transgenic mice (80). For these reasons, we wanted to determine if the 5E6Tg mice have altered NK and T cell receptor expression.

1. Evaluation of NK receptor repertoire in Tg mice

As described by the receptor calibration model (discussed in Chapter 1), the ability of NK cells to alter their Ly49 receptor repertoire to different MHC environments may be a required process to maintain a balance between activating and inhibitory signals. For example, Ly49A/C double transgenic mice have a reduction in expression of other inhibitory Ly49 receptors (87) . The interaction with MHC class I molecules provides signals to NK cells to help calibrate receptor expression as a way to limit development of hypoactive NK cells, with excessive inhibitory receptors. In turn, we hypothesized that blocking MHC-Ly49 interactions during NK development may alter NK receptor expression, possibly by upregulating the expression of other inhibitory Ly49s.

To determine if the 5E6Tg mice have an altered NK repertoire, splenocytes from transgenic mice and B6 mice were stained for NK1.1 and a panel of additional NK receptors and assessed by flow cytometry. Table 3.1 shows that NK1.1+ cells from 5E6Tg mice have similar expression of Ly49G2, Ly49A, Ly49D, and CD94 when compared to B6 controls.

Ly49C/I expression was evaluated using various mAbs, 5E6, 8H7 and IF8. We observed a reduction of Ly49C/I expression on splenic NK cells in 5E6Tg mice (24.07%) compared to B6 mice (34.68%). Though these values were not statistically significant (p=0.06), we speculate that this reduction may be due to masking of the receptors by 5E6Tg antibody or by receptor internalization. When splenocytes were placed in culture for 3 days with IL-2, we observed that receptor expression returned to levels comparable to B6 controls (data not shown).

2. Evaluation of T cell development by assessing T cell receptor expression in Tg mice

Since a small subset of T cells in B6 mice express Ly49C/I receptors, we wanted to determine if the 5E6Tg antibody affected T cell receptor repertoire. Ly49A transgenic mice develop severe inflammatory disorders. Since proper T cell signaling relies on T cell and MHC interactions, studies in Ly49ATg mice suggests that Ly49 receptors could participate in T cell development by attenuating TCR signaling thresholds. Therefore, we felt it necessary to explore the effects on T cell thymic development in 5E6Tg mice.

Thymocytes and splenocytes from transgenic mice and B6 controls were stained for CD3, CD4 and CD8 (Table 3.2). In addition, we wanted to know if Ly49C/I expression would be altered, therefore, cells were also stained with 5E6, 8H7 and IF8 to assess expression of Ly49C/I (Table 2). From staining data, CD4, CD8 and Ly49C/I expression on splenic CD3+ cells of the 5E6Tg mice was similar to B6 controls. In the thymus, we observed a slightly higher percent of single CD4+ cells and CD8+ cells compared to controls (p=0.05, p=0.07), while the percent of CD4+CD8+ cells was reduced compared to controls

(p=0.03). The reason for this was not fully determined. However, since RT-PCR analysis indicated a low level of transgenic transcript in the thymus, localized production of 5E6 mAb or effects of transgene integration may alter thymic development.

3. Tg mice do not exhibit autoreactivity

Our phenotypic assessment of the 5E6Tg mice suggests these mice appear to have normal numbers and subsets of NK cells and T cells. Next, we wanted to assess if NK cell function in 5E6Tg mice was similar to controls. Given that inhibitory receptor function of Ly49C/I in the transgenic mice is being blocked, we wanted to determine if NK cells are still tolerant to syngeneic cells. Previously, we have seen that B6 mice treated with 5E6 $F(ab)'_2$ accept B6 BM grafts (unpublished data). Therefore, we have no reason to believe that 5E6Tg mice would display autoreactivity. In Figure 3.10, we conducted a syngeneic BMT which showed that transgenic mice accepted B6 BM equivalent to B6 controls. Two PCR (+) mice displayed less IUdR uptake in the group. This may be attributed to poor injections during BMT assay. From these results, we concluded that NK cells of transgenic mice do not exhibit autoreactivity towards marrow stem cells. In addition, freshly isolated splenocytes from transgenic mice and B6 mice were activated *in vivo* with poly IC and used as effectors in a ^{51}Cr release assay. Their ability to lyse YAC-1 cells, a NK sensitive target, and B16-F10 cells ($H2^b$), a NK resistant target (271) were comparable to controls (Figure 3.11). This demonstrates that bulk NK cells from transgenic mice behave similar to controls *in vitro* and exhibits no autoreactivity.

C. 5E6Tg mice response to tumor challenge

From evaluation of the 5E6Tg mice, it appears that mice are phenotypically and functionally comparable to B6 controls except for the presence of 5E6 transgene. Next, we wanted to determine if the presence of the 5E6Tg mAb had any effects *in vivo* in regards to a tumor challenge. Previously, 5E6 $F(ab)'_2$ treatments were shown to augment NK cell anti-tumor effects using the leukemia cell line, C1498 (225). In our studies, we wanted to test the ability of transgenic NK cells to inhibit growth of another type of tumor, B16-F10 melanoma. B16-F10 (B16) melanoma cells are poorly immunogenic (271). This cell line allows us to evaluate the efficacy of antibody treatment for metastatic tumors. As discussed in Chapter 1, in reviewing many clinical trials, melanoma is commonly studied for response to immunotherapies, since it appears to be resistant to conventional chemotherapies (272). In addition, adoptive transfer of NK cells to the site of melanoma can contribute to tumor eradication (273). When mice are injected (iv) with B16 tumor cells they develop lung metastases. These tumor nodules can be counted to quantitate tumor burden. 5E6Tg mice and B6 control mice were injected (iv) with $3x10^5$ B16 cells. After 17 days, mice were sacrificed and lungs were fixed in 10% formaldehyde. When lung metastases were counted, 5E6Tg mice had statistically significant reduction ($p=0.017$) in tumor burden compared to B6 controls (Figure 3.12). This effect is NK mediated since depletion of NK cells from transgenic mice results on the development of tumors too numerous to count, over 200 tumor nodules (data not shown). From this tumor challenge, we conclude that the presence of the 5E6Tg can augment NK mediated tumor surveillance of a metastatic tumor.

III. Conclusions/Discussion

Augmenting NK activity for tumor clearance has broad implications for the creation of additional immunotherapies. An analogous example to NK inhibitory receptor blockade is that of CTLA-4 receptor blockade in T cells. But anti-CTLA-4 mAb treatment caused T cell autoimmunity, which is common when T cell negative regulation is altered (220;223;224). NK cells have not exhibited autoimmunity *in vivo* even when their inhibitory receptors have been blocked (194;195;225). And it appears that they have a fascinating way of developing or maintaining tolerance, discussed in Chapter 1. Therefore, augmenting NK activity has advantages and great potential since it can enhance tumor clearance and avoid harmful effects such as autoimmunity.

Previously, the concept of NK inhibitory receptor blockade for augmenting NK anti-tumor properties was proven effective in murine leukemia models (225). We wanted to evaluate further how 5E6 receptor blockade can enhance tumor clearance. Our goal was to better characterize the effects of NK inhibitory receptor blockade and test it in other tumor models. In order to pursue these studies, we developed a transgenic model to answer questions regarding the effects of receptor blockade *in vivo*.

The experiments presented in this section provide evidence that a 5E6 mAb transgenic model was developed. PCR and RT-PCR results show that the transgene was integrated into the mouse genome and was transcribed into RNA. From additional results presented, it appears that the 2888 founder line makes low but functional levels of antibody sufficient to block Ly49C/I receptors on NK cells. Because antibody was not detected in the serum of mice by competitive binding assays or ELISA, we provided functional evidence

that antibody was likely bound to Ly49I+ cells because FVB.I mice containing the 5E6 mAb transgene were now able to reject B6 BMC. The blockade of Ly49C/I and MHC class I interactions did not appear to significantly alter NK or T cell development. In addition, questions regarding autoimmunity were answered using the transgenic model. If continuous receptor blockade occurred in these mice, it did not drive NK cells to be autoreactive since these mice did not reject syngeneic BMC. If the function of inhibitory receptors for "self" MHC is blocked in 5E6Tg mice, the NK cells did not attack normal BMCs. The 5E6 transgene did not appear to induce autoimmunity in 5E6Tg mice.

In addition, we tested another tumor model in the transgenic mice. Upon challenge with B16 metastatic tumors, there were fewer tumor nodules in 5E6Tg mice than compared to B6 controls. These results support the notion that NK cells can be more effective in tumor surveillance of metastatic and leukemic tumors when inhibitory receptors are not engaged.

Overall, we expected much greater 5E6 mAb levels in mice. It would have been interesting to have compared a transgenic founder line that expressed high levels of 5E6 mAb (if one existed) to our 2888 founder line. If these mice had similarities in receptor repertoire and development of NK and T cells and maintained tolerance, we could have more definitively concluded that the effects of blocking Ly49I-MHC interactions only influence NK cytolytic activity in tumor clearance. This is important because it would have added to our growing knowledge of NK development and function. In addition, in developing effective immunotherapies, limiting side effects are required for the success of a reagent. The transgenic model differs from the antibody treatment model in that the transgene is presumably active throughout life. But, using the evidence accumulated from both studies,

2888 founder line and other studies when NK cells were treated with blocking antibody in adult mice, we would predict that 5E6 Fab treatment has minimal side effects. The reasons for this are, first, our approach utilizes mAbs which have very specific targets and secondly, the target is expressed mainly on NK cells which have multiple ways of maintaining tolerance *in vivo*. To apply the approach of blocking negative signals in humans requires the identification of a blocking mAb that binds human inhibitory NK receptors. The results of our studies do not rule out any potential side effects that may be found in clinical trials, but it provides greater hope of success.

Table 3.1: NK receptor expression in 5E6Tg mice

	Percent of splenic NK1.1+ cells		
NK receptor	**B6**	**5E6 Transgenic**	**p values**
Ly49A	25.38 ± 5.31	24.30 ± 4.03	0.86
Ly49G2 (4D11)	31.26 ± 2.82	34.61 ± 1.54	0.26
Ly49D	20.36 ± 3.43	18.54 ± 3.85	0.71
CD94	46.27 ± 2.17	47.73 ± 1.91	0.59
Ly49C/I (5E6)	34.68 ± 3.69	24.07 ± 4.0	0.06
Ly49I (8H7)	28.93 ± 2.82	24.75 ± 3.17	0.33
Ly49C/I/H (1F8)	36.56 ± 4.0	27.71 ± 3.17	0.08

Experiments from multiple experiments were pooled, n=6-19 mice
P values indicate results of student t-test

Table 3.2: T cell receptor expression in 5E6Tg mice

	Percent of CD3+ cells					
	Splenic			**Thymic**		
T cell receptor	**B6**	**Tg**	**P values**	**B6**	**Tg**	**P values**
$CD4^+$	53.23 ± 3.94	48.32 ± 2.47	0.25	34.45 ± 3.17	42.35 ±2.38	0.05
$CD8^+$	6.32 ± 1.95	7.48 ± 0.83	0.30	10.13 ± 0.64	14.04 ±1.70	0.07
$CD4^+CD8^+$	1.21 ± 0.29	2.18 ± 0.59	0.18	51.98 ± 0.644	40.44 ±3.52	0.03
$CD4^-CD8^-$	39.22 ± 3.44	42.00 ± 2.34	0.47	3.02 ± 0.52	4.07 ±0.39	0.17
Ly49C/I (5E6)	1.58 ± 0.45	1.33 ± 0.33	0.65	0.39 ± 0.11	0.59 ± 0.24	0.45
Ly49I (8H7)	0.91 ± 0.22	1.12 ± 0.28	0.56	0.91 ± 0.31	1.68 ± 0.44	0.17
Ly49C/I/H (1F8)	4.23 ± 0.9	4.49 ± 0.77	0.83	0.40 ± 0.24	0.16 ± 0.1	0.32

Experiments from multiple experiments were pooled, n=7-18 mice, except staining with IF8 where n=3
P values indicate results of student t-test

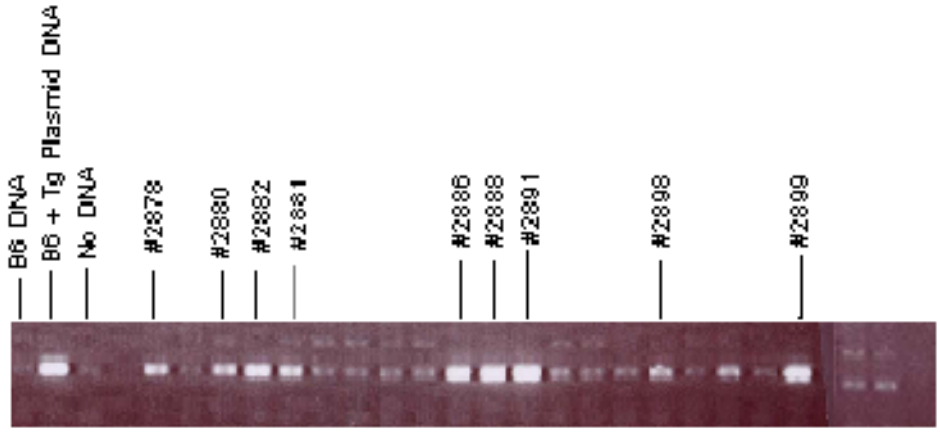


Figure 3.1: PCR screen analysis of original founder mice. Tail DNA was extracted from 22 pups and screened by PCR using CMV promoter specific primers. Expected PCR product was 499 bp. Positive samples are labeled with a number. Samples with negative PCR results are not labeled.

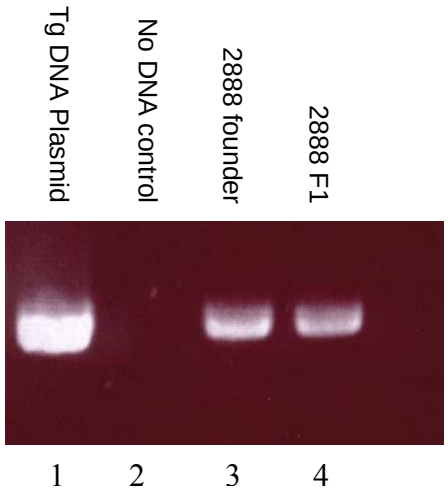


Figure 3.2: 5E6Tg has germline transmission indicated by presence of transgene detected in F1 progeny of founder 2888. Gel displays PCR amplification of B6 DNA mixed with transgenic plasmid DNA as positive control (lane 1), B6 tail DNA (lane 2), 5E6 Tg mouse founder (lane 3), F1 mouse (lane 4). PCR product 499 bp.

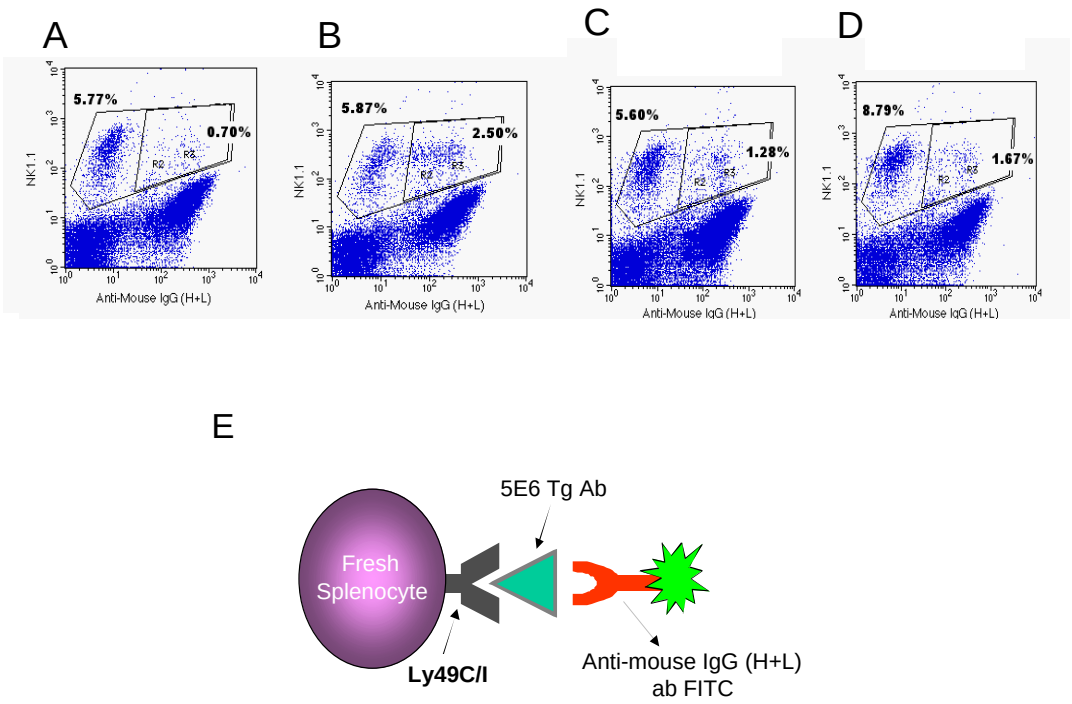


Figure 3.3: Presence of mouse antibody bound to NK cells of transgenic mice. (A) B6 splenocytes stained with anti-mouse IgG secondary ab and then with NK1.1, 5.77% represents entire NK1.1+ population and included 0.70% (B) B6 splenocytes spiked with 250ng of 5E6 F(ab')$_2$ ab, prior to staining. 2.5% of NK1.1+ cells are stained with anti-mouse IgG ab indicating that 5E6 ab fragments are bound to the cell surface, while entire NK1.1+ population was 5.87%. This is consistent with the expected Ly49C/I subsets for NK cells. (C,D) 5E6 Tg PCR+ Mouse (F1 progeny). NK1.1 staining was 5.60% and 8.79% of splenocytes. Percent of NK1.1+ cells bound by antibody is 1.28% and 1.87% compared to 2.5% in positive control (B). (E) Detection of recombinant 5E6 Ab bound to Ly49C/I+ cells in the transgenic mice was assayed by staining fresh splenocytes with antibodies for NK1.1, CD3, B220 (not shown) and anti-mouse IgG (H+L) specific secondary ab which should detect surface bound 5E6 antibody.

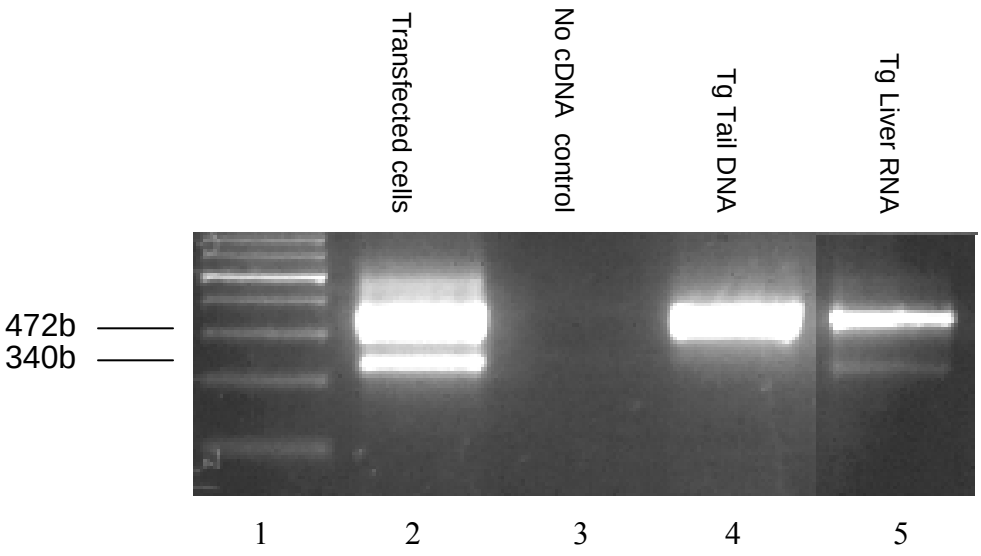


Figure 3.4: Detection of 5E6 transgenic mRNA by RT-PCR analysis. 100bp molecular weight ladder (Lane 1), amplification of cDNA from transient transfected 293T (lane 2), no DNA or RNA control, H_2O control (lane 3), tail DNA from PCR positive mouse (lane 4), cDNA from liver of 5E6Tg mouse (lane 5). 472bp band corresponded to amplification of non-spliced DNA. 340bp band results from amplification of spliced cDNA excluding vector specific intron or intervening sequence.

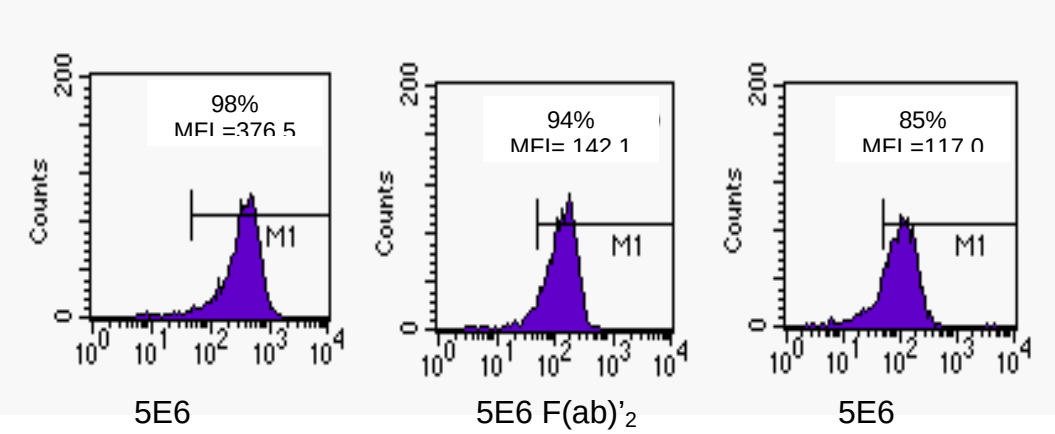
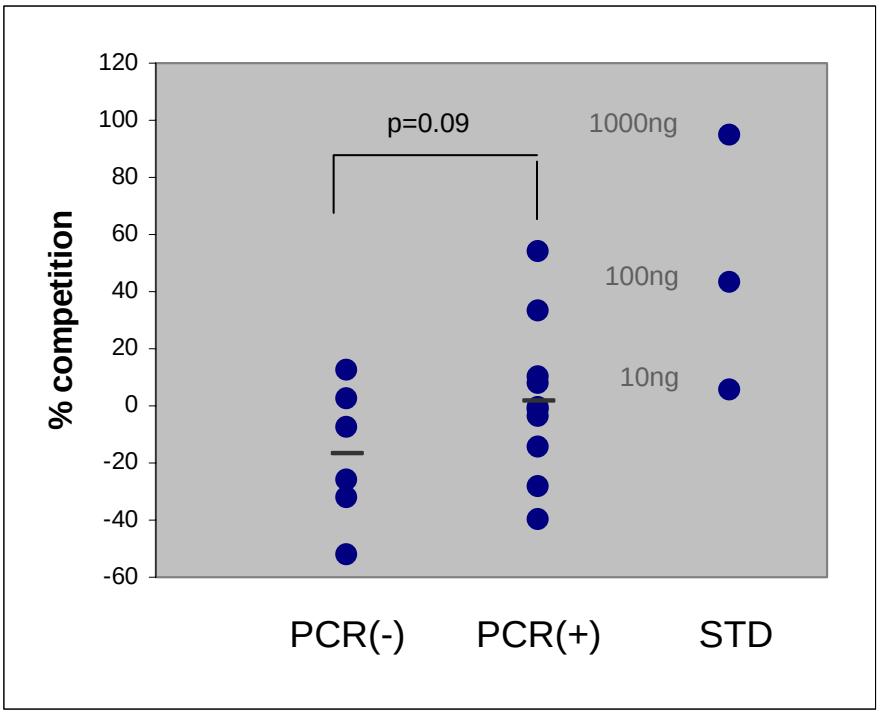


Figure 3.5: Ly49I transfectants staining with 5E6 whole ab, 5E6 F(ab)'$_2$ and 5E6 Fab antibody fragments. 500 ng of each antibody was used to stain BW/I cells and then stained with secondary antibody that detects bound IgG H+L. Staining of Ly49I transfectants showed a slight decrease in staining with antibody fragments compared to whole mAb.

A



B

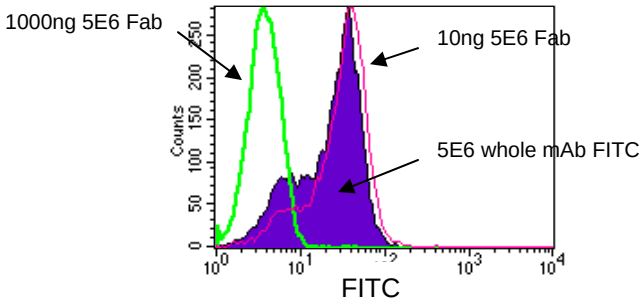


Figure 3.6 : 5E6Tg antibody is not detectable in serum of transgenic mice. (A) Serum from several PCR(-) and PCR(+) mice was tested for ability to compete for binding with conjugated 5E6 whole to BW/I cells. Changes in MFI were assessed to determine percent of competition. (B) Histogram of standards which displays reduced MFI when 1000 ng 5E6 Fab was mixed with FITC conjugated 5E6 mAb. Purple filled histogram represents 5E6 FITC only, Pink histogram represents 5E6 FITC+ 10ng 5E6 Fab, Green histogram represents 5E6 FITC + 1000ng of 5E6 Fab. Standard using 100 ng 5E6 Fab has intermediate MFI, not shown.

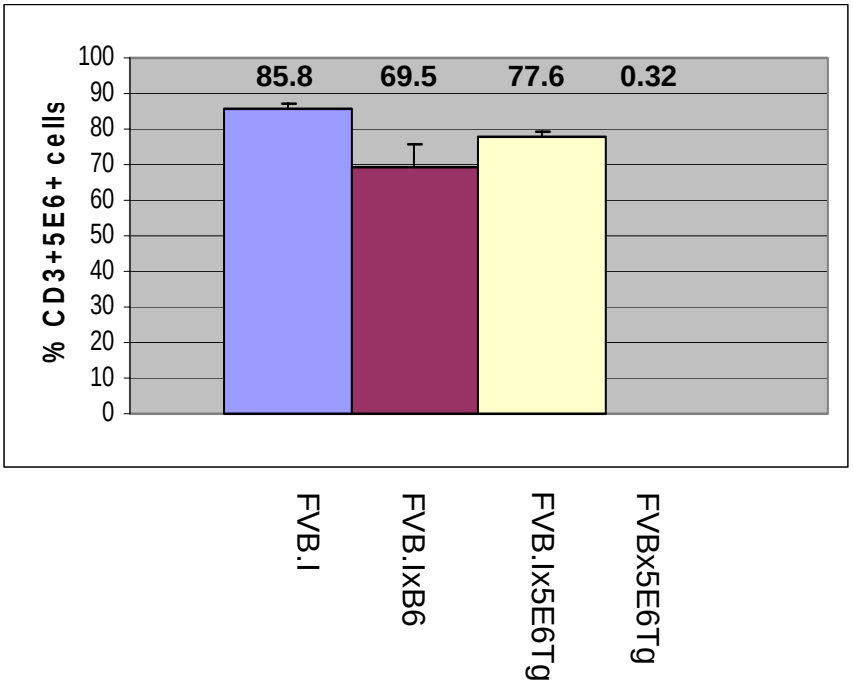


Figure 3.7: Mice heterozygous for Ly49I transgene express Ly49I on most CD3+ cells. After RBC lysis, peripheral blood was stained with CD3 mAb and 5E6 mAb. Percent of double positive cells are presented.

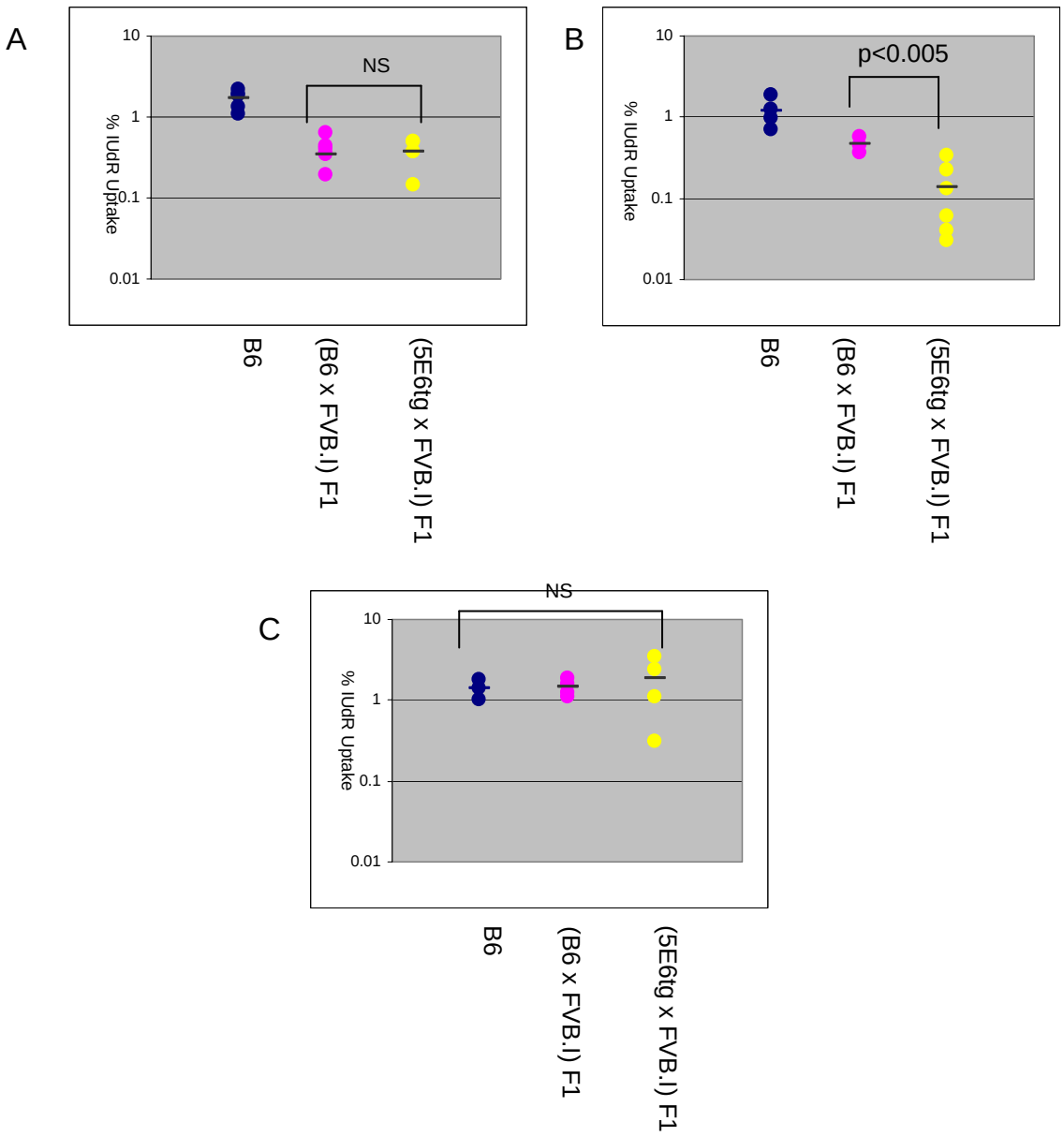


Figure 3.8: Bone marrow transplantation assay testing the effects of the presence of 5E6 transgene in FVB.Ly49I F1 transgenic mice (A) with no antibody treatment, (FVB.I x B6) and (FVB.I x 5E6tg) F1 do not reject B6 bone marrow. (B) With T cell depletion, (FVB.I x 5E6tg) F1 mice reject B6 bone marrow. (C) With NK depletion, mice are unable to reject B6 bone marrow in all groups. 2-3x10^6 B6 BMC were injected (iv) into lethally irradiated recipients. Five days post-BMT, mice were assessed for splenic [^{125}I]udR uptake. Statistically significant in a 2-tailed t-test indicated by p value or NS, for not statistically significant.

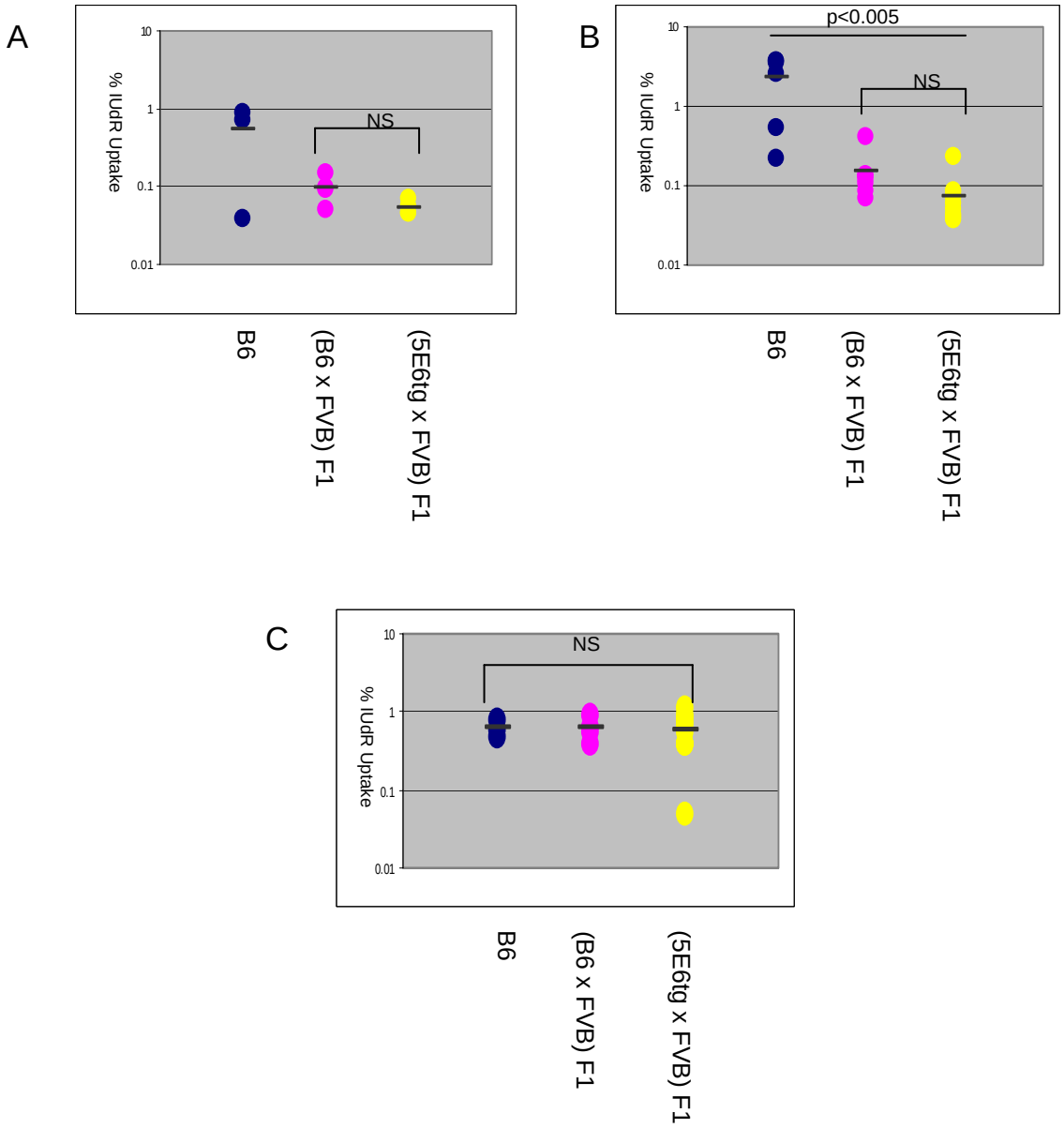


Figure3.9: FVB F1 mice reject B6 bone marrow as a demonstration of hybrid resistance (A) with no antibody treatment or (B) With T cell depletion, (FVB x B6) and (FVB x 5E6tg) F1 reject B6 bone marrow. (C) Bone marrow rejection is NK mediated as with NK depletion, mice are unable to reject B6 bone marrow in all groups. $2x10^6$ B6 BMC were injected (iv) into lethally irradiated recipients. Five days post-BMT, mice were assessed for splenic [^{125}I]udR uptake. Statistically significant in a 2-tailed t-test indicated by p value or NS, for not statistically significant.

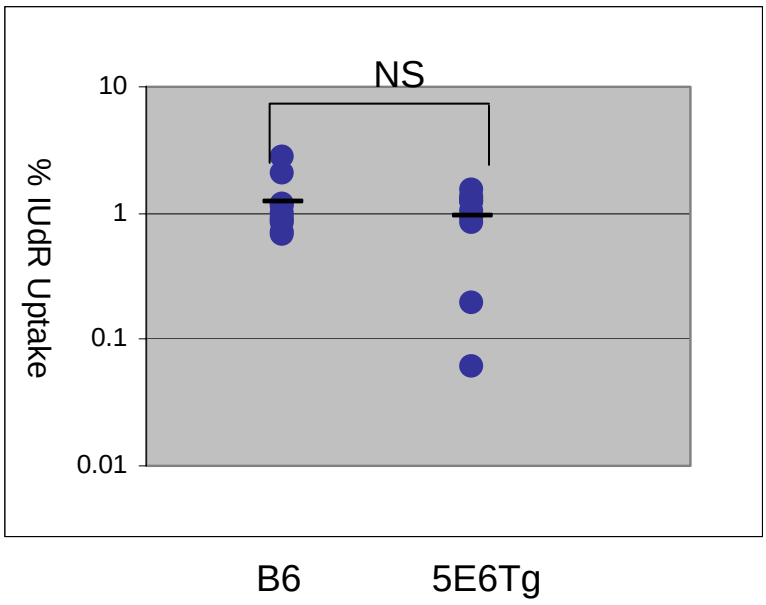


Figure 3.10: 5E6Tg mice do not display autoreactivity to syngeneic BMCs. $2x10^6$ B6 BMC were injected (iv) into lethally irradiated recipients. Five days post-BMT, mice were assessed for splenic [125I]dU uptake. Differences between the two groups is not statistically significant (NS) in two tailed t-test.

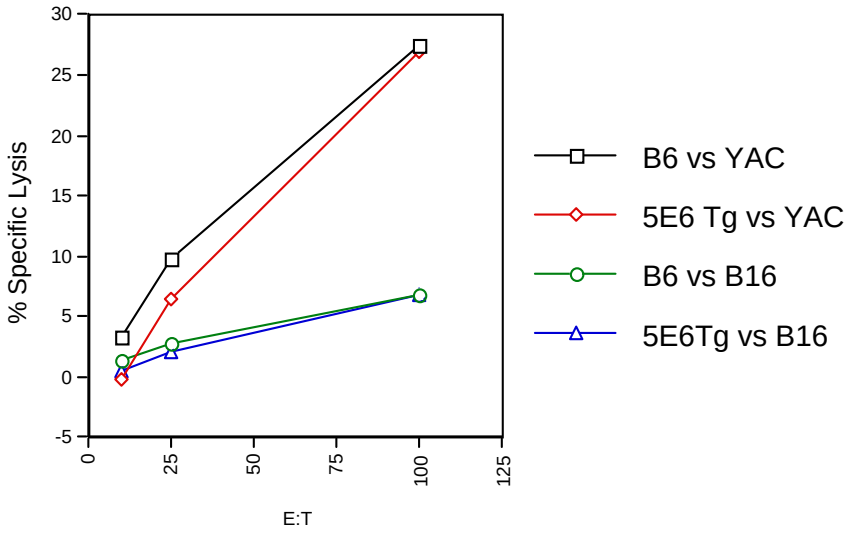


Figure 3.11: Activated splenocytes of transgenic mice do not display altered cytolytic activity. Splenocytes from 5E6 Tg mice and B6 mice were activated with Poly IC one day prior to being used as effectors in 1 hour ^{51}Cr release assay. YAC and B16 melanoma cells were used as targets. Graph shows percentage of specific lysis. Error bars indicate standard deviations.

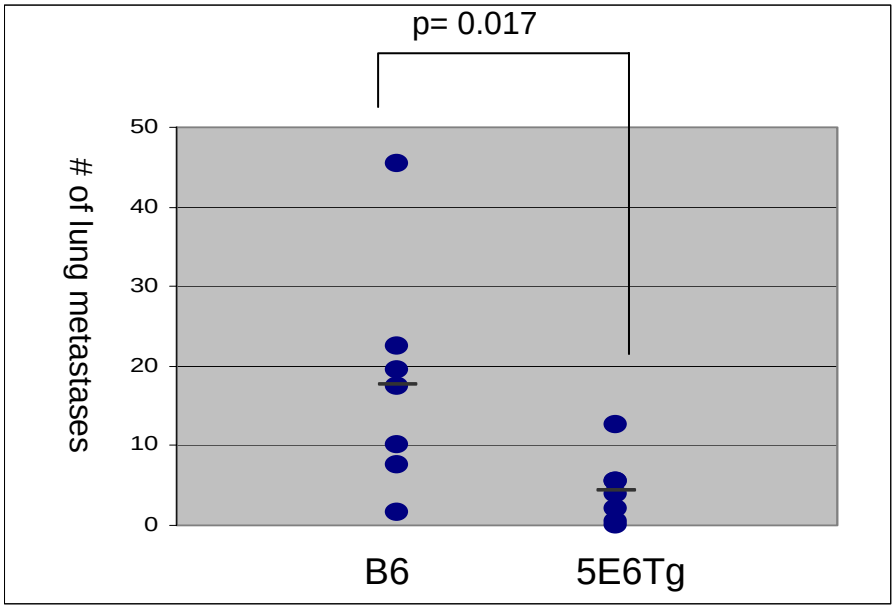


Figure 3.12: 5E6Tg mice have improved resistance to B16 melanoma tumor challenge. $3x10^5$ B16 melanoma cells were injected (iv) into 5E6Tg and B6 mice. 17 days later lungs were harvested and tumor nodules in lung were counted. Data represented is pooled from 2 experiments and is statistically significant in a 1-tailed t-test indicated by p value of 0.017. When statistical significant was determined excluding the highest data point in B6 group, p= 0.014.

CHAPTER FOUR

Study of nascent CML tumor model to enhance NK cells role in tumor surveillance

I. Introduction

As discussed in Chapter 1 and 3, using two *in vivo* experimental models, our lab has shown that blocking negative signals on NK cells allows for increased tumor susceptibility to NK mediated lysis. With high dose tumor challenges of C1498 leukemia or B16 melanoma cells, the presence of anti-Ly49C/I, 5E6 non-depleting mAb allowed significant reduction of tumor burden compared to controls. To test the effects of blocking inhibitory signals further, we decided to utilize a nascent tumor model in which the tumor is initiated from otherwise normal hematopoietic progenitor cells.

Chronic myelogenous leukemia (CML) is a commonly studied tumor and tumor model in mice and humans. CML is a fatal, myeloproliferative disease characterized by the accumulation of predominately granulocytes in peripheral blood, BM and spleen(274). Patients experience leukocytosis, high white blood counts (WBCs) and symptoms such as fever, splenomegaly, weight loss and joint pain (275). In humans, higher than 95% of CML cases are associated with a chromosomal translocation called the Philadelphia chromosome. The reciprocal translocation between chromosome 9 and 22 creates 2 gene products, BCR/ABL and ABL/BCR (275-278). Though the functional activity of ABL/BCR is unknown, the BCR/ABL protein is a constitutively active tyrosine kinase with widespread effects due to its ability to phosphorylate a large number of substrates. BCR/ABL transforms

cells by transducing signals in various pathways that interfere with basic cellular processes such as regulation of proliferation, adherence, and apoptosis (278).

It has been reported that in untreated CML patients, the mean survival is only 31 months from initial onset of symptoms. Survival rates among treated patients varies from 1 year to 20 years, and can depend on the stage when treatment was begun (276). There are three clinical phases of CML. In the chronic phase, there is a massive expansion of the myeloid cell compartment, while other hematopoietic cells are able to function and differentiate normally. The accelerated phase reflects disease progression in which a greater appearance of immature granulocytes is detected in the blood. The blast phase is the final stage (lasting weeks to months) when additional oncogenic changes occur in transformed cells and an accumulation of immature granulocytes cells is found in the blood (279;280).

BCR/ABL+ cells appear to be resistant to cytotoxic drugs and radiation (275), therefore BMT are commonly used as a treatment option. For patients that do not receive BMT due to unavailable donors, IFNα treatment is recommended (276). In more recent years, a powerful new drug has been available for treatment of CML. Imatinib mesylate (ST1571 or Gleevac) is a tyrosine kinase inhibitor that specifically targets BCR/ABL. Imatinib mesylate competes for binding with ATP at ATP-binding sites of the tyrosine kinase domains of BCR/ABL. When the drug was tested in clinical trials on patients that had failed IFNα treatment, many patients achieved rapid (within 3 months) hematological remission (the normalization of the blood counts), in some trials up to 95% of patients showed remission (279;281). Because of the specificity of imatinib mesylate, side effects are mild

and rarely require cessation of therapy (276). The drug was approved by the FDA in December of 2002 as the first-line treatment for newly diagnosed CML patients (279).

In mice, there are several models of CML utilizing different forms of the BCR/ABL oncogene. The products of BCR/ABL translocation exist in p210, p190, and p230 forms arising from distinct breakpoints of the translocation (259). One well-established model of CML in BALB/c mice involves BMT of p210 transduced BMC into heavily irradiated syngeneic hosts. BALB/c donor mice are treated with 5-FU to kill rapidly dividing cells and enrich for and initiate cell division of pluripotent stem cells. 5-FU treated BMC are then retrovirally transduced with the BCR/ABL/p210 oncogene and injected into lethally irradiated mice. We have utilized this model for our studies and replicated characteristics of the disease as described in the literature (259;282;283). Common features of the CML-like disease in mice are elevated WBCs (15,000-500,000 cells/mm^3 compared to the normal 5000 cells/mm^3), accumulation of Mac-1+ and Gr-1+ cells in peripheral blood and death within 3 weeks of initial BMT due to fulminant granulocytosis. At autopsy, mice have splenomegaly with spleen weights of 0.8 -1.0 grams (259;283;284). Disease progression can be slowed but not halted by dilution of transduced BMC (259;283;285) or by treatment with imatinib mesylate (286).

II. Results

A. Establishing murine CML

Figure 4.1 shows several characteristics of murine CML which is similar to the chronic phase of CML seen in humans (283). For detection of BCR/ABL expressing cells (in collaboration with R.L. Ilaria at UT Southwestern), we used, a bicistronic retroviral DNA construct

containing an IRES that allows for BCR/ABL/p210 and green fluorescent protein (GFP) (p210/GFP) to be translated from the same transcript. Cells expressing BCR/ABL will also be GFP+ (274). As a control, the vector without BCR/ABL was used (GFP-only). Both constructs are depicted in Figure 2.9. Figure 4.1 shows an experiment in which a BMT was conducted on two groups of young adult BALB/c mice, (as described in Chapter 2) where one group received BMCs transduced with p210/GFP (p210+ BMC) and the second group received BMCs transduced with GFP only vector after irradiation. To assess disease progression, eight days post-BMT peripheral blood was drawn and GFP expression was evaluated by flow cytometry. At this early timepoint, mice given p210+ BMC had already begun to exhibit signs of CML. 50% of the live lymphocytes in their peripheral blood were GFP+ as compared to a very low level of GFP detected in GFP only group (Figure 4.1A). At Day 16, it was confirmed that other characteristics of CML were being displayed by p210+ BMT mice. Mice receiving p210+ BMC had developed grossly enlarged spleens, weighing an average of 0.667g (Figure 4.1). Mice in the control group had spleen sizes similar to that of healthy untreated mice (0.1g). In Figure 4.1 C-D, BM and splenocytes were assessed for expression of GFP. Similar to what was seen in peripheral blood, p210+ BMT mice had significantly higher levels of GFP expression than controls. Since granulocytosis is a hallmark of CML, we wanted to confirm that the GFP+ cells in mice receiving p210+ BMC were indeed granulocytes. Peripheral blood cells at day 16 post-BMT were stained with CD11b (Mac-1), a marker for granulocytes. The majority of GFP+ cells from p210+ BMT mice were CD11b+, whereas in control mice CD11b+ cells were not GFP positive and therefore reflect the phenotype of healthy mice.

B. Characterization of NK function in a murine CML model

We wanted to utilize this murine tumor model to better understand the contribution of NK cells in tumor surveillance of CML. Most of the reported studies assessing the role of NK cells in CML have been conducted *in vitro* with experimental target cell lines and effectors obtained from patients with advanced disease. Our goal was to add to the knowledge about NK cells and their response to CML, using primary target and effector cells from mice.

There is strong evidence that NK cells can preferentially kill BCR/ABL+ targets. The human tumor cell line, K562, commonly used as an NK sensitive cell line, is BCR/ABL+ and was derived from a CML patient. In addition, NK resistant leukemic cell lines can be made sensitive to human NK cells from CD34+ cord blood cells when transfected to express BCR/ABL (287).

NK cells have been implicated in participating in IS of CML because they play a role in GVL in CML patients after autologous BMT (177;288). However, the number of NK cells progressively decreases with advanced CML. NK cells isolated from CML patients display impaired lytic capacity. However, treatment with NK activating cytokines (IL-2 or IL-15 or interferon) can restore NK function. Because of these reported effects on NK cells in the CML patients, it has been suggested NK cells could play a role in tumor surveillance of CML(177;177;178;288;288;289). In addition, tumor cells may be escaping IS because aspects of the disease weaken NK cell function.

In support of this idea, it has been found that NK differentiation is altered in CML patients. Upon further study, it was shown these changes to NK cells were directly attributed

to presence of BCR/ABL and the predominance of surrounding myeloid cells (288). In addition, transformation of NK-92, a human NK cell line, with BCR/ABL showed decreased ability to lyse K562 targets and an increased ability to acquire inhibitory KIR receptors (290).

Evidence from these studies has suggested that BCR/ABL expression on targets or NK cells affects NK cytotoxicity directly. We wanted to test an alternative hypothesis to explain the reduced ability of NK cells in IS of CML. We wanted to determine if BCR/ABL+ tumor cells underwent changes that made them less susceptible to NK lysis as the disease progressed.

It has been thought that CML is a disease of stem cells but more recent evidence has shown that BCR/ABL can transform cells downstream from the pluripotent hematopoietic stem cell. BCR/ABL mRNA transcripts are absent from CD34+ Lin- progenitor cells but are found in cells of the myeloid lineage (291;292). We considered that NK cells may be effective in IS during the early stages of CML by killing these early myeloid CML precursors before they become leukemic granulocytes.

To investigate this hypothesis, we tested NK cytotoxicity against primary cells categorized as late stage CML targets and CML precursors. BM and splenocytes from mice demonstrating symptoms of CML were used as targets in a ^{51}Cr release assays. Cells used as targets were taken from a sacrificed mouse 23 days after BMT with p210+BMC. This mouse had a spleen weight of 0.6 grams and GFP expression in the BM (42%) and splenocytes (37%). BMC and splenocytes from a healthy untreated mouse were used as controls and YAC cells were used as a positive control for killing. Effectors cells were taken from a

healthy syngeneic mouse that was stimulated *in vivo* by administration of poly IC one day prior to the assay. The lytic capacity of poly IC activated splenocytes is believed to be mostly mediated by NK cells (293). Targets and effector cells were co-cultured for one hour. The results of this experiment showed that effector splenocytes, containing activated NK cells, were functional as they effectively killed YAC targets (Figure 4.2). But these effectors were unable to lyse BCR/ABL+ cells from late stage CML mice or healthy syngeneic cells. It appeared that BCR/ABL+ granulocytic cells from CML mice are resistant to NK lysis.

To follow up on this finding, we wanted to see if CML precursors were susceptible to NK lysis. To develop CML precursors, we retrovirally transduced BMC with the p210/GFP construct. We used the same protocol that was used to establish CML *in vivo*. In addition, as a control, we transduced BMC with the GFP-only construct with transduction efficiency around 20%. ^{51}Cr release assays were preformed using p210+ or GFP+ only transduced BM. Similar to the previous experiment, effectors were poly IC activated splenocytes from a syngeneic mouse. The effector and targets were incubated for one hour. In figure 4.3, the results of this assay show that NK cells can preferentially lyse p210+ BMC cells, but not BMC that express only GFP. Since both groups of BMC were treated similar prior to the assay, we concluded that the expression of BCR/ABL made these BMC sensitive to lysis. From the results of these two experiments, we deduced that NK cells are able to lyse CML precursors but not BCR/ABL+ more differentiated myeloid cells of advanced CML disease. Therefore, simple expression of BCR/ABL does not cause susceptibility to NK cells. Rather, the change in properties of the progenitor cells may be more important.

C. Effects of blocking NK inhibitory receptors on CML progression

Since it appears that NK cells are implicated in IS of CML, probably early in disease, we wanted to determine if blocking negative signals on NK cells could enhance IS and result in prolonged survival of mice with CML. The retrovirally transduction protocol to establish CML has been well established in BALB/c mice. However, B6 mice are more resistant to this protocol for establishing CML. Therefore for these experiments, we used BALB/c mice or (BALB.NK1.1 x B6) F1 mice.

To block negative signals in BALB/c mice ($H2^d$), we used the 4D11 mAb which block the activity of Ly49G2 receptor whose cognate ligand are $H2D^d$ and $H2K^d$. After establishing CML in BALB/c mice (n=6), mice were treated (ip) with 2-3 doses of 200ug of 4D11 $F(ab)'_2$ (n=3) and disease progression was monitored by assessing GFP expression in peripheral blood. Compared to mice that did not received mAb treatment, we did not see any effect of mAb treatment on disease progression or survival.

We hypothesized that a more sustained antibody level or a longer treatment period may be required to demonstrate an effect in this CML model. Therefore, we utilized the 5E6Tg mice (described in Chapter 3) to test this hypothesis. 5E6Tg mice of B6 background were crossed to BALB.NK1.1 mice (BALB/c mice congenic for the NKC of B6 origin). Using the F1 progeny, CML was established using standard protocol (described in Chapter 2), which exhibited similar disease to that of the BALB/c mice. BMCs from (BALB.NK1.1 x B6) F1 donor mice were transduced with p210/GFP and injected into (5E6Tg x BALB.NK1.1) F1 and control (B6 x BALB.NK1.1) F1 mice. Mice were monitored for GFP expression in peripheral blood to assess disease progression at different timepoints. As

shown in Figure 4.4A, at Day 5, both groups of mice had very low GFP expression in peripheral blood (close to 0%). At Day 8, GFP expression in control (B6 x BALB.NK1.1) F1 mice appeared to indicate disease progression with 25% GFP. However, (5E6Tg x BALB.NK1.1) F1 mice maintained low GFP expression in peripheral blood cells (3%). By day 14, both groups had similar levels of GFP+ cells in their blood and both groups of mice succumbed to CML at the same rate, 25-28 days post-BMT. Figure 4.4B displays results from a second experiment which shows similar results. Although 5E6Tg mice did not experience prolonged survival, it did appear as if 5E6Tg mice had a delayed onset of CML compared to controls.

II. Conclusions/Discussion

Studies using experimental cell lines showed that NK resistant targets can be made sensitive to NK lysis by expression of BCR/ABL (287). Our study evaluated two different BCR/ABL+ targets to assess why NK cells are not more effective in IS during CML disease progression. We showed that BCR/ABL+GFP+ cells from late stage CML are more resistant to NK lysis than cells that are the precursors for the disease.

This is supported by reports in the literature that have noted some myeloid leukemic blasts, cell lines or primary cells, are resistant to LAK killing (294). Some of the proposed reasons for this are that myeloid blast cells cause suppression of NK cell function by secreting reactive oxygen species and/or PGE_2. PGE_2 is made by myeloid cells such as monocytes and macrophages and inhibits NK activity, in part, by affecting NK cell synthesis of IFN-gamma (295-297). In fact, it has been shown that monocytes can be stimulated to make more PGE_2 when cultured with transformed cells such as K562 (298).

Another reason for the change in susceptibility of BCR/ABL+ targets is that the tumor cells may undergo further alteration to become fully transformed and developing alterations in immune recognition components. Earlier reports claimed that BCR/ABL expression was sufficient to transform tumors. But more recently, evidence suggests that additional mutations or modifications of BCR/ABL+ cells are needed to get full transformation and tumorigencity (299-301).

While expression of BCR/ABL in various cell lines or primary cells stimulates growth, these cells still maintained their ability to differentiate, comparable to the chronic phase of CML where myeloid cells have enhance proliferation, but are also still able to differentiate. In the blast phase crisis, differentiation is arrested while immature myeloid cells continue to accumulate. It is believed that a second event occurs causing complete transformation. This is further demonstrated in studies that have detected BCR/ABL gene expression in cells of healthy people that do not show signs of CML. In fact, in these studies, investigators were attempting to identify BCR/ABL- controls, and surprisingly found that many samples from healthy individuals had low but detectable levels of BCR/ABL mRNA as determined by RT-PCR. Samples from children and umbilical cord blood were also evaluated but found to be mostly negative for BCR/ABL transcripts (300;302).

In a recent study by I. L. Weissman's group, the requirement of oncogenic cooperatively for full transformation by BCR/ABL was evaluated. By using transgenic mice, it was shown that forced expression of BCR/ABL and bcl-2 (another oncogene) in pre-myeloid cells can trigger a blast crisis, whereas the single oncogene transgenic did not exhibit a blast phase. Other oncogenes, c-myc or N-ras, were also combined with BCR/ABL,

but did not provide the same synergistic effect. These findings were correlated to clinical data that show that patients in CML blast crisis have higher expression of bcl-2 compared to those in the chronic phase of CML. Taken together, it appears that blast phase of CML is a consequence of an accumulation of oncogenic mutations (300).

In turn, these findings can help us interpret the data regarding the susceptibility of BCR/ABL+ targets to NK cells. The expression of BCR/ABL alone may allow expression of ligands for NK activating receptors. As the disease progresses, those BCR/ABL+ cells that survive IS probably develop additional mutations that allow them to further transform and evade the NK response. Part of the immune evasion mechanism could include secretion of immune suppressive agents such as reactive oxygen species or PGE2. But in addition, the cells may be acquiring mutations in other cellular components that affect their ability to be lysed by NK cells.

As a way to prevent growth of BCR/ABL+ cells, imatinib mesylate has been effective in causing remission in many patients. But, studies in the murine models show that imatinib mesylate is not curative, even though it prolongs survival significantly. In addition, imatinib mesylate resistance has been reported in 25% of mice receiving treatment (286). The few cases of resistance reported in humans have been addressed by increasing the dose of imatinib mesylate (276). As suggested by the concept of "immunoediting", tumors adapt to their environment and respond to selective survival pressure by developing resistant clones. The use of imatinib mesylate adds another layer of selective pressure on the CML tumor cells and therefore the clones that grow will develop resistance to imatinib mesylate.

The need for novel immunotherapies for the treatment of CML is still required. Since CML tumors express MHC class I and are engaging NK inhibitory receptors, blocking the negative signals on NK cells may make them more susceptible to NK lysis. Using the 5E6Tg mice, we demonstrated that blocking mAb or the 5E6 transgene might indeed slow the progression of the disease. This supports our claims that NK cells appear more effective in the early stages of CML development and/or when CML tumor burden is low. BMT or imatinib mesylate treatment for CML is considered curative even though residual tumor cells may survive. These treatments may induce equilibrium between the tumors and the immune response. The effects of mAb treatment to block NK inhibitory receptors may be optimal when used with standard treatments to prevent relapse or at early signs of tumor escape.

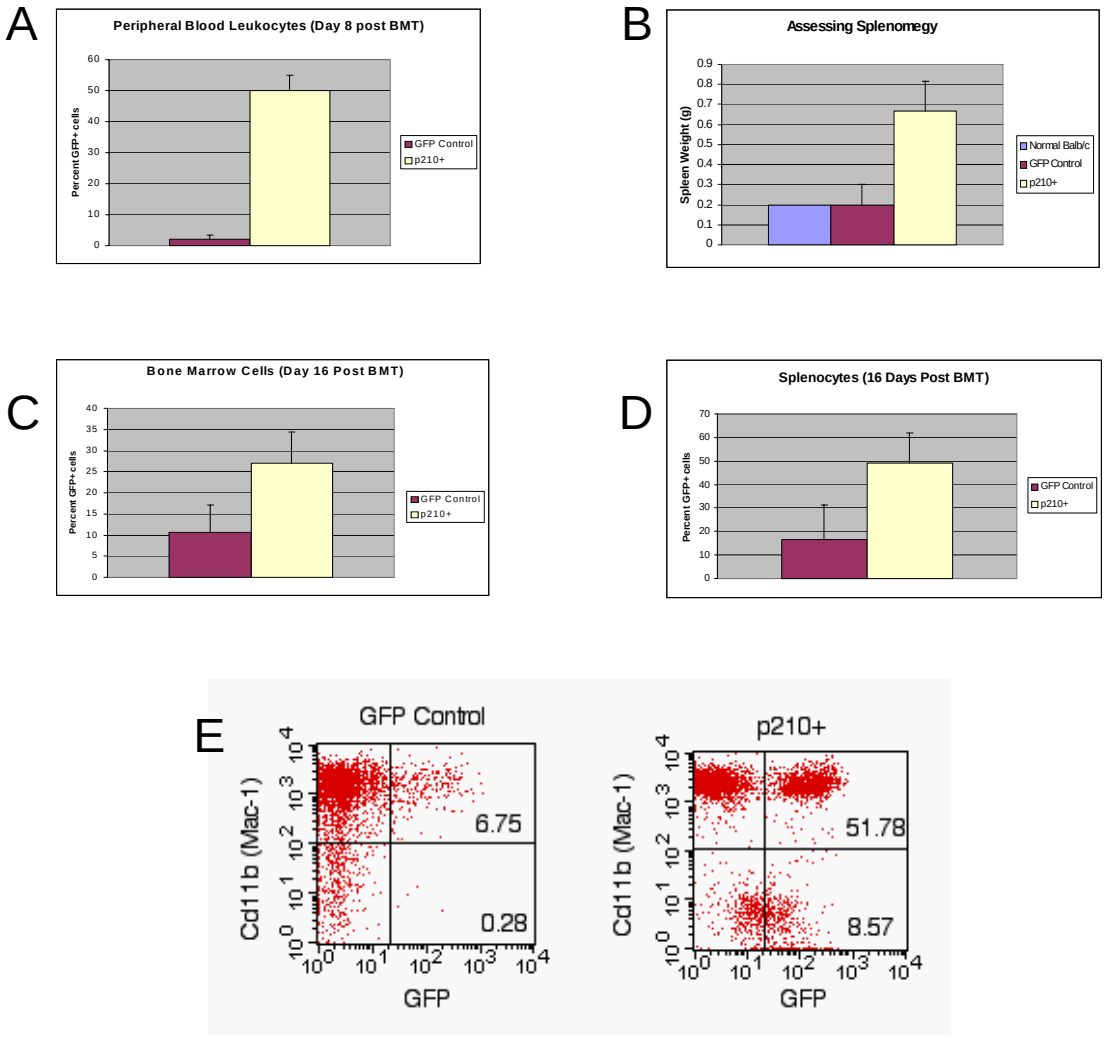


Figure 4.1: Diagnosis of CML-like disease in mice. (A) Peripheral blood leukocytes from mice 8 days post BMT was assessed for GFP expression. (B) Day 16 mice spleen weights. Normal mouse spleens weigh between 0.1-0.2g. (C, D) Bone marrow and splenocytes were assessed for expression of GFP on 16 days post BMT. (E) Day 16 peripheral blood was stained with CD11b (Mac-1) marker for granulocytes which accumulate in blood due to CML. Majority of GFP+ cells from p210+ mice are Mac-1+.

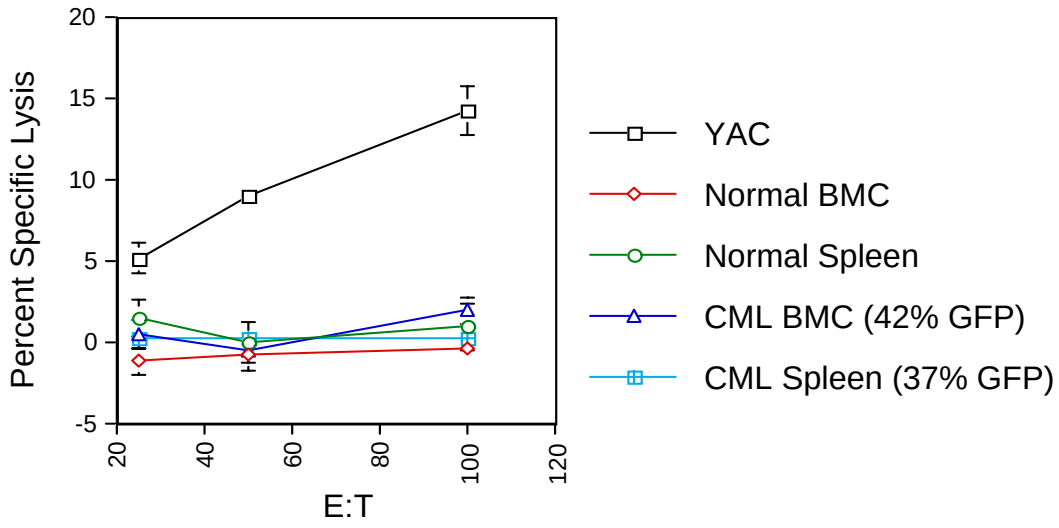


Figure 4.2: Late stage CML tumor cells are resistant to lysis by activated splenocytes. Poly IC treated splenocytes from BALB/c mice were used as effectors in a 1 hour ^{51}Cr release assay. As a positive control, YAC cells were used as NK sensitive targets. Bone marrow cells and splenocytes from untreated BALB/c mouse were used negative killing control and bone marrow and splenocytes from Day 23 (late stage) CML were tested for sensitivity to NK lysis. Graph shows percentage of specific lysis. Error bars indicate standard deviations.

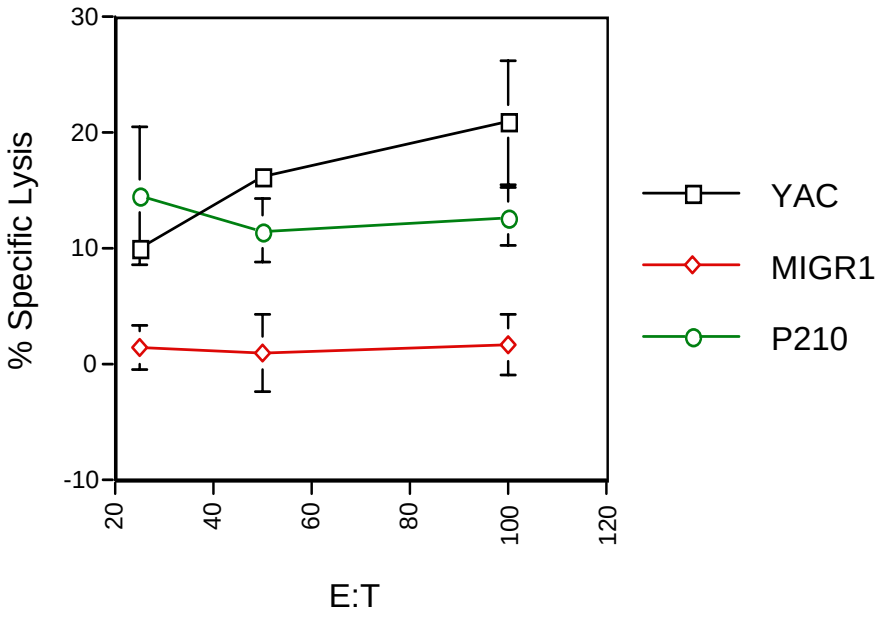


Figure 4.3: CML precursor BMCs are preferentially lysed by activated splenocytes. Poly IC treated splenocytes from BALB/c mice were used as effectors in a 1 hour ^{51}Cr release assay. As a positive control, YAC cells were used as NK sensitive targets. Bone marrow transduced with p210/GFP or GFP only were tested for NK lysis. Graph shows percentage of specific lysis. Error bars indicate standard deviations.

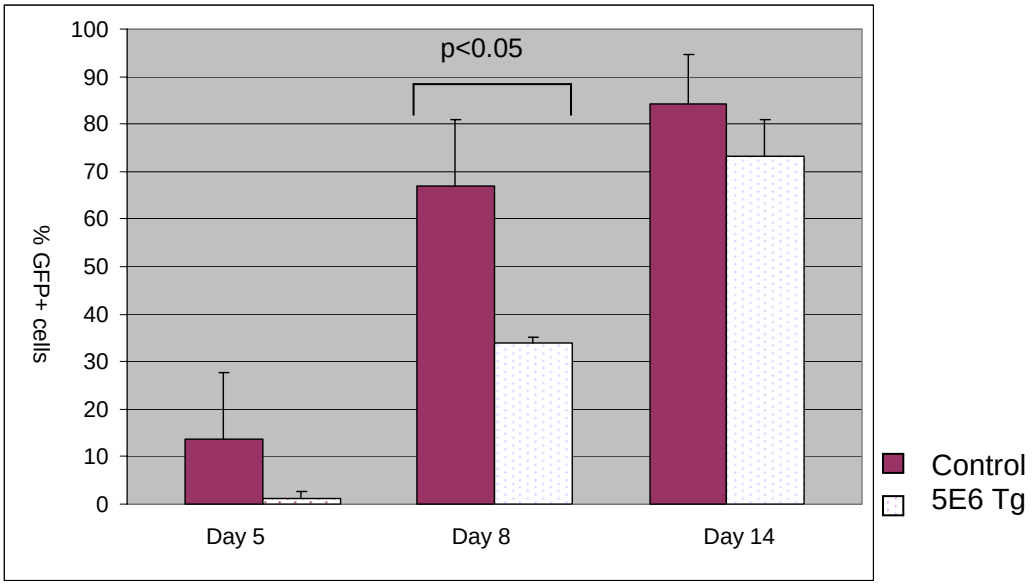


Figure 4.4: 5E6Tg mice exhibit delayed onset of CML. (BALB.NK1.1 x 5E6tg) F1 mice display resistance to CML at Day 8. By Day 14, CML progression is similar to controls. Lethally irradiated (BALB.NK1.1 xB6)F1 or (BALB.NK1.1 x 5E6tg) F1 mice were infused with p210/GFP transduced BMC and monitored for GFP expression in peripheral blood. GFP indicates CML disease progression. Statistically significant in a 1-tailed t-test indicated by p value. Experiment has n=5 per group.

CHAPTER FIVE

Discussion and Significance

I. Summary

The goal of this thesis project was to develop a model to further study how blocking inhibitory Ly49 receptors *in vivo* enhances anti-tumor immunity of NK cells. We chose to develop a transgenic model that would produce non-depleting 5E6 (anti-Ly49C/I) mAb in order to study this further. Using this model, we were able to address some important questions regarding NK cells in regards to development, tolerance and tumor clearance (as discussed in Chapter 3 and 4). But in order to investigate these questions several obstacles of our transgenic system needed to be overcome.

Once the transgenic mice were created and several founders were identified by PCR, we had difficulty developing effective screens to determine which founder would be studied. In the experimental design of the transgenic, we put an epitope tag on the mAb gene to help distinguish the transgenic antibody from endogenous antibody. And therefore, we planned to quantitate serum antibody titers in this fashion. But, our choice of epitope tags (His) was poor since commercial antibodies for His detection proved to be inadequate for this task. We learned that mAb structure influences serum clearance. Smaller antibody molecules have reduced half-life compared to whole mAb (303). We realized that serum detection would be difficult but, we still expected one of the founder lines would make antibody in such excess that we could detect it. We had hoped to compare a high expresser founder line with a low expresser founder line, to see how mAb concentration affected the phenotype of the mice. Because serum detection was difficult, we chose to use the flow cytometry screen where we

attempted to detect antibody bound to NK cells directly ex vivo (Figure 3.3). And later we developed the competitive binding assay which also confirmed that serum antibody levels were below detectable levels (Figure 3.6).

The low to no antibody levels may have been attributed to our choice of promoters and transgenic construct design. We had evaluated other antibody transgenic models and we believed that the CMV promoter would give us the best chances for success. But the limitation with using the CMV promoter is that we could not predict patterns for the transgenic mRNA transcription. If we had used a tissue specific promoter, such as albumin, our screens for expression would have been more targeted and easier to assess. In addition, we chose to use express our transgene as part of a bicistronic construct. Generally, in bicistronic constructs, the two genes are not transcribed in equal proportions. More of the first gene product is made than the second gene product. Therefore, 5E6Tg protein production may have been limited by LC translation, since it occupied the second part of the cistron. A more effective option would have been to use a one gene construct, where both genes were fused together to make the protein of interest. Expression of non-depleting 5E6 mAb may have been better expressed as a scFv. The 5E6 scFv was not developed at the time the transgenic mice were generated, but may be an option for future studies.

An alternative explanation of why antibody was not detected in the 5E6Tg mice is that no antibody was ever made. This would mean that even though transgenic DNA was integrated and transcribed to make transgenic RNA, the protein product of transgene was not produced. A consequence in developing the transgenic mice using our DNA construct with CMV promoter is that the transgenic DNA incorporates into the mouse genome randomly.

Random integration of transgene can alter the phenotype of mice by affecting other endogenous gene expression or function. In the 5E6Tg mice, we observed several results that showed statistical significant differences between 5E6Tg mice and B6 controls. Since we were unable to confirm transgene expression by determine antibody in the serum of mice, we can not rule out that these results were due to random transgene incorporation. To substantiate that our results were not due to random integration, another founder line would have had to be evaluated. Since no other founder line was available for study, we can not entirely confirm that results seen using the 5E6Tg mice were not a consequence of procedure to make the mice.

For future studies, there are other ways to develop an *in vivo* model for studying blocking negative signals to improve NK tumor clearance. Now that lentiviral vectors are more readily available, mice can be locally infected with a lentivirus engineered to express the 5E6 mAb fragment. Similar studies have been conducted as described in (304). In addition, R.M. Chu has developed a method which incorporates DNA into muscle by electroporation (personal communication). This technique has been tested with the IL-6 gene which resulted in transient expression at high levels for one week.. Another *in vivo* method to allow sustained protein levels involves using nanoparticle technology. Studies have been done using IL-2, which have demonstrated the usefulness of this technology in providing a slow release of cytokines (PV. Kulkarni, personal communication). These methods would allow the study of improved tumor clearance by NK cells but may not be ideal for studying the effects of Ly49-MHC interactions in NK and T cells development, if used in adult mice, which is why we chose to develop a transgenic model.

Our conclusions regarding the effect of 5E6 transgene expression in mice were that it did not dramatically affect NK or T cell development of receptor repertoires. Some of the reasons for this could be that such low antibody levels were not sufficient to cause an effect. Or perhaps, the specific Ly49 receptor that was blocked did not affect development. Data regarding Ly49 transgenic mice led us to this latter consideration. Studies of the Ly49C and A transgenic mice reported that there were changes in T cell receptor profiles and function (68;69). The results were opposite in studies using the Ly49I transgenic mice which did not exhibit these changes (80). As discussed in Chapter 1, the strength of binding that Ly49 receptors exhibit with their cognate ligand may influence functional characteristics of these receptors. The Ly49 receptors, though they share genetic and structural similarities, can bind their ligands in different fashions shown by their crystal structures (305). Ly49A was found to be a powerful regulator of T cell function, because it can rescue CTLA-4-/- mice from lymphoproliferative disease (75). Whereas, Ly49I has weaker interactions than Ly49A with its ligands (65). This is predicted to affect its strength of inhibition (72). Therefore, studies in the Ly49 transgenic mice provides insight into what may happen in the circumstances when the Ly49 receptor interactions are blocked instead of activated , like in the 5E6Tg mice. We speculate that if these blocking studies were conducted with anti-Ly49A mAb, the effect on NK and T cell development may have been different.

The expression of inhibitory receptors restricts NK activity to prevent autoimmune effects. NK cells have been implicated in some autoimmune diseases, as they can modulate the response of other immune cells (306). But, NK cells themselves do not appear to show alloreactivity or GVHD *in vivo* as seen in NK mismatched BMT (172;173). In fact, as

discussed in Chapter 1, NK cells appear to have an interesting way to become tolerant to new MHC environments after BMTs. The understanding of the nature of this tolerance development has only partially been elucidated. It is those same mechanisms that allowed NK cells in the 5E6Tg to remain tolerant to 'self' shown by syngeneic BMTs (Figure 3.10). Because NK cells can maintain tolerance even when 50% of the cells have inhibitory receptors blocked (like in the 5E6Tg mice, or when mAb is administered), the use of NK cells as a base of immunotherapies is very appealing.

Our assessment in tumor challenges using NK cells with inhibitory blocking antibody supports this claim. Previous data showed the efficacy of 5E6 mAb treatment in an *in vivo* C1498 leukemia model and in syngeneic and allogeneic purging models (194;195;225). We have only begun to explore enhanced NK activity for tumor clearance in the 5E6Tg mice. We showed using the B16 melanoma model and a nascent tumor model of CML that 5E6 mAb transgenic mice have enhanced NK anti-tumor functions. Future experiments can be done to try to demonstrate more pronounced effects of blocking Ly49C/I in CML.

Due to the fashion in which the CML model is established, recipient mice undergo lethal irradiation and their radio-resistant NK cells are only available for approximately 5-7 days post BMT. Then NK cells do not return to normal numbers or function until approximately day 14. We believe that during days 5 and 14 there is no IS occurring and the tumor grows exponentially. Therefore, to demonstrate a more substantial effect of NK inhibitory receptor blockade, a variation of experimental design is needed. We propose that if mice receive an adoptive transfer of NK cells on Day 5, the blocking antibody might continue to modulate the activity of NK cells and a greater effect on CML progression will

be observed. In a preliminary experiment (n=3), 5 days after undergoing p210+ BMT, mice received adoptive transfer of NK cells. One group of mice received 4D11 F(ab)'$_2$ (anti-Ly49G2) treatment (twice a week for 3 weeks). In this experiment, 2 of 3 CML mice receiving 4D11 antibody displayed dramatically improved survival (60+ days), while the control group succumbed to disease within 3 weeks. This experimental design should maintain levels of NK cell function. In future studies we would like to utilize the 5E6Tg mice in these adoptive transfer experiment, which does not require continuous administration of blocking inhibitory receptor mAb. Our studies indicate that NK cells appear to be effective when tumor burden is low. Since imatinib mesylate can maintain low tumor burden in mice, studies with imatinib mesylate and blocking negative signals may provide interesting results and implications for combinational therapies.

To establish CML in the 5E6Tg mice, we used F1 progeny of B6 x BALB.NK1.1 mice. These mice are $H2^{b/d}$ and possess inhibitory receptors for both MHC molecules. But, in the transgenic, we are only blocking Ly49C/I which sees $H2^{b}$, therefore, if we combined the transgenic mice with the administration of 4D11 (anti-Ly49G2 which sees $H2^{d}$) we could block a majority of the inhibitory receptors.

We have considered expanding the effect of blocking inhibitory signals by finding a receptor target that is on all NK cells. By blocking inhibitory signals on all NK cells compared to just on 50%, as we have done so far, may improve our tumor challenge results dramatically. The challenge has been finding antibodies that block all NK inhibitory receptors. One way to accomplish this is to use a cocktail of mAb for Ly49s or develop a pan-Ly49 mAb that see host MHC antigens. In addition, recently, it has been determined that

2B4 has inhibitory function when engaged by its ligand CD48 (98). 2B4 is expressed on all activated NK cells. There is potential to enhance NK tumor rejection of CD48+ tumors when 2B4 is blocked by mAb. Another approach is to block all inhibitory receptor function by targeting a common downstream signaling molecule. SHP-1 and SHP-2 are phosphatases that are recruited to ITIMS of intracellular portion of Ly49 inhibitory receptors. These phosphatases are responsible for shutting off signaling pathway thus resulting in NK inhibition. In collaboration with C. Wuelfing, our lab has developed a dominant negative SHP mutant (dnSHP). This recombinant protein is fused to tat molecule to facilitate cellular import (307). Once inside a cell the dnSHP should compete for binding with phosphatases that are recruited to ITIMs of inhibitory receptors. The advantage of targeting phosphatase activity is that SHP is a common signaling intermediate in inhibitory receptor function on NK cells. A feature of the tat fusion proteins is that they move by passive diffusion in and out of the cell until equilibrium is reached, therefore, to use tat fusion to block SHP activity, cells and medium must be loaded with adequate concentration of fusion protein (*in vitro* this could range from nM to mM concentrations). Using tat-dnSHP approach may be feasible in an *ex vivo* purging model. But using it as *in vivo* treatment option may not be ideal. First, it will require a massive amount of protein and secondly, it has the potential to cause many adverse effects as seen in the moth-eaten mice (SHP knockout mice) (308).

We have had success in improving NK rejection of tumors with blockade of only a subset of the NK cells (194;195). Whether blockade of all NK inhibitory receptors is necessary from improved NK activity has not been evaluated. In fact, there is some evidence that NK cells participate in cross-talk, by some unknown mechanism. It is thought that NK

cells can influence the function of other NK cells that do not express the same inhibitory receptors (309). A demonstration of this concept was shown in BMT studies with large BMC inoculums. Generally, NK cells are unable to reject large inoculums (25×10^6) of allogeneic BMC. When a subset of NK cells that contains inhibitory Ly49 for donor class I antigens is depleted, these mice can now reject the large inoculum of BMC, even though the absolute number of NK cells *in vivo* is greatly reduced. This implies that some subsets of NK cells are inhibiting other NK cells from rejection. We hypothesize that a similar effect could be occurring when mAb are used to block Ly49 inhibitory signals. So, in addition to enhancing tumor rejection capacity of Ly49C/I+ cells, receptor blockade with 5E6 mAb could be affecting Ly49C/I- cells (309). Further studies of these concepts are ongoing in our laboratory.

II. Implications

The field of immunotherapy has been develop innovative technologies and has helped to advance our knowledge of the immune system, in general. It provides promise of new therapies to cure or treat cancer, but so far has fallen short. Translating exciting effects seen in animal studies into the human system has been the greatest obstacle. One reason for these difficulties is that the knowledge base of basic science is divergent from knowledge base in the clinical setting. In order to successfully translate investigational immunotherapies into clinic, a broader understanding of basic research in humans is needed (310).

Translating the concept of blocking NK inhibitory signals to improve tumor rejection may encounter similar difficulties. It is conceivable that human NK inhibitory receptors,

KIRs, can be blocked and initiate enhanced tumor rejection by NK cells. Since the MHC environment which dictates KIR expression is highly polymorphic in humans, finding the right target molecules for mAb that are conserved will be difficult.

Recently, there have been reports of cancer cells expressing HLA-G, the non-classical class I molecule involved with fetal-maternal tolerance. The expression of HLA-G by tumor cells results in inhibition of NK response by CD94/NKG2A. Studies have shown that HLA-G expressing melanoma cell line are resistant to NK and CTL lysis (142-145;307). But blocking HLA-G or CD94/NKG2A with a specific mAb reverses inhibition. Since CD94 is more conserved and non-polymorphic between mouse and humans, than Ly49 and KIRs, this approach has great potential for translation into a human immunotherapy.

In general, immunotherapy approaches may fail because the diversity of the human immune system and cancer cells is so vast that designing a therapy to fit all cases would be difficult. If cancers could be further phenotyped, it may be possible to customize immunotherapies and specifically target those tumors. This has begun to be explored by Genitope (Redwood City, CA). The company has ongoing clinical trials in which they develop cancer vaccines to tumor antigens defined from individual patient's tumors. The vaccine is combined with adjuvant and an approved mAb therapy. Rituximab plus the Genitope personalized vaccine have been combined for treatment of patients with Follicular Non-Hodgkin's Lymphoma and has advanced into Phase 3 trials (311).

In order for these personalized therapies to be a feasible option, clinicians and basic scientists need further collaboration and exchange of knowledge. Customizing cancer treatment will force clinicians to evaluate individual patients differently than the manner in

which they are trained. Patients will not be able to be placed into generalized categories with the same treatment protocol. In addition, basic researchers need better insight into patient manifestation of disease and how associated complications can affect the treatment course.

Some investigators have already lost hope in finding better treatments for cancer, as they have commented in the literature (120;310). The competition between the immune response and cancer cells is ongoing and constantly evolving. They believe that as better immunotherapies are developed, selective pressure on the tumor will increase and result in more immune escape variants. Maybe eliminating cancer altogether is not realistic but perhaps the goal should be to develop therapies to maintain the equilibrium between cancer and the immune response and therefore cancer can be treated as a chronic disease. Cancer cells will continue to evolve and develop immune escape variants, but meanwhile researchers will persevere to find ways to improve our immune response through acquiring a better understanding of the immune system and cancer development, and that too is evolution.

Reference List

1 Bartek, J. and Lukas, J. 2001. Mammalian G1- and S-phase checkpoints in response to DNA damage. *Curr.Opin.Cell Biol.* 13:738-747.

2 Nojima, H. 2004. G1 and s-phase checkpoints, chromosome instability, and cancer. *Methods Mol.Biol.* 280:3-50.

3 Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., and Schreiber, R. D. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat.Immunol.* 3:991-998.

4 Foley, E. J. 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* 13:835-837.

5 Burnet, F. M. 1964. Immunological factors in the process of carcinogenesis. *British Medical Bulletin* 20:154-158.

6 Burnet, F. M. 1971. Immunological surveillance in neoplasia. *Transplant.Rev.* 7:3-25.

7 Schneck, S. A. and Penn, I. 1971. De-novo brain tumours in renal-transplant recipients. *Lancet* 1:983-986.

8 Gatti, R. A. and Good, R. A. 1971. Occurrence of malignancy in immunodeficiency diseases. A literature review. *Cancer* 28:89-98.

9 Hellstrom, I. E., Hellstrom, K. E., Pierce, G. E., and Bill, A. H. 1968. Demonstration of cell-bound and humoral immunity against neuroblastoma cells. *Proc.Natl.Acad.Sci.U.S.A* 60:1231-1238.

10 Stutman, O. 1974. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science* 183:534-536.

11 Pardoll, D. 2001. T cells and tumours. *Nature* 411:1010-1012.

12 Ikehara, S., Pahwa, R. N., Fernandes, G., Hansen, C. T., and Good, R. A. 1984. Functional T cells in athymic nude mice. *Proc.Natl.Acad.Sci.U.S.A* 81:886-888.

13 Maleckar, J. R. and Sherman, L. A. 1987. The composition of the T cell receptor repertoire in nude mice. *J.Immunol.* 138:3873-3876.

14 Dighe, A. S., Richards, E., Old, L. J., and Schreiber, R. D. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity.* 1:447-456.

15 Kaplan, D. H., Shankaran, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., and Schreiber, R. D. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc.Natl.Acad.Sci.U.S.A* 95:7556-7561.

16 Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., and Schreiber, R. D. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410:1107-1111.

17 van den Broek, M. E., Kagi, D., Ossendorp, F., Toes, R., Vamvakas, S., Lutz, W. K., Melief, C. J., Zinkernagel, R. M., and Hengartner, H. 1996. Decreased tumor surveillance in perforin-deficient mice. *J.Exp.Med.* 184:1781-1790.

18 Smyth, M. J., Taniguchi, M., and Street, S. E. 2000. The anti-tumor activity of IL-12: mechanisms of innate immunity that are model and dose dependent. *J.Immunol.* 165:2665-2670.

19 Trinchieri, G. 1989. Biology of Natural Killer Cells. *Adv.Immunol.* 47.

20 Herberman, R. B., Nunn, M. E., Holden, H. T., and Lavrin, D. H. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int.J.Cancer* 16:230-239.

21 Herberman, R. B., Nunn, M. E., and Lavrin, D. H. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int.J.Cancer* 16:216-229.

22 Kiessling, R., Klein, E., and Wigzell, H. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur.J.Immunol.* 5:112-117.

23 Kiessling, R., Klein, E., Pross, H., and Wigzell, H. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur.J.Immunol.* 5:117-121.

24 Weissler, J. C., Nicod, L. P., Lipscomb, M. F., and Toews, G. B. 1987. Natural killer cell function in human lung is compartmentalized. *Am.Rev.Respir.Dis.* 135:941-949.

25 Lowin, B., Peitsch, M. C., and Tschopp, J. 1995. Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. *Curr.Top.Microbiol.Immunol.* 198:1-24.

26 Trambas, C. M. and Griffiths, G. M. 2003. Delivering the kiss of death. *Nat.Immunol.* 4:399-403.

27 Wulfing, C., Purtic, B., Klem, J., and Schatzle, J. D. 2003. Stepwise cytoskeletal polarization as a series of checkpoints in innate but not adaptive cytolytic killing. *Proc.Natl.Acad.Sci.U.S.A* 100:7767-7772.

28 Arase, H., Saito, T., Phillips, J. H., and Lanier, L. L. 2001. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). *J.Immunol.* 167:1141-1144.

29 Vales-Gomez, M., Reyburn, H., and Strominger, J. 2000. Interaction between the human NK receptors and their ligands. *Crit Rev.Immunol.* 20:223-244.

30 van der Vliet, H. J., Molling, J. W., von Blomberg, B. M., Nishi, N., Kolgen, W., van den Eertwegh, A. J., Pinedo, H. M., Giaccone, G., and Scheper, R. J. 2004. The immunoregulatory role of CD1d-restricted natural killer T cells in disease. *Clin.Immunol.* 112:8-23.

31 Kiessling, R., Klein, E., and Wigzell, H. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur.J.Immunol.* 5:112-117.

32 Kiessling, R., Klein, E., Pross, H., and Wigzell, H. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur.J.Immunol.* 5:117-121.

33 Herberman, R. B., Nunn, M. E., and Lavrin, D. H. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int.J.Cancer* 16:216-229.

34 Karre, K., Klein, G. O., Kiessling, R., Klein, G., and Roder, J. C. 1980. In vitro NK-activity and in vivo resistance to leukemia: studies of beige, beige//nude and wild-type hosts on C57BL background. *Int.J.Cancer* 26:789-797.

35 Talmadge, J. E., Meyers, K. M., Prieur, D. J., and Starkey, J. R. 1980. Role of NK cells in tumour growth and metastasis in beige mice. *Nature* 284:622-624.

36 Haliotis, T., Ball, J. K., Dexter, D., and Roder, J. C. 1985. Spontaneous and induced primary oncogenesis in natural killer (NK)-cell-deficient beige mutant mice. *Int.J.Cancer* 35:505-513.

37 Cudkowicz, G. and Bennett, M. 1971. Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F 1 hybrid mice. *J.Exp.Med.* 134:1513-1528.

38 Bennett, M. 1987. Biology and genetics of hybrid resistance. *Adv.Immunol.* 41:333-445.

39 Cudkowicz, G. and Bennett, M. 1971. Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice. *J.Exp.Med.* 134:83-102.

40 Bennett, M. 1973. Prevention of marrow allograft rejection with radioactive strontium: evidence for marrow-dependent effector cells. *J.Immunol.* 110:510-516.

41 Kiessling, R., Hochman, P. S., Haller, O., Shearer, G. M., Wigzell, H., and Cudkowicz, G. 1977. Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. *Eur.J.Immunol.* 7:655-663.

42 Varla-Leftherioti, M. 2004. Role of a KIR/HLA-C allorecognition system in pregnancy. *J.Reprod.Immunol.* 62:19-27.

43 Yokoyama, W. M., Kim, S., and French, A. R. 2004. The dynamic life of natural killer cells. *Annu.Rev.Immunol.* 22:405-429.

44 French, A. R. and Yokoyama, W. M. 2004. Natural killer cells and autoimmunity. *Arthritis Res.Ther.* 6:8-14.

45 Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P., and Salazar-Mather, T. P. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu.Rev.Immunol.* 17:189-220.

46 Bukowski, J. F., Woda, B. A., Habu, S., Okumura, K., and Welsh, R. M. 1983. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. *J.Immunol.* 131:1531-1538.

47 Ljunggren, H. G. and Karre, K. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol.Today* 11:237-244.

48 Lanier, L. L. 1997. Natural killer cells: from no receptors to too many. *Immunity.* 6:371-378.

49 Lanier, L. L. and Phillips, J. H. 1996. Inhibitory MHC class I receptors on NK cells and T cells. *Immunol.Today* 17:86-91.

50 Lanier, L. L. 1998. NK cell receptors. *Annu.Rev.Immunol.* 16:359-393.

51 Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. *Annu.Rev.Immunol.* 17:875-904.

52 Yokoyama, W. M. 1998. Natural killer cell receptors. *Curr.Opin.Immunol.* 10:298-305.

53 Yokoyama, W. M. and Plougastel, B. F. 2003. Immune functions encoded by the natural killer gene complex. *Nat.Rev.Immunol.* 3:304-316.

54 Vales-Gomez, M., Reyburn, H., and Strominger, J. 2000. Molecular analyses of the interactions between human NK receptors and their HLA ligands. *Hum.Immunol.* 61:28-38.

55 Makrigiannis, A. P., Pau, A. T., Saleh, A., Winkler-Pickett, R., Ortaldo, J. R., and Anderson, S. K. 2001. Class I MHC-binding characteristics of the 129/J Ly49 repertoire. *J.Immunol.* 166:5034-5043.

56 Makrigiannis, A. P. and Anderson, S. K. 2001. The murine Ly49 family: form and function. *Arch.Immunol.Ther.Exp.(Warsz.)* 49:47-50.

57 Raulet, D. H., Held, W., Correa, I., Dorfman, J. R., Wu, M. F., and Corral, L. 1997. Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. *Immunol.Rev.* 155:41-52.

58 Moretta, A., Bottino, C., Vitale, M., Pende, D., Biassoni, R., Mingari, M. C., and Moretta, L. 1996. Receptors for HLA class-I molecules in human natural killer cells. *Annu.Rev.Immunol.* 14:619-648.

59 Dorfman, J. R. and Raulet, D. H. 1996. Major histocompatibility complex genes determine natural killer cell tolerance. *Eur.J.Immunol.* 26:151-155.

60 Mason, L. H., Ortaldo, J. R., Young, H. A., Kumar, V., Bennett, M., and Anderson, S. K. 1995. Cloning and functional characteristics of murine large granular lymphocyte-1: a member of the Ly-49 gene family (Ly-49G2). *J.Exp.Med.* 182:293-303.

61 Sentman, C. L., Hackett, J., Jr., Kumar, V., and Bennett, M. 1989. Identification of a subset of murine natural killer cells that mediates rejection of Hh-1d but not Hh-1b bone marrow grafts. *J.Exp.Med.* 170:191-202.

62 Sentman, C. L., Kumar, V., and Bennett, M. 1991. Rejection of bone marrow cell allografts by natural killer cell subsets: 5E6+ cell specificity for Hh-1 determinant 2 shared by H-2d and H-2f. *Eur.J.Immunol.* 21:2821-2828.

63 Brennan, J., Lemieux, S., Freeman, J. D., Mager, D. L., and Takei, F. 1996. Heterogeneity among Ly-49C natural killer (NK) cells: characterization of highly related receptors with differing functions and expression patterns. *J.Exp.Med.* 184:2085-2090.

64 Yu, Y. Y., George, T., Dorfman, J. R., Roland, J., Kumar, V., and Bennett, M. 1996. The role of Ly49A and 5E6(Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blasts. *Immunity.* 4:67-76.

65 Hanke, T., Takizawa, H., McMahon, C. W., Busch, D. H., Pamer, E. G., Miller, J. D., Altman, J. D., Liu, Y., Cado, D., Lemonnier, F. A., Bjorkman, P. J., and Raulet, D. H. 1999. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity.* 11:67-77.

66 Vivier, E. and Anfossi, N. 2004. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat.Rev.Immunol.* 4:190-198.

67 Peacock, C. D., Xu, W., Stepp, S. E., and Welsh, R. M. 2002. Dynamics of Ly49 expressing cytotoxic lymphocyte subsets in response to virus infection. *Microbes.Infect.* 4:1481-1490.

68 Held, W., Cado, D., and Raulet, D. H. 1996. Transgenic expression of the Ly49A natural killer cell receptor confers class I major histocompatibility complex (MHC)-specific inhibition and prevents bone marrow allograft rejection. *J.Exp.Med.* 184:2037-2041.

69 Fahlen, L., Khoo, N. K., Daws, M. R., and Sentman, C. L. 1997. Location-specific regulation of transgenic Ly49A receptors by major histocompatibility complex class I molecules. *Eur.J.Immunol.* 27:2057-2065.

70 Oberg, L., Eriksson, M., Fahlen, L., and Sentman, C. L. 2000. Expression of Ly49A on T cells alters the threshold for T cell responses. *Eur.J.Immunol.* 30:2849-2856.

71 Oberg, L., Eriksson, M., Fahlen, L., and Sentman, C. L. 2000. Expression of Ly49A on T cells alters the threshold for T cell responses. *Eur.J.Immunol.* 30:2849-2856.

72 Chalifour, A., Roger, J., Lemieux, S., and Duplay, P. 2003. Receptor/ligand avidity determines the capacity of Ly49 inhibitory receptors to interfere with T-cell receptor-mediated activation. *Immunology* 109:58-67.

73 Hanke, T. and Raulet, D. H. 2001. Cumulative inhibition of NK cells and T cells resulting from engagement of multiple inhibitory Ly49 receptors. *J.Immunol.* 166:3002-3007.

74 Natarajan, K., Dimasi, N., Wang, J., Margulies, D. H., and Mariuzza, R. A. 2002. MHC class I recognition by Ly49 natural killer cell receptors. *Mol.Immunol.* 38:1023-1027.

75 Chambers, C. A., Kang, J., Wu, Y., Held, W., Raulet, D. H., and Allison, J. P. 2002. The lymphoproliferative defect in CTLA-4-deficient mice is ameliorated by an inhibitory NK cell receptor. *Blood* 99:4509-4516.

76 Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A., and Sharpe, A. H. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* 3:541-547.

77 Cambiaggi, A., Verthuy, C., Naquet, P., Romagne, F., Ferrier, P., Biassoni, R., Moretta, A., Moretta, L., and Vivier, E. 1997. Natural killer cell acceptance of H-2 mismatch bone marrow grafts in transgenic mice expressing HLA-Cw3 specific killer cell inhibitory receptor. *Proc.Natl.Acad.Sci.U.S.A* 94:8088-8092.

78 Liu, J., Morris, M. A., Nguyen, P., George, T. C., Koulich, E., Lai, W. C., Schatzle, J. D., Kumar, V., and Bennett, M. 2000. Ly49I NK cell receptor transgene inhibition of rejection of H2b mouse bone marrow transplants. *J.Immunol.* 164:1793-1799.

79 Morris, M. A., Koulich, E., Liu, J., Arora, V., George, T. C., Schatzle, J. D., Kumar, V., and Bennett, M. 2002. Definition of additional functional ligands for Ly49I(B6) using FVBLy49I(B6) transgenic mice and B6 natural killer cell effectors. *Transplantation* 74:1449-1454.

80 Morris, M. A., Liu, J., Arora, V., George, T. C., Klem, J., Schatzle, J. D., Kumar, V., and Bennett, M. 2002. B6 strain Ly49I inhibitory gene expression on T cells in FVB.Ly49IB6 transgenic mice fails to prevent normal T cell functions. *J.Immunol.* 169:3661-3666.

81 Dimasi, N., Sawicki, M. W., Reineck, L. A., Li, Y., Natarajan, K., Margulies, D. H., and Mariuzza, R. A. 2002. Crystal structure of the Ly49I natural killer cell receptor reveals variability in dimerization mode within the Ly49 family. *J.Mol.Biol.* 320:573-585.

82 Coles, M. C., McMahon, C. W., Takizawa, H., and Raulet, D. H. 2000. Memory CD8 T lymphocytes express inhibitory MHC-specific Ly49 receptors. *Eur.J.Immunol.* 30:236-244.

83 Dorfman, J. R. and Raulet, D. H. 1996. Major histocompatibility complex genes determine natural killer cell tolerance. *Eur.J.Immunol.* 26:151-155.

84 Held, W., Dorfman, J. R., Wu, M. F., and Raulet, D. H. 1996. Major histocompatibility complex class I-dependent skewing of the natural killer cell Ly49 receptor repertoire. *Eur.J.Immunol.* 26:2286-2292.

85 Karlhofer, F. M., Hunziker, R., Reichlin, A., Margulies, D. H., and Yokoyama, W. M. 1994. Host MHC class I molecules modulate in vivo expression of a NK cell receptor. *J.Immunol.* 153:2407-2416.

86 Salcedo, M., Diehl, A. D., Olsson-Alheim, M. Y., Sundback, J., Van Kaer, L., Karre, K., and Ljunggren, H. G. 1997. Altered expression of Ly49 inhibitory receptors on natural killer cells from MHC class I-deficient mice. *J.Immunol.* 158:3174-3180.

87 Fahlen, L., Lendahl, U., and Sentman, C. L. 2001. MHC class I-Ly49 interactions shape the Ly49 repertoire on murine NK cells. *J.Immunol.* 166:6585-6592.

88 Raulet, D. H., Vance, R. E., and McMahon, C. W. 2001. Regulation of the natural killer cell receptor repertoire. *Annu.Rev.Immunol.* 19:291-330.

89 Dorfman, J. R., Zerrahn, J., Coles, M. C., and Raulet, D. H. 1997. The basis for self-tolerance of natural killer cells in beta2-microglobulin- and TAP-1- mice. *J.Immunol.* 159:5219-5225.

90 Johansson, M. H., Bieberich, C., Kase-Sjostrom, A., Yoshioka, T., Hoglund, E., Christy, B. A., Scangos, G., Karre, K., Jay, G., and Hoglund, P. 2000. Differential effects on T cell and NK cell development by tissue-specific expression of H-2D(d) transgene. *Eur.J.Immunol.* 30:525-533.

91 Johansson, M. H., Hoglund, E., Nakamura, M. C., Ryan, J. C., and Hoglund, P. 1998. Alpha1/alpha2 domains of H-2D(d), but not H-2L(d), induce "missing self" reactivity in vivo--no effect of H-2L(d) on protection against NK cells expressing the inhibitory receptor Ly49G2. *Eur.J.Immunol.* 28:4198-4206.

92 Zhao, Y., Ohdan, H., Manilay, J. O., and Sykes, M. 2003. NK cell tolerance in mixed allogeneic chimeras. *J.Immunol.* 170:5398-5405.

93 McVicar, D. W. and Burshtyn, D. N. 2001. Intracellular signaling by the killer immunoglobulin-like receptors and Ly49. *Sci.STKE.* 2001:RE1.

94 Brown, M. H., Boles, K., van der Merwe, P. A., Kumar, V., Mathew, P. A., and Barclay, A. N. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J.Exp.Med.* 188:2083-2090.

95 Schatzle, J. D., Sheu, S., Stepp, S. E., Mathew, P. A., Bennett, M., and Kumar, V. 1999. Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. *Proc.Natl.Acad.Sci.U.S.A* 96:3870-3875.

96 Mooney, J. M., Klem, J., Wulfing, C., Mijares, L. A., Schwartzberg, P. L., Bennett, M., and Schatzle, J. D. 2004. The Murine NK Receptor 2B4 (CD244)

Exhibits Inhibitory Function Independent of Signaling Lymphocytic Activation Molecule-Associated Protein Expression. *J.Immunol.* 173:3953-3961.

97 Garni-Wagner, B. A., Witte, P. L., Tutt, M. M., Kuziel, W. A., Tucker, P. W., Bennett, M., and Kumar, V. 1990. Natural killer cells in the thymus. Studies in mice with severe combined immune deficiency. *J.Immunol.* 144:796-803.

98 Lee, K. M., McNerney, M. E., Stepp, S. E., Mathew, P. A., Schatzle, J. D., Bennett, M., and Kumar, V. 2004. 2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells. *J.Exp.Med.* 199:1245-1254.

99 Mason, L. H., Willette-Brown, J., Anderson, S. K., Gosselin, P., Shores, E. W., Love, P. E., Ortaldo, J. R., and McVicar, D. W. 1998. Characterization of an associated 16-kDa tyrosine phosphoprotein required for Ly-49D signal transduction. *J.Immunol.* 160:4148-4152.

100 Diefenbach, A. and Raulet, D. H. 2002. The innate immune response to tumors and its role in the induction of T-cell immunity. *Immunol.Rev.* 188:9-21.

101 Diefenbach, A., Jamieson, A. M., Liu, S. D., Shastri, N., and Raulet, D. H. 2000. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat.Immunol.* 1:119-126.

102 Johansson, M. H., Bieberich, C., Jay, G., Karre, K., and Hoglund, P. 1997. Natural killer cell tolerance in mice with mosaic expression of major histocompatibility complex class I transgene. *J.Exp.Med.* 186:353-364.

103 Ohlen, C., Kling, G., Hoglund, P., Hansson, M., Scangos, G., Bieberich, C., Jay, G., and Karre, K. 1989. Prevention of allogeneic bone marrow graft rejection by H-2 transgene in donor mice. *Science* 246:666-668.

104 Yu, Y. Y., Forman, J., Aldrich, C., Blazar, B., Flaherty, L., Kumar, V., and Bennett, M. 1994. Natural killer cells recognize common antigenic motifs shared by H-2Dd, H-2Ld and possibly H-2Dr molecules expressed on bone marrow cells. *Int.Immunol.* 6:1297-1306.

105 Bennett, M. 1972. Rejection of marrow allografts: importance of H-2 homozygosity of donor cells. *Transplantation* 14:289-298.

106 Raziuddin, A., Longo, D. L., Mason, L., Ortaldo, J. R., Bennett, M., and Murphy, W. J. 1998. Differential effects of the rejection of bone marrow allografts by the depletion of activating versus inhibiting Ly-49 natural killer cell subsets. *J.Immunol.* 160:87-94.

107 George, T. C., Mason, L. H., Ortaldo, J. R., Kumar, V., and Bennett, M. 1999. Positive recognition of MHC class I molecules by the Ly49D receptor of murine NK cells. *J.Immunol.* 162:2035-2043.

108 Furukawa, H., Iizuka, K., Poursine-Laurent, J., Shastri, N., and Yokoyama, W. M. 2002. A ligand for the murine NK activation receptor Ly-49D: activation of tolerized NK cells from beta 2-microglobulin-deficient mice. *J.Immunol.* 169:126-136.

109 Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B., and Lanier, L. L. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296:1323-1326.

110 Scalzo, A. A., Fitzgerald, N. A., Wallace, C. R., Gibbons, A. E., Smart, Y. C., Burton, R. C., and Shellam, G. R. 1992. The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J.Immunol.* 149:581-589.

111 Xu, J., Dallas, P. B., Lyons, P. A., Shellam, G. R., and Scalzo, A. A. 1992. Identification of the glycoprotein H gene of murine cytomegalovirus. *J.Gen.Virol.* 73 (Pt 7):1849-1854.

112 Lee, S. H., Girard, S., Macina, D., Busa, M., Zafer, A., Belouchi, A., Gros, P., and Vidal, S. M. 2001. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nat.Genet.* 28:42-45.

113 Webb, J. R., Lee, S. H., and Vidal, S. M. 2002. Genetic control of innate immune responses against cytomegalovirus: MCMV meets its match. *Genes Immun.* 3:250-262.

114 Daniels, K. A., Devora, G., Lai, W. C., O'Donnell, C. L., Bennett, M., and Welsh, R. M. 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J.Exp.Med.* 194:29-44.

115 Lee, S. H., Zafer, A., de Repentigny, Y., Kothary, R., Tremblay, M. L., Gros, P., Duplay, P., Webb, J. R., and Vidal, S. M. 2003. Transgenic expression of the activating natural killer receptor Ly49H confers resistance to cytomegalovirus in genetically susceptible mice. *J.Exp.Med.* 197:515-526.

116 Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J. H., Lanier, L. L., and Spies, T. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285:727-729.

117 Diefenbach, A., Jensen, E. R., Jamieson, A. M., and Raulet, D. H. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413:165-171.

118 Moretta, A., Biassoni, R., Bottino, C., Mingari, M. C., and Moretta, L. 2000. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. *Immunol.Today* 21:228-234.

119 Dunn, G. P., Old, L. J., and Schreiber, R. D. 2004. The Three Es of Cancer Immunoediting. *Annu.Rev.Immunol.* 22:329-360.

120 Malmberg, K. J. 2004. Effective immunotherapy against cancerA question of overcoming immune suppression and immune escape? *Cancer Immunol.Immunother.*

121 Street, S. E., Trapani, J. A., MacGregor, D., and Smyth, M. J. 2002. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J.Exp.Med.* 196:129-134.

122 Algarra, I., Garcia-Lora, A., Cabrera, T., Ruiz-Cabello, F., and Garrido, F. 2004. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. *Cancer Immunol.Immunother.* 53:904-910.

123 Khong, H. T. and Restifo, N. P. 2002. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat.Immunol.* 3:999-1005.

124 Elder, G. J., Hersey, P., and Branley, P. 1997. Remission of transplanted melanoma--clinical course and tumour cell characterisation. *Clin.Transplant.* 11:565-568.

125 Suranyi, M. G., Hogan, P. G., Falk, M. C., Axelsen, R. A., Rigby, R., Hawley, C., and Petrie, J. 1998. Advanced donor-origin melanoma in a renal transplant recipient: immunotherapy, cure, and retransplantation. *Transplantation* 66:655-661.

126 Terabe, M. and Berzofsky, J. A. 2004. Immunoregulatory T cells in tumor immunity. *Curr.Opin.Immunol.* 16:157-162.

127 Shibata, M., Nezu, T., Kanou, H., Abe, H., Takekawa, M., and Fukuzawa, M. 2002. Decreased production of interleukin-12 and type 2 immune responses are marked in cachectic patients with colorectal and gastric cancer. *J.Clin.Gastroenterol.* 34:416-420.

128 Lauerova, L., Dusek, L., Simickova, M., Kocak, I., Vagundova, M., Zaloudik, J., and Kovarik, J. 2002. Malignant melanoma associates with Th1/Th2 imbalance that coincides with disease progression and immunotherapy response. *Neoplasma* 49:159-166.

129 Spaner, D. E. 2004. Amplifying cancer vaccine responses by modifying pathogenic gene programs in tumor cells. *J.Leukoc.Biol.* 76:338-351.

130 Ohm, J. E., Gabrilovich, D. I., Sempowski, G. D., Kisseleva, E., Parman, K. S., Nadaf, S., and Carbone, D. P. 2003. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood* 101:4878-4886.

131 Gabrilovich, D. I., Chen, H. L., Girgis, K. R., Cunningham, H. T., Meny, G. M., Nadaf, S., Kavanaugh, D., and Carbone, D. P. 1996. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat.Med.* 2:1096-1103.

132 Paliogianni, F. and Boumpas, D. T. 1996. Prostaglandin E2 inhibits the nuclear transcription of the human interleukin 2, but not the Il-4, gene in human T cells by targeting transcription factors AP-1 and NF-AT. *Cell Immunol.* 171:95-101.

133 Walker, W. and Rotondo, D. 2004. Prostaglandin E2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-gamma synthesis. *Immunology* 111:298-305.

134 Lin, D. T., Subbaramaiah, K., Shah, J. P., Dannenberg, A. J., and Boyle, J. O. 2002. Cyclooxygenase-2: a novel molecular target for the prevention and treatment of head and neck cancer. *Head Neck* 24:792-799.

135 Klampfer, L., Huang, J., Corner, G., Mariadason, J., Arango, D., Sasazuki, T., Shirasawa, S., and Augenlicht, L. 2003. Oncogenic Ki-ras inhibits the expression of interferon-responsive genes through inhibition of STAT1 and STAT2 expression. *J.Biol.Chem.* 278:46278-46287.

136 Hu, W. and Kavanagh, J. J. 2003. Anticancer therapy targeting the apoptotic pathway. *Lancet Oncol.* 4:721-729.

137 Krishnakumar, S., Kandalam, M., Mohan, A., Iyer, A., Venkatesan, N., Biswas, J., and Shanmugam, M. P. 2004. Expression of Fas ligand in retinoblastoma. *Cancer* 101:1672.

138 Enjoji, M., Yamaguchi, K., Nakashima, M., Ohta, S., Kotoh, K., Fukushima, M., Kuniyoshi, M., Tanaka, M., Nakamuta, M., Watanabe, T., and Nawata, H. 2004. Serum levels of soluble molecules associated with evasion of immune surveillance: a study in biliary disease. *Liver Int.* 24:330-334.

139 Garcia-Lora, A., Algarra, I., and Garrido, F. 2003. MHC class I antigens, immune surveillance, and tumor immune escape. *J.Cell Physiol* 195:346-355.

140 Doubrovina, E. S., Doubrovin, M. M., Vider, E., Sisson, R. B., O'Reilly, R. J., Dupont, B., and Vyas, Y. M. 2003. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *J.Immunol.* 171:6891-6899.

141 Cooper, M. A., Fehniger, T. A., Fuchs, A., Colonna, M., and Caligiuri, M. A. 2004. NK cell and DC interactions. *Trends Immunol.* 25:47-52.

142 Paul, P., Rouas-Freiss, N., Khalil-Daher, I., Moreau, P., Riteau, B., Le Gal, F. A., Avril, M. F., Dausset, J., Guillet, J. G., and Carosella, E. D. 1998. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc.Natl.Acad.Sci.U.S.A* 95:4510-4515.

143 Polakova, K., Bandzuchova, E., Hofmeister, V., Weiss, E. H., Hutter, H., and Russ, G. 2003. Binding analysis of HLA-G specific antibodies to hematopoietic cells isolated from leukemia patients. *Neoplasma* 50:331-338.

144 Bukur, J., Malenica, B., Huber, C., and Seliger, B. 2003. Altered expression of nonclassical HLA class Ib antigens in human renal cell carcinoma and its association with impaired immune response. *Hum.Immunol.* 64:1081-1092.

145 Ibrahim, e. C., Aractingi, S., Allory, Y., Borrini, F., Dupuy, A., Duvillard, P., Carosella, E. D., Avril, M. F., and Paul, P. 2004. Analysis of HLA antigen expression in benign and malignant melanocytic lesions reveals that upregulation of HLA-G expression correlates with malignant transformation, high inflammatory infiltration and HLA-A1 genotype. *Int.J.Cancer* 108:243-250.

146 National Cancer Institute. Chemotherapy and You:
A Guide to Self-Help During Cancer Treatment. 6-1-2004.
Ref Type: Report

147 Ko, L., Liu, Y., and Kong, Q. 1999. Current perspective in immunotherapy. *Ann.Thorac.Surg.* 68:S28-S33.

148 Ko, E. C., Wang, X., and Ferrone, S. 2003. Immunotherapy of malignant diseases. Challenges and strategies. *Int.Arch.Allergy Immunol.* 132:294-309.

149 Waller, E. K. and Ernstoff, M. S. 2003. Modulation of antitumor immune responses by hematopoietic cytokines. *Cancer* 97:1797-1809.

150 Atkins, M. B., Regan, M., and McDermott, D. 2004. Update on the Role of Interleukin 2 and Other Cytokines in the Treatment of Patients with Stage IV Renal Carcinoma. *Clin.Cancer Res.* 10:6342S-6346S.

151 Tagliaferri, P., Caraglia, M., Budillon, A., Marra, M., Vitale, G., Viscomi, C., Masciari, S., Tassone, P., Abbruzzese, A., and Venuta, S. 2004. New pharmacokinetic and pharmacodynamic tools for interferon-alpha (IFN-alpha) treatment of human cancer. *Cancer Immunol.Immunother.*

152 Sabel, M. S. and Sondak, V. K. 2003. Pros and cons of adjuvant interferon in the treatment of melanoma. *Oncologist.* 8:451-458.

153 Choueiri, T. K., Hutson, T. E., and Bukowski, R. M. 2003. Evolving role of pegylated interferons in metastatic renal cell carcinoma. *Expert.Rev.Anticancer Ther.* 3:823-829.

154 Rosenberg, S. A., Mule, J. J., Spiess, P. J., Reichert, C. M., and Schwarz, S. L. 1985. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. *J.Exp.Med.* 161:1169-1188.

155 Fyfe, G., Fisher, R. I., Rosenberg, S. A., Sznol, M., Parkinson, D. R., and Louie, A. C. 1995. Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. *J.Clin.Oncol.* 13:688-696.

156 Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H., and White, D. E. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J.Natl.Cancer Inst.* 86:1159-1166.

157 Atkins, M. B. 2002. Interleukin-2: clinical applications. *Semin.Oncol.* 29:12-17.

158 McIntosh, J. K., Mule, J. J., Krosnick, J. A., and Rosenberg, S. A. 1989. Combination cytokine immunotherapy with tumor necrosis factor alpha, interleukin 2, and alpha-interferon and its synergistic antitumor effects in mice. *Cancer Res.* 49:1408-1414.

159 Rosenberg, S. A., Lotze, M. T., Yang, J. C., Linehan, W. M., Seipp, C., Calabro, S., Karp, S. E., Sherry, R. M., Steinberg, S., and White, D. E. 1989. Combination therapy with interleukin-2 and alpha-interferon for the treatment of patients with advanced cancer. *J.Clin.Oncol.* 7:1863-1874.

160 Nelson, B. H. 2004. IL-2, regulatory T cells, and tolerance. *J.Immunol.* 172:3983-3988.

161 Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C., Seipp, C. A., and . 1988. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N.Engl.J.Med.* 319:1676-1680.

162 Belardelli, F., Ferrantini, M., Parmiani, G., Schlom, J., and Garaci, E. 2004. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? *Cancer Res.* 64:6827-6830.

163 Lorenz, E., Uphoff, D., Reid, T. R., and Shelton, E. 1951. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J.Natl.Cancer Inst.* 12:197-201.

164 Anasetti, C. 2004. Advances in the prevention of graft-versus-host disease after hematopoietic cell transplantation. *Transplantation* 77:S79-S83.

165 Barao, I. and Murphy, W. J. 2003. The immunobiology of natural killer cells and bone marrow allograft rejection. *Biol.Blood Marrow Transplant.* 9:727-741.

166 Young, N. T. 1999. Kir genes, killer cells and clinical transplantation. *Transplantation* 68:1626-1628.

167 Velardi, A., Ruggeri, L., Alessandro, Moretta, and Moretta, L. 2002. NK cells: a lesson from mismatched hematopoietic transplantation. *Trends Immunol.* 23:438-444.

168 Giebel, S., Locatelli, F., Lamparelli, T., Velardi, A., Davies, S., Frumento, G., Maccario, R., Bonetti, F., Wojnar, J., Martinetti, M., Frassoni, F., Giorgiani, G., Bacigalupo, A., and Holowiecki, J. 2003. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood* 102:814-819.

169 Young, N. T. 2004. Immunobiology of natural killer lymphocytes in transplantation. *Transplantation* 78:1-6.

170 Voutsadakis, I. A. 2003. NK cells in allogeneic bone marrow transplantation. *Cancer Immunol.Immunother.* 52:525-534.

171 Farag, S. S., Fehniger, T. A., Ruggeri, L., Velardi, A., and Caligiuri, M. A. 2002. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* 100:1935-1947.

172 Ruggeri, L., Capanni, M., Casucci, M., Volpi, I., Tosti, A., Perruccio, K., Urbani, E., Negrin, R. S., Martelli, M. F., and Velardi, A. 1999. Role of natural killer cell

alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 94:333-339.

173 Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W. D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., Martelli, M. F., and Velardi, A. 2002. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295:2097-2100.

174 Farag, S. S., Fehniger, T. A., Ruggeri, L., Velardi, A., and Caligiuri, M. A. 2002. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* 100:1935-1947.

175 Asai, O., Longo, D. L., Tian, Z. G., Hornung, R. L., Taub, D. D., Ruscetti, F. W., and Murphy, W. J. 1998. Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation. *J.Clin.Invest* 101:1835-1842.

176 Mule, J. J., Shu, S., Schwarz, S. L., and Rosenberg, S. A. 1984. Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2. *Science* 225:1487-1489.

177 Cervantes, F., Pierson, B. A., McGlave, P. B., Verfaillie, C. M., and Miller, J. S. 1996. Autologous activated natural killer cells suppress primitive chronic myelogenous leukemia progenitors in long-term culture. *Blood* 87:2476-2485.

178 Pierson, B. A. and Miller, J. S. 1996. CD56+bright and CD56+dim natural killer cells in patients with chronic myelogenous leukemia progressively decrease in number, respond less to stimuli that recruit clonogenic natural killer cells, and exhibit decreased proliferation on a per cell basis. *Blood* 88:2279-2287.

179 Burns, L. J., Weisdorf, D. J., DeFor, T. E., Vesole, D. H., Repka, T. L., Blazar, B. R., Burger, S. R., Panoskaltsis-Mortari, A., Keever-Taylor, C. A., Zhang, M. J., and Miller, J. S. 2003. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. *Bone Marrow Transplant.* 32:177-186.

180 Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E., Ettinghausen, S. E., Matory, Y. L., Skibber, J. M., Shiloni, E., and Vetto, J. T. 1985. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N.Engl.J.Med.* 313:1485-1492.

181 Rosenberg, S. A., Lotze, M. T., Muul, L. M., Chang, A. E., Avis, F. P., Leitman, S., Linehan, W. M., Robertson, C. N., Lee, R. E., and Rubin, J. T. 1987. A progress report on the treatment of 157 patients with advanced cancer using

lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N.Engl.J.Med.* 316:889-897.

182 Fisher, R. I., Coltman, C. A., Jr., Doroshow, J. H., Rayner, A. A., Hawkins, M. J., Mier, J. W., Wiernik, P., McMannis, J. D., Weiss, G. R., and Margolin, K. A. 1988. Metastatic renal cancer treated with interleukin-2 and lymphokine-activated killer cells. A phase II clinical trial. *Ann.Intern.Med.* 108:518-523.

183 Bar, M. H., Sznol, M., Atkins, M. B., Ciobanu, N., Micetich, K. C., Boldt, D. H., Margolin, K. A., Aronson, F. R., Rayner, A. A., and Hawkins, M. J. 1990. Metastatic malignant melanoma treated with combined bolus and continuous infusion interleukin-2 and lymphokine-activated killer cells. *J.Clin.Oncol.* 8:1138-1147.

184 Mittelman, A., Savona, S., Gafney, E., Penichet, K. O., Lin, B. Y., Levitt, D., Ahmed, T., Arlin, Z. A., Baskind, P., and Needleman, D. 1989. Treatment of patients with advanced cancer using multiple long-term cultured lymphokine-activated killer (LAK) cell infusions and recombinant human interleukin-2. *J.Biol.Response Mod.* 8:468-478.

185 Margolin, K. A., Aronson, F. R., Sznol, M., Atkins, M. B., Ciobanu, N., Fisher, R. I., Weiss, G. R., Doroshow, J. H., Bar, M. H., and Hawkins, M. J. 1991. Phase II trial of high-dose interleukin-2 and lymphokine-activated killer cells in Hodgkin's disease and non-Hodgkin's lymphoma. *J.Immunother.* 10:214-220.

186 Thompson, J. A., Shulman, K. L., Benyunes, M. C., Lindgren, C. G., Collins, C., Lange, P. H., Bush, W. H., Jr., Benz, L. A., and Fefer, A. 1992. Prolonged continuous intravenous infusion interleukin-2 and lymphokine-activated killer-cell therapy for metastatic renal cell carcinoma. *J.Clin.Oncol.* 10:960-968.

187 Weiss, G. R., Margolin, K. A., Aronson, F. R., Sznol, M., Atkins, M. B., Dutcher, J. P., Gaynor, E. R., Boldt, D. H., Doroshow, J. H., and Bar, M. H. 1992. A randomized phase II trial of continuous infusion interleukin-2 or bolus injection interleukin-2 plus lymphokine-activated killer cells for advanced renal cell carcinoma. *J.Clin.Oncol.* 10:275-281.

188 Rosenberg, S. A., Lotze, M. T., Yang, J. C., Topalian, S. L., Chang, A. E., Schwartzentruber, D. J., Aebersold, P., Leitman, S., Linehan, W. M., and Seipp, C. A. 1993. Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. *J.Natl.Cancer Inst.* 85:622-632.

189 Cascinelli, N., Belli, F., Marchini, S., Marolda, R., Prada, A., Sciorelli, G., Villani, F., Gambacorti-Passerini, C., Galazka, A., and Parmiani, G. 1989. A phase II study of the administration of recombinant interleukin 2 (rIL-2) plus

lymphokine activated killer (LAK) cells in stage IV melanoma patients. *Tumori* 75:233-244.

190 Rosenberg, S. A., Spiess, P., and Lafreniere, R. 1986. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* 233:1318-1321.

191 Anderson, T. M., Ibayashi, Y., Holmes, E. C., and Golub, S. H. 1987. Modification of natural killer activity of lymphocytes infiltrating human lung cancers. *Cancer Immunol.Immunother.* 25:65-68.

192 Quattrocchi, K. B., Miller, C. H., Cush, S., Bernard, S. A., Dull, S. T., Smith, M., Gudeman, S., and Varia, M. A. 1999. Pilot study of local autologous tumor infiltrating lymphocytes for the treatment of recurrent malignant gliomas. *J.Neurooncol.* 45:141-157.

193 Reichert, T. E., Strauss, L., Wagner, E. M., Gooding, W., and Whiteside, T. L. 2002. Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. *Clin.Cancer Res.* 8:3137-3145.

194 Koh, C. Y., Ortaldo, J. R., Blazar, B. R., Bennett, M., and Murphy, W. J. 2003. NK-cell purging of leukemia: superior antitumor effects of NK cells H2 allogeneic to the tumor and augmentation with inhibitory receptor blockade. *Blood* 102:4067-4075.

195 Koh, C. Y., Raziuddin, A., Welniak, L. A., Blazar, B. R., Bennett, M., and Murphy, W. J. 2002. NK inhibitory-receptor blockade for purging of leukemia: effects on hematopoietic reconstitution. *Biol.Blood Marrow Transplant.* 8:17-25.

196 Klingemann, H. G., Wong, E., and Maki, G. 1996. A cytotoxic NK-cell line (NK-92) for ex vivo purging of leukemia from blood. *Biol.Blood Marrow Transplant.* 2:68-75.

197 Maki, G. 2001. Ex vivo purging of stem cell autografts using cytotoxic cells. *J.Hematother.Stem Cell Res.* 10:545-551.

198 Alvarnas, J. C. and Forman, S. J. 2004. Graft purging in autologous bone marrow transplantation: a promise not quite fulfilled. *Oncology (Huntingt)* 18:867-876.

199 Villamor, N., Montserrat, E., and Colomer, D. 2003. Mechanism of action and resistance to monoclonal antibody therapy. *Semin.Oncol.* 30:424-433.

200 von Mehren, M., Adams, G. P., and Weiner, L. M. 2003. Monoclonal antibody therapy for cancer. *Annu.Rev.Med.* 54:343-369.

201 Wels, W., Biburger, M., Muller, T., Dalken, B., Giesubel, U., Tonn, T., and Uherek, C. 2004. Recombinant immunotoxins and retargeted killer cells: employing engineered antibody fragments for tumor-specific targeting of cytotoxic effectors. *Cancer Immunol.Immunother.* 53:217-226.

202 Benjamini E., Coico R., and Sunshine G. 2000. *Immunology, a short course*, 4th edn.

203 Janeway, C., Travers, P., Walport, M., and Capra JD 1999. *Immunobiology*.

204 Little, M., Kipriyanov, S. M., Le Gall, F., and Moldenhauer, G. 2000. Of mice and men: hybridoma and recombinant antibodies. *Immunol.Today* 21:364-370.

205 Weiner, L. M. 2000. Bispecific antibodies in cancer therapy. *Cancer J.* 6 Suppl 3:S265-S271.

206 Spiegelberg, H. L. and Weigle, W. O. 1965. The catabolism of homologous and heterologous 7S gamma globulin fragments. *J.Exp.Med.* 121:323-338.

207 Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. 1992. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* 52:3402-3408.

208 Weiner, L. M. and Adams, G. P. 2000. New approaches to antibody therapy. *Oncogene* 19:6144-6151.

209 Adams, G. P., Schier, R., McCall, A. M., Simmons, H. H., Horak, E. M., Alpaugh, R. K., Marks, J. D., and Weiner, L. M. 2001. High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. *Cancer Res.* 61:4750-4755.

210 Segal, D. M., Weiner, G. J., and Weiner, L. M. 2001. Introduction: bispecific antibodies. *J.Immunol.Methods* 248:1-6.

211 Ludwig, D. L., Pereira, D. S., Zhu, Z., Hicklin, D. J., and Bohlen, P. 2003. Monoclonal antibody therapeutics and apoptosis. *Oncogene* 22:9097-9106.

212 Presta, L. 2003. Antibody engineering for therapeutics. *Curr.Opin.Struct.Biol.* 13:519-525.

213 Davies, N. P., Rosewell, I. R., Richardson, J. C., Cook, G. P., Neuberger, M. S., Brownstein, B. H., Norris, M. L., and Bruggemann, M. 1993. Creation of mice expressing human antibody light chains by introduction of a yeast artificial chromosome containing the core region of the human immunoglobulin kappa locus. *Biotechnology (N.Y.)* 11:911-914.

214 Jakobovits, A., Green, L. L., Hardy, M. C., Maynard-Currie, C. E., Tsuda, H., Louie, D. M., Mendez, M. J., Abderrahim, H., Noguchi, M., and Smith, D. H. 1995. Production of antigen-specific human antibodies from mice engineered with human heavy and light chain YACs. *Ann.N.Y.Acad.Sci.* 764:525-535.

215 Smith, K. A., Nelson, P. N., Warren, P., Astley, S. J., Murray, P. G., and Greenman, J. 2004. Demystified...recombinant antibodies. *J.Clin.Pathol.* 57:912-917.

216 Leach, D. R., Krummel, M. F., and Allison, J. P. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271:1734-1736.

217 Varga, E. M., Nouri-Aria, K., Till, S. J., and Durham, S. R. 2003. Immunomodulatory treatment strategies for allergic diseases. *Curr.Drug Targets.Inflamm.Allergy* 2:31-46.

218 Chambers, C. A., Kuhns, M. S., Egen, J. G., and Allison, J. P. 2001. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu.Rev.Immunol.* 19:565-594.

219 McCoy, K., Camberis, M., and Gros, G. L. 1997. Protective immunity to nematode infection is induced by CTLA-4 blockade. *J.Exp.Med.* 186:183-187.

220 Hurwitz, A. A., Sullivan, T. J., Krummel, M. F., Sobel, R. A., and Allison, J. P. 1997. Specific blockade of CTLA-4/B7 interactions results in exacerbated clinical and histologic disease in an actively-induced model of experimental allergic encephalomyelitis. *J.Neuroimmunol.* 73:57-62.

221 Perrin, P. J., Maldonado, J. H., Davis, T. A., June, C. H., and Racke, M. K. 1996. CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. *J.Immunol.* 157:1333-1336.

222 Egen, J. G., Kuhns, M. S., and Allison, J. P. 2002. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat.Immunol.* 3:611-618.

223 Hodi, F. S., Mihm, M. C., Soiffer, R. J., Haluska, F. G., Butler, M., Seiden, M. V., Davis, T., Henry-Spires, R., MacRae, S., Willman, A., Padera, R., Jaklitsch, M. T., Shankar, S., Chen, T. C., Korman, A., Allison, J. P., and Dranoff, G. 2003. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc.Natl.Acad.Sci.U.S.A* 100:4712-4717.

224 Phan, G. Q., Yang, J. C., Sherry, R. M., Hwu, P., Topalian, S. L., Schwartzentruber, D. J., Restifo, N. P., Haworth, L. R., Seipp, C. A., Freezer, L. J., Morton, K. E., Mavroukakis, S. A., Duray, P. H., Steinberg, S. M., Allison, J.

P., Davis, T. A., and Rosenberg, S. A. 2003. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc.Natl.Acad.Sci.U.S.A* 100:8372-8377.

225 Koh, C. Y., Blazar, B. R., George, T., Welniak, L. A., Capitini, C. M., Raziuddin, A., Murphy, W. J., and Bennett, M. 2001. Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. *Blood* 97:3132-3137.

226 Farag, S. S., Fehniger, T. A., Ruggeri, L., Velardi, A., and Caligiuri, M. A. 2002. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* 100:1935-1947.

227 Hara, T., Nishimura, H., Hasegawa, Y., and Yoshikai, Y. 2001. Thymus-dependent modulation of Ly49 inhibitory receptor expression on NK1.1+gamma/delta T cells. *Immunology* 102:24-30.

228 Daniels, K. A., Devora, G., Lai, W. C., O'Donnell, C. L., Bennett, M., and Welsh, R. M. 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J.Exp.Med.* 194:29-44.

229 Ortaldo, J. R., Winkler-Pickett, R., Mason, A. T., and Mason, L. H. 1998. The Ly-49 family: regulation of cytotoxicity and cytokine production in murine CD3+ cells. *J.Immunol.* 160:1158-1165.

230 Zajac, A. J., Vance, R. E., Held, W., Sourdive, D. J., Altman, J. D., Raulet, D. H., and Ahmed, R. 1999. Impaired anti-viral T cell responses due to expression of the Ly49A inhibitory receptor. *J.Immunol.* 163:5526-5534.

231 Yu, Y. Y., Kumar, V., and Bennett, M. 1992. Murine natural killer cells and marrow graft rejection. *Annu.Rev.Immunol.* 10:189-213.

232 Gavilondo-Cowley, J. V., Coloma, M. J., Vazquez, J., Ayala, M., Macias, A., Fry, K. E., and Larrick, J. W. 1990. Specific amplification of rearranged immunoglobulin variable region genes from mouse hybridoma cells. *Hybridoma* 9:407-417.

233 Spiegelberg, H. L. and Weigle, W. O. 1965. he Catabolism of Homologous and Heterologous 7S Gamma Globulin Fragments *J.Exp.Med.* 121:323-338.

234 McCool, D., Birshtein, B. K., and Painter, R. H. 1985. Structural requirements of immunoglobulin G for binding to the Fc gamma receptors of the human tumor cell lines U937, HL-60, ML-1, and K562. *J.Immunol.* 135:1975-1980.

235 Kim, J. K., Tsen, M. F., Ghetie, V., and Ward, E. S. 1994. Identifying amino acid residues that influence plasma clearance of murine IgG1 fragments by site-directed mutagenesis. *Eur.J.Immunol.* 24:542-548.

236 Tao, M. H. and Morrison, S. L. 1989. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J.Immunol.* 143:2595-2601.

237 Shinde, S., Gee, R., Santulli-Marotto, S., Bockenstedt, L. K., Clarke, S. H., and Mamula, M. J. 1999. T cell autoimmunity in Ig transgenic mice. *J.Immunol.* 162:7519-7524.

238 Santulli-Marotto, S., Retter, M. W., Gee, R., Mamula, M. J., and Clarke, S. H. 1998. Autoreactive B cell regulation: peripheral induction of developmental arrest by lupus-associated autoantigens. *Immunity.* 8:209-219.

239 Okamoto, M., Murakami, M., Shimizu, A., Ozaki, S., Tsubata, T., Kumagai, S., and Honjo, T. 1992. A transgenic model of autoimmune hemolytic anemia. *J.Exp.Med.* 175:71-79.

240 Mertsching, E., Burdet, C., and Ceredig, R. 1995. IL-7 transgenic mice: analysis of the role of IL-7 in the differentiation of thymocytes in vivo and in vitro. *Int.Immunol.* 7:401-414.

241 Wirth, S., Guidotti, L. G., Ando, K., Schlicht, H. J., and Chisari, F. V. 1995. Breaking tolerance leads to autoantibody production but not autoimmune liver disease in hepatitis B virus envelope transgenic mice. *J.Immunol.* 154:2504-2515.

242 Sutherland, R. M., Brady, J. L., Georgiou, H. M., Thomas, H. E., and Lew, A. M. 2000. Protective effect of CTLA4Ig secreted by transgenic fetal pancreas allografts. *Transplantation* 69:1806-1812.

243 Lane, P., Gerhard, W., Hubele, S., Lanzavecchia, A., and McConnell, F. 1993. Expression and functional properties of mouse B7/BB1 using a fusion protein between mouse CTLA4 and human gamma 1. *Immunology* 80:56-61.

244 Lane, P., Burdet, C., Hubele, S., Scheidegger, D., Muller, U., McConnell, F., and Kosco-Vilbois, M. 1994. B cell function in mice transgenic for mCTLA4-H gamma 1: lack of germinal centers correlated with poor affinity maturation and class switching despite normal priming of CD4+ T cells. *J.Exp.Med.* 179:819-830.

245 Lane, P. 1995. Blocking CD28/B7 with soluble competitors: immunological phenotype of mCTLA4-H gamma 1 transgenic mice. *Res.Immunol.* 146:176-179.

246 Lane, P. 1996. Development of B-cell memory and effector function. *Curr.Opin.Immunol.* 8:331-335.

247 Lane, P., Haller, C., and McConnell, F. 1996. Evidence that induction of tolerance in vivo involves active signaling via a B7 ligand-dependent mechanism: CTLA4-Ig protects V beta 8+ T cells from tolerance induction by the superantigen staphylococcal enterotoxin B. *Eur.J.Immunol.* 26:858-862.

248 Kawashima, I., Yoshida, Y., Taya, C., Shitara, H., Yonekawa, H., Karasuyama, H., Tada, N., Furukawa, K., and Tai, T. 2003. Expansion of natural killer cells in mice transgenic for IgM antibody to ganglioside GD2: demonstration of prolonged survival after challenge with syngeneic tumor cells. *Int.J.Oncol.* 23:381-388.

249 Zhan, Y., Brady, J. L., Johnston, A. M., and Lew, A. M. 2000. Predominant transgene expression in exocrine pancreas directed by the CMV promoter. *DNA Cell Biol.* 19:639-645.

250 Zhan, Y., Corbett, A. J., Brady, J. L., Sutherland, R. M., and Lew, A. M. 2000. CD4 help-independent induction of cytotoxic CD8 cells to allogeneic P815 tumor cells is absolutely dependent on costimulation. *J.Immunol.* 165:3612-3619.

251 Yuan, D., Bibi, R., and Dang, T. 2004. The role of adjuvant on the regulatory effects of NK cells on B cell responses as revealed by a new model of NK cell deficiency. *Int.Immunol.* 16:707-716.

252 Kabat, E. A., Wu, T. T, Perry, H. M., Gottesman, K. S., and Foeller, C. Sequences of Proteins of Immunological Interest, 5th Edition. NIH Publication No. 91-3242. 1991. US Dept of Health and Human Services, Public Health Service, National Institutes of Health.

253 Sambrook, J., Fritsch, E. J., and Maniatis T. 1989. *Molecular Cloning: a laboratory manual, second edition* Cold Spring Harbor Laboratory Press.

254 Hackett, J., Jr., Bennett, M., and Kumar, V. 1985. Origin and differentiation of natural killer cells. I. Characteristics of a transplantable NK cell precursor. *J.Immunol.* 134:3731-3738.

255 Bennett, M. 1971. Graft-versus-host reactions in mice. I. Kinetic and immunogenetic studies of alloantigen-sensitive units of lymphoid tissue. *Transplantation* 11:158-169.

256 Bennett, M., Taylor, P. A., Austin, M., Baker, M. B., Schook, L. B., Rutherford, M., Kumar, V., Podack, E. R., Mohler, K. M., Levy, R. B., and Blazar, B. R.

1998. Cytokine and cytotoxic pathways of NK cell rejection of class I-deficient bone marrow grafts: influence of mouse colony environment. *Int.Immunol.* 10:785-790.

257 Bennett, M. 1971. Graft-versus-host reactions in mice. I. Kinetic and immunogenetic studies of alloantigen-sensitive units of lymphoid tissue. *Transplantation* 11:158-169.

258 Davenport, C., Kumar, V., and Bennett, M. 1995. Rapid rejection of H2k and H2k/b bone marrow cell grafts by CD8+ T cells and NK cells in irradiated mice. *J.Immunol.* 155:3742-3749.

259 Li, S., Ilaria, R. L., Jr., Million, R. P., Daley, G. Q., and Van Etten, R. A. 1999. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J.Exp.Med.* 189:1399-1412.

260 Shinde, S., Gee, R., Santulli-Marotto, S., Bockenstedt, L. K., Clarke, S. H., and Mamula, M. J. 1999. T cell autoimmunity in Ig transgenic mice. *J.Immunol.* 162:7519-7524.

261 Okamoto, M., Murakami, M., Shimizu, A., Ozaki, S., Tsubata, T., Kumagai, S., and Honjo, T. 1992. A transgenic model of autoimmune hemolytic anemia. *J.Exp.Med.* 175:71-79.

262 Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 41:521-530.

263 Foecking, M. K. and Hofstetter, H. 1986. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. *Gene* 45:101-105.

264 Ronchese, F., Hausmann, B., Hubele, S., and Lane, P. 1994. Mice transgenic for a soluble form of murine CTLA-4 show enhanced expansion of antigen-specific CD4+ T cells and defective antibody production in vivo. *J.Exp.Med.* 179:809-817.

265 Milenic, D. E., Yokota, T., Filpula, D. R., Finkelman, M. A., Dodd, S. W., Wood, J. F., Whitlow, M., Snoy, P., and Schlom, J. 1991. Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. *Cancer Res.* 51:6363-6371.

266 Murphy, W. J., Kumar, V., and Bennett, M. 1987. Acute rejection of murine bone marrow allografts by natural killer cells and T cells. Differences in kinetics and target antigens recognized. *J.Exp.Med.* 166:1499-1509.

267 Daniels, K. A., Devora, G., Lai, W. C., O'Donnell, C. L., Bennett, M., and Welsh, R. M. 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J.Exp.Med.* 194:29-44.

268 Daniels, K. A., Devora, G., Lai, W. C., O'Donnell, C. L., Bennett, M., and Welsh, R. M. 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J.Exp.Med.* 194:29-44.

269 Kambayashi, T., Assarsson, E., Michaelsson, J., Berglund, P., Diehl, A. D., Chambers, B. J., and Ljunggren, H. G. 2000. Emergence of CD8+ T cells expressing NK cell receptors in influenza A virus-infected mice. *J.Immunol.* 165:4964-4969.

270 Peacock, C. D., Lin, M. Y., Ortaldo, J. R., and Welsh, R. M. 2000. The virus-specific and allospecific cytotoxic T-lymphocyte response to lymphocytic choriomeningitis virus is modified in a subpopulation of CD8(+) T cells coexpressing the inhibitory major histocompatibility complex class I receptor Ly49G2. *J.Virol.* 74:7032-7038.

271 Fidler, I. J. and Bucana, C. 1977. Mechanism of tumor cell resistance to lysis by syngeneic lymphocytes. *Cancer Res.* 37:3945-3956.

272 Xu, D., Gu, P., Pan, P. Y., Li, Q., Sato, A. I., and Chen, S. H. 2004. NK and CD8+ T cell-mediated eradication of poorly immunogenic B16-F10 melanoma by the combined action of IL-12 gene therapy and 4-1BB costimulation. *Int.J.Cancer* 109:499-506.

273 Yang, Q., Hokland, M. E., Bryant, J. L., Zhang, Y., Nannmark, U., Watkins, S. C., Goldfarb, R. H., Herberman, R. B., and Basse, P. H. 2003. Tumor-localization by adoptively transferred, interleukin-2-activated NK cells leads to destruction of well-established lung metastases. *Int.J.Cancer* 105:512-519.

274 Zhang, X. and Ren, R. 1998. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. *Blood* 92:3829-3840.

275 Faderl, S., Talpaz, M., Estrov, Z., O'Brien, S., Kurzrock, R., and Kantarjian, H. M. 1999. The biology of chronic myeloid leukemia. *N.Engl.J.Med.* 341:164-172.

276 Clarkson, B., Strife, A., Wisniewski, D., Lambek, C. L., and Liu, C. 2003. Chronic myelogenous leukemia as a paradigm of early cancer and possible curative strategies. *Leukemia* 17:1211-1262.

277 Duffy, K. M. 2003. Innovations in the management of leukemia: role of biologic therapies. *Cancer Nurs.* 26:26S-31S.

278 Kantarjian, H., Melo, J. V., Tura, S., Giralt, S., and Talpaz, M. 2000. Chronic Myelogenous Leukemia: Disease Biology and Current and Future Therapeutic Strategies. *Hematology.(Am.Soc.Hematol.Educ.Program.)*90-109.

279 Deininger, M. W. and Druker, B. J. 2003. Specific targeted therapy of chronic myelogenous leukemia with imatinib. *Pharmacol.Rev.* 55:401-423.

280 Jaiswal, S., Traver, D., Miyamoto, T., Akashi, K., Lagasse, E., and Weissman, I. L. 2003. Expression of BCR/ABL and BCL-2 in myeloid progenitors leads to myeloid leukemias. *Proc.Natl.Acad.Sci.U.S.A* 100:10002-10007.

281 Mauro, M. J., Druker, B. J., and Maziarz, R. T. 2004. Divergent clinical outcome in two CML patients who discontinued imatinib therapy after achieving a molecular remission. *Leuk.Res.* 28 Suppl 1:S71-S73.

282 Zhang, X. and Ren, R. 1998. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. *Blood* 92:3829-3840.

283 Daley, G. Q., Van Etten, R. A., and Baltimore, D. 1990. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* 247:824-830.

284 Zhang, X. and Ren, R. 1998. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. *Blood* 92:3829-3840.

285 Zhang, X. and Ren, R. 1998. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. *Blood* 92:3829-3840.

286 Wolff, N. C. and Ilaria, R. L., Jr. 2001. Establishment of a murine model for therapy-treated chronic myelogenous leukemia using the tyrosine kinase inhibitor STI571. *Blood* 98:2808-2816.

287 Baron, F., Turhan, A. G., Giron-Michel, J., Azzarone, B., Bentires-Alj, M., Bours, V., Bourhis, J. H., Chouaib, S., and Caignard, A. 2002. Leukemic target susceptibility to natural killer cytotoxicity: relationship with BCR-ABL expression. *Blood* 99:2107-2113.

288 Nakajima, H., Zhao, R., Lund, T. C., Ward, J., Dolan, M., Hirsch, B., and Miller, J. S. 2002. The BCR/ABL transgene causes abnormal NK cell differentiation and can be found in circulating NK cells of advanced phase chronic myelogenous leukemia patients. *J.Immunol.* 168:643-650.

289 Pierson, B. A. and Miller, J. S. 1997. The role of autologous natural killer cells in chronic myelogenous leukemia. *Leuk.Lymphoma* 27:387-399.

290 Chiorean, E. G., Dylla, S. J., Olsen, K., Lenvik, T., Soignier, Y., and Miller, J. S. 2003. BCR/ABL alters the function of NK cells and the acquisition of killer immunoglobulin-like receptors (KIRs). *Blood* 101:3527-3533.

291 Passegue, E., Jamieson, C. H., Ailles, L. E., and Weissman, I. L. 2003. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc.Natl.Acad.Sci.U.S.A* 100 Suppl 1:11842-11849.

292 Jaiswal, S., Traver, D., Miyamoto, T., Akashi, K., Lagasse, E., and Weissman, I. L. 2003. Expression of BCR/ABL and BCL-2 in myeloid progenitors leads to myeloid leukemias. *Proc.Natl.Acad.Sci.U.S.A* 100:10002-10007.

293 Zarling, J. M. and Kung, P. C. 1980. Monoclonal antibodies which distinguish between human NK cells and cytotoxic T lymphocytes. *Nature* 288:394-396.

294 Vitale, A., Guarini, A., Latagliata, R., Cignetti, A., and Foa, R. 1998. Cytotoxic effectors activated by low-dose IL-2 plus IL-12 lyse IL-2-resistant autologous acute myeloid leukaemia blasts. *Br.J.Haematol.* 101:150-157.

295 Walker, W. and Rotondo, D. 2004. Prostaglandin E2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-gamma synthesis. *Immunology* 111:298-305.

296 Hall, T. J., Chen, S. H., Brostoff, J., and Lydyard, P. M. 1983. Modulation of human natural killer cell activity by pharmacological mediators. *Clin.Exp.Immunol.* 54:493-500.

297 Hellstrand, K., Brune, M., Dahlgren, C., Hansson, M., Hermodsson, S., Lindner, P., Mellqvist, U. H., and Naredi, P. 2000. Alleviating oxidative stress in cancer immunotherapy: a role for histamine? *Med.Oncol.* 17:258-269.

298 Leung, K. H., Fischer, D. G., and Koren, H. S. 1983. Erythromyeloid tumor cells (K562) induce PGE synthesis in human peripheral blood monocytes. *J.Immunol.* 131:445-449.

299 Gishizky, M. L. and Witte, O. N. 1992. Initiation of deregulated growth of multipotent progenitor cells by bcr-abl in vitro. *Science* 256:836-839.

300 Jaiswal, S., Traver, D., Miyamoto, T., Akashi, K., Lagasse, E., and Weissman, I. L. 2003. Expression of BCR/ABL and BCL-2 in myeloid progenitors leads to myeloid leukemias. *Proc.Natl.Acad.Sci.U.S.A* 100:10002-10007.

301 Scherle, P. A., Dorshkind, K., and Witte, O. N. 1990. Clonal lymphoid progenitor cell lines expressing the BCR/ABL oncogene retain full differentiative function. *Proc.Natl.Acad.Sci.U.S.A* 87:1908-1912.

302 Biernaux, C., Loos, M., Sels, A., Huez, G., and Stryckmans, P. 1995. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. *Blood* 86:3118-3122.

303 Spiegelberg, H. L. and Weigle, W. O. 1965. The Catabolism of Homologous and Heterologous 7S Gamma Globulin Fragments. *J.Exp.Med.* 121:323-338.

304 Seppen, J., Barry, S. C., Harder, B., and Osborne, W. R. 2001. Lentivirus administration to rat muscle provides efficient sustained expression of erythropoietin. *Blood* 98:594-596.

305 Dimasi, N., Sawicki, M. W., Reineck, L. A., Li, Y., Natarajan, K., Margulies, D. H., and Mariuzza, R. A. 2002. Crystal structure of the Ly49I natural killer cell receptor reveals variability in dimerization mode within the Ly49 family. *J.Mol.Biol.* 320:573-585.

306 French, A. R. and Yokoyama, W. M. 2004. Natural killer cells and autoimmunity. *Arthritis Res.Ther.* 6:8-14.

307 Yoon, J. S., Jung, Y. T., Hong, S. K., Kim, S. H., Shin, M. C., Lee, D. G., Shin, W. S., Min, W. S., and Paik, S. Y. 2004. Characteristics of HIV-Tat Protein Transduction Domain. *J.Microbiol.* 42:328-335.

308 Zhang, J., Somani, A. K., and Siminovitch, K. A. 2000. Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. *Semin.Immunol.* 12:361-378.

309 Raziuddin, A., Longo, D. L., Bennett, M., Winkler-Pickett, R., Ortaldo, J. R., and Murphy, W. J. 2002. Increased bone marrow allograft rejection by depletion of NK cells expressing inhibitory Ly49 NK receptors for donor class I antigens. *Blood* 100:3026-3033.

310 Steinman, R. M. and Mellman, I. 2004. Immunotherapy: bewitched, bothered, and bewildered no more. *Science* 305:197-200.

311 Genitope Corporation home page. www.genitope.com . 2004.
Ref Type: Electronic Citation

VITA

Namita Gandhi was born in Washington DC (USA) on December 31, 1974, to Anilkumar Venilal Gandhi and Gita Anilkumar Gandhi. In June 1992, Namita graduated from high school from Thomas S. Wootton High School in Rockville, MD and in September of that year entered University of Maryland at College Park, MD. She earned her Bachelor of Science degree in Biology with a concentration in Neurobiology and Physiology in May 1996. While in college, Namita worked part-time as a research assistant at Walter Reed Army Institute of Research in Silver Spring, MD. She analyzed data from behavioral biological studies on effects of sleep deprivation. After college, Namita worked in clinical research field in which her duties involved monitoring ongoing clinical trials, performing patient data review activities and working with clinical investigators to ensured regulatory and protocol compliance. Between August 1996 and July 1999, Namita preformed these activities while employed by clinical research organizations (CROs): Commonwealth Clinical Research Services, Inc. (Ashland, VA), Trilogy Consulting Corporation (Boulder, CO) and MedTrials, Inc. (Dallas, TX). She entered graduate school in Biomedical Sciences at University of Texas Southwestern Medical Center at Dallas in August 1999.

This dissertation was written and typed by Namita A. Gandhi

Permanent Address: 14022 Welland Terrace
North Potomac, MD 20878