DEFINING HOW POLYMORPHISMS AT THE SLAM FAMILY LOCUS

AFFECT NK AND T CELL FUNCTION

APPROVED BY SUPERVISORY COMMITTEE

John Schatzle, Ph.D

James Forman, D.M.D, Ph.D

Mike Bennett, Ph.D

Edward K Wakeland, Ph.D

Diego Castrillon, M.D., Ph.D

ACKNOWLEDGEMENTS

I have several people I want to thank for their time, patients, guidance, support and encouragement during the course of my graduate work at UT Southwestern. I would not be the scientist I am today if it were not for my mentor John Schatzle. You have taught me how to use my innate doubting Thomas skills for the better, so that I always examine things critically and objectively. Your patients and willingness to let me figure things out on my own have allowed me to learn valuable skills and how to trouble shoot very effectively. Graduate school is inherently a painful process and you have made it more then tolerable. It has been a pleasure working with you and I can honestly say that after completing graduate school in addition to the PhD at the end of my name I have also gained a dear friend. THANKS

Next, I would like to thank my dissertation committee: Drs. Jim Forman, Mike Bennett, Edward Wakeland, and Diego Castrillon. Collectively you represent over 150 years of scientific knowledge, which when discussing ideas for my project is a beautiful thing but when preparing for a defense is very intimidating. I have been very fortunate because many of you have been more like a mentor then a committee member to me. I want to thank you all for the time and energy you

have spent over the years serving on my committee. Jim thank you for always asking me tough questions and making me think big picture. Mike thank you for teaching me all your in vivo expertise and for our countless scientific conversations. Every time I talk to you I learn something new. Ward thank you for all your guidance and for exposing me to a geneticist view of immunology. All of my genetics knowledge I owe to you. Diego, I want to thank you for agreeing to serve as my outside committee member and for giving me a new perspective on my project.

The Bennett-Schatzle lab has always been a great lab and environment to work in because of the people who work there. I want to thank both past and present lab members (Jennifer Klem, Gene Devora, Mesha Taylor, Maggie Morris, Maria Johansson, Verra Arora, Wayne Lai, Beth Cameron, and Leslie Moody) for their support and friendship. Jennifer you taught me all I know about flow cytometry and I have learned more from you over the years both scientifically and personally then you will ever know. I cannot thank you enough. Gene thank you for all our candid conversations and advice during my first years in lab. Beth and Leslie thank you for your friendship and always making me laugh. I'm going to miss our ice cream runs to Milwaukee Joe's.

Karen Kazemzadeh, thank you for letting us use your office as a safe haven from science and for all your encouragement. I'd also like to thank Marilyn Gibson for administrative support and working her magic when I needed a reagent ASAP.

I would like to thank the faculty members of the immunology program because I have knocked on many doors over the years and I have never been turned away. I would especially like to thank Dorothy Yuan and Borna Mehrad for all your insightful suggestions and advice. I would also like to thank David Farrar, Christoph Wülfing, and Nicolai van Oers for always answering my questions. I have valued and learned from your advice and scientific discussion over the past years. I have knocked on your doors numerous times with questions and in need of reagents and you have always made time for me no matter how busy you were and been very generous. Thank you all very much, I really appreciate your time and efforts.

There are several friends I would like to thank for their support and encouragement. I have been so fortunate and blessed to have so many great friends. If it weren't for them I would not have been able to hold onto what little sanity I possess. First I would like to thank Cheryl and West Garr for exposing me to the world of science and for all your guidance during these later stages of my graduate career. I would like to thank my fellow classmates: Jessica Bartlett,

Stacey Glasgow, Jonathan Lawson, and last but certainly not least Jeff Milush. Thank you all for sharing countless beers, coffees, and ice creams. You have all given me encouragement when my science was not working and I will always treasure our friendship. I would like to thank the "Fab Four": Steve Davies, Ivan Maskey, Lisa Pitcher, and Maria Monteverde. You guys have been such great friends and my family away from family. You guys always bring a smile to my face, make me laugh, and keep everything in perspective. Finally, I'd like to thank Emily and Ryan Genor and Steve Cochran for being the voice in the back of my head saying hurry up and finish. You guys never let me quit and always provided me with a nice escape from Texas and lab.

Finally, I'd like to thank my family. Words cannot even describe how blessed and fortunate I am to have such a supportive and caring family. You have always supported me no matter how crazy my idea. I want to thank my mom, dad, grandma Harriet and grandpa Chris for instilling in me such a strong work ethic. It is because of you guys that I know I can do anything if I just work hard enough. Thanks for always being there for me. I couldn't have done this without you.

DEFINING HOW POLYMORPHISMS AT THE SLAM FAMILY LOCUS AFFECT NK AND T CELL FUNCTION

by

JILL MARIE MOONEY

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, 2006

DEFINING HOW POLYMORPHISMS AT THE SLAM FAMILY LOCUS AFFECT NK AND T CELL FUNCTION

Jill Marie Mooney

The University of Texas Southwestern Medical Center at Dallas

Supervising Professor: John D. Schatzle, Ph.D

Sequence analysis of the SLAM family of receptors identified two stable haplotypes found in common laboratory strains of mice. The *b* haplotype is found in all C57-derived mouse strains, and the *z* haplotype is found in all non-C57 derived mouse strains, including many autoimmune mouse strains. The SLAM

family of receptors, which includes CD244, Ly108, CD84, CD48, CD229, and Cs1, are involved in regulating several immune functions such as cellular activation, cytokine secretion, cytotoxicity, and apoptosis. Thus, it is not surprising that dysregulation of this family of receptors is associated with disease states such as systemic lupus erythematosus (SLE) and X-linked lymphoproliferative disease (XLP). The antinuclear autoantibody (ANA) causative locus, *Sle1b*, in the NZM2410 mouse model of lupus has been shown to contain the entire SLAM family (SF) locus. Whereas, XLP is caused by mutations in a critical adaptor protein, SAP, leading to defective SF signaling. This dissertation is based on the premise that polymorphisms at the SF locus alter SF function in lymphocytes. To address this, we compared lymphocyte function in B6 mice, which contain the *b* haplotype of the SF, and B6.*Sle1b* mice that are congenic for the 900 kb interval surrounding the *z* haplotype of the SF. Thus, differences in lymphocyte function can be directly attributed to polymorphism at the SF locus. These congenic mouse strains were used in two studies: 1) to characterize the function of CD244 in NK cells, and 2) to characterize CD4+ T cell function. We show that polymorphisms in CD244 alter receptor function, where engagement of the *z* allele of CD244 results in increased cytotoxicity dependent on SAP expression. In contrast, engagement of the *b* allele of CD244 predominately results in inhibition of cytotoxicity, independent of SAP expression. These studies may explain previously conflicting data describing

CD244 function. Our studies characterizing CD4+ T cell function determined that polymorphisms at the SF locus result in altered SF expression, decreased IFN-γ, IL-4, IL-5, IL-6, and IL-10 secretion, increased and prolonged CD40L expression, and altered SAP expression. In conclusion, we show that polymorphisms at the SF locus alter NK and T cell lymphocyte function.

TABLE OF CONTENTS

PUBLICATIONS

Morrison BE, Park SJ, **Mooney JM**, and Mehrad B (2003). Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. *J.Clin. Invest.* 112(12):1862-70

Mooney JM, Klem J, Wülfing C, Mijares LA, Schwartzberg PL, Bennett M, and JD Schatzle (2004). The murine NK receptor 2B4 (CD244) exhibits inhibitory function independent of signaling lymphocytic activation molecule-associated protein (SAP) expression. *J. Immunol.* 173:3953-3961

Calpe S, Erds E, Laio G, Wang N, Rietdij S, Scholtz B, **Mooney JM**, Lee CH, Shin MS, Rajnavolgyi E, Schatzle JD, Morse H, Terhorst C, and A Lanyi (2006). Identification and characterization of two related murine genes, *eat-2a* and *eat-2b*, encoding single SH2-domain adapters. *Immunogenetics,* (E published ahead of print DOI 10.1007/s00251-005-0056-3)

Mooney JM, Chan A, Bennett M, Wakeland EK, and JD Schatzle (2005). Polymorphic alleles of CD244 exhibit divergent function in murine NK cells. (Paper submitted)

Mooney JM, Chan A, Nguyen C, Wakeland EK, and JD Schatzle. SLAM family haplotypes regulates CD4+ T cell cytokine secretion. (In preparation)

FIGURES

TABLES

ABBREVIATIONS

3BP2:	c-Abl-src homology 3 domain binding protein-2
α-GC:	alpha-galactosyl ceramide
a.a.:	amino acid
ADCC:	antibody directed cell cytotoxicity
AICD:	activation induced cell death
AID:	activation-induced cytidine deaminase
ANA:	antinuclear autoantibody
APC:	allophycocyanin
APC:	antigen presenting cell
ASC:	antibody secreting cell
ASFV:	African swine fever virus
β2m:	beta 2 microglobulin
BCR:	B cell receptor
BLAME:	B lymphocyte activator macrophage expressed
C2-Ig:	constant-2 immunoglobulin
^{51}Cr:	chromium
CD244L:	CD244 long
CD244S:	CD244 short
cDNA:	complementary deoxyribonucleic acid

CFSE:	carboxyfluorescein diacetate, succinimidyl ester
CLR:	c-type lectin receptors
CMV:	cytomegalovirus
CRACC:	CD2-like receptor-activating cytotoxic cell
CS1:	CD2 subset 1
CTL:	cytotoxic T lymphocyte
CVID:	common variable immunodeficiency disease
DAG:	diacylglycerol
DAP-10/12:	DNAX-activating protein of 10/12 kDa
DC:	dendritic cell
DETC:	dendritic epidermal gamma/delta T cell
DN:	double negative
Dok1:	downstream of multiple tyrosine kinases 1
DP:	double positive
EAE:	experimental autoimmune encephalomyelitis
EAT-2a/b:	EWS/FLI1 activated transcript 2a/b
EBV:	Epstein-Barr virus
ER:	endoplasmic reticulum
ERK:	extracellular signal-regulated kinase
ES:	embryonic stem cell
Fab:	antibody fragment

FACS:	fluorescence activated cell sorting
FasL:	Fas ligand
FcR:	Fc receptor
FCS:	fetal calf serum
FIM:	fulminant infectious mononucleosis
FITC:	fluorescein isothiocyanate
GATA-3:	GATA binding protein 3
GC:	germinal center
GEM:	glycolipid enriched microdomains
GFP:	green fluorescence protein
GM-CSF:	granulocyte-macrophage colony stimulating factor
GPI:	glycosylphosphatidylinositol
Grb2:	growth factor receptor binding protein 2
GSK-3:	glycogen synthase kinase-3
iGb3:	isoglobotrihexosylceraminde
HLA:	human leukocyte antigen
HRP:	horseradish peroxidase
ICOS:	inducible co-stimulator
IFNα/β:	interferon alpha/beta
IFNγ:	interferon gamma
Ig:	immunoglobulin

Ig-SF:	immunoglobulin super-family
IL:	interleukin
IRES:	internal ribosome entry site
ITAM:	immunoreceptor tyrosine-based activation motif
ITIM:	immunoreceptor tyrosine-based inhibition motif
ITK:	interlekin-2 inducible T cell kinase
ITSM:	immunoreceptor tyrosine-based switch motif
kDa:	kilodalton
KIR:	killer cell immunoglobulin-like receptor
LAK:	lymphokine activated killer
LAT:	linker for activation of T cells
LCMV:	lymphocytic choriomeningitis virus
LPS:	lipopolysaccharide
mAb:	monoclonal antibody
MAPK:	mitogen activated protein kinase
MAR-1:	mouse activating receptor-1
MBP:	myelin basic protein
MCMV:	murine cytomegalovirus
MEK:	MAPK kinase
MFI:	mean fluorescence intensity

MγHV-68: murine gammaherpesvirus-68

MHC: major histocompatibility complex

MIP-1α/β: macrophage inflammatory protiein 1 α/β

MLR: mixed lymphocyte reaction

MS: multiple sclerosis

MULT1: mouse UL-16 binding protein-like transcript-1

NCR: natural cytotoxicity receptor

NK: natural killer

NKR: natural killer receptor

NKT: natural killer T cell

NTB-A: NK-T-B-antigen

NZM: New Zealand mixed

ORF: open reading frame

PAK1: p21-activated kinase 1

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PE: phycoerythrin

PI3K: phosphoinositide 3-kinase

PLCγ: phospholipase C gamma

PMA: phorbol myristate acetate

PolyI:C: polyinosinic:polycytidylic acid

RACE:	rapid amplification of cDNA ends
Rae-1:	retinoic acid early inducible gene
RANTES:	regulated on activation-normal T cell expressed and secreted
RLK:	resting lymphocyte kinase
RNK:	rat natural killer
RT-PCR:	reverse transcriptase-polymerase chain reaction
SAP:	SLAM-associated protein
SCID:	severe combined immune deficiency
SCF:	stem cell factor
SDS-PAGE:	sodium dodecylsulfate polyacrylamide gel electrophoresis
SF:	SLAM family
SH2/3:	Src homology 2/3
SHIP:	SH2 domain-containing inositol-5-phosphatase
SHP-1/2	SH2 domain-containing tyrosine phosphatase-1/2
SLAM:	signaling lymphocytic activation molecule
SLE:	systemic lupus erythematosus
SLP-76:	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SNP:	single nucleotide polymorphisms
SP:	single positive
SPF:	specific pathogen free
ssDNA:	single stranded deoxyribonucleic acid

STAT-1/6:	signal transducers and activators of transcription-1/6
Syk:	spleen tyrosine kinase
TAP:	transporters associated with antigen processing
Tbet:	T-box transcription factor
TCR:	T cell receptor
TEC:	tyrosine kinase expressed in hepatocellular carcinoma
T_{FH}:	follicular B helper T cell
Tg:	transgenic
TGFβ:	transforming growth factor beta
Th:	T helper
TLR:	Toll like receptor
TNFα:	tumor necrosis factor alpha
TNFR:	tumor necrosis factor receptor
TRAIL-R1:	tumor necrosis factor-related apoptosis-inducing ligand receptor 1
TRAIL-R2:	tumor necrosis factor-related apoptosis-inducing ligand receptor 2
TRAMP:	tumor necrosis factor-receptor-related apoptosis-mediated protein
ULBPs:	UL-16 binding proteins
V-Ig:	variable-immunoglobulin
XLP:	X-linked lymphoproliferative disease
ZAP-70:	zeta-associated protein of 70 kilodalton

INTRODUCTION

The immune system is composed of an intricate network of tissues, cells, and molecules that collectively work to protect against pathogen invasion. The immune system responds to the surrounding environment via several mechanisms, including cell-cell contact and secreting soluble factors. Dysregulation of this network results in immune system failure that can ultimately cause death. Understanding how a functional immune system maintains a pathogen free existence in a pathogen plagued world can lead to the development of treatments for disease states where the immune system is impaired. The CD2/SLAM family of receptors play a critical role in maintaining a functioning immune system. This dissertation will focus on describing how the CD2/SLAM family functions in normal immune responses and how altered CD2/SLAM family function can result in disease.

I. GENERAL IMMUNOBIOLOGY

A. Innate Immune Response

The innate immune response is the first line of defense against pathogen invasion and does not require previous exposure to pathogens to be activated. The innate immune response includes epithelial barriers, phagocytic cells, NK cells, the complement system, and cytokines. The innate immune response is

immediately activated upon pathogen recognition by dendritic cells (DCs), natural killer cells (NK), natural killer T cells (NKT), macrophages, and neutrophils. The function of NK and NKT cells will be described in detail below. Receptors of the innate immune response have evolved to recognize structures conserved among many pathogens, for example LPS and CpG rich regions. Toll like receptors are an example of these pattern recognition receptors found in the innate immune response. Innate immune receptors are encoded by genes that do not undergo rearrangement (1). Engagement of innate immune receptors leads to activation of effector functions such as cytotoxicity, phagocytosis, and the production of cytokines. Pathogen clearance at the site of infection by innate immune cells results in termination of the immune response. Pathogen burden dictates the extent of the immune response. If the innate response in unable to clear the pathogen, signals are initiated for the adaptive immune response; these innate immune signals influence how the adaptive immune response responds to the pathogen (2). The innate immune response occurs immediately upon pathogen invasion, while the adaptive immune response occurs over several days to weeks.

B. Adaptive Immune Response

Relative to the innate immune response, the adaptive immune response is delayed, occurring over several days or weeks after pathogen invasion. This delay is the result of a need for the expansion of a limited responder pool of

pathogen specific effector cells. The adaptive immune response is mediated by B and T cells. These cells acquire a pathogen-specific response via gene rearrangement to express B cell (BCR) and T cell (TCR) receptors that recognize pathogenic antigens. The TCR recognizes pathogenic encoded peptides presented on the surface of an infected cell by major histocompatibility complex molecules (MHC in rodents, or human leukocyte antigen, HLA in humans). Engagement of TCRs that recognize pathogen antigens results in clonal expansion and MHC-dependent cytotoxicity and/or cytokine production. Engagement of the BCR leads to production of pathogen specific antibodies and presentation of pathogen derived peptides. The adaptive immune response, unlike the innate immune response, is able to establish memory. Upon encountering the pathogen again, the adaptive immune response can respond immediately and vigorously (2).

II. NATURAL KILLER CELLS (NK)

A. Background and Immunobiology

Natural Killer cells are of hematopoietic origin, and like T and B cells arise from a pluripotent hematopoietic stem cell in the bone marrow. Upon maturation in the bone marrow, NK cells migrate out and circulate through the blood and lymphoid organs; where they make up approximately 5% of circulating lymphocytes (3, 4). NK cells lack distinct markers such as a TCR or BCR expressed on T and B cells, respectively. In general, NK cells are described as

having a large granular morphology and express surface receptors in a germ line configuration; in other words NK cell receptors do not undergo rearrangement like T and B cells (5). NK cells are generally defined by the expression of surface markers NK1.1 and DX5 in the mouse or CD56 in humans. However, these markers are not exclusive to NK cells and are expressed on other lymphocytes populations.

NK cells function as the first line of defense against some viral infections and play a critical role in clearing transformed or cancerous cells (6, 7). As key members of the innate immune response, NK cells mediate cytotoxicity and cytokine secretion. Once fully mature, NK cells are poised to kill, but inhibitory signals keep NK cytotoxicity in check, preventing normal cells from being killed. This occurs through interactions between self-MHC ligands and inhibitory receptors on the NK cell surface (8). Down-modulation of self-MHC via viral infection or cellular transformation makes these infected or transformed cells susceptible to NK killing, or 'targets' of NK killing. The mechanisms of NK function will be discussed in detail below. Briefly, cytotoxicity is mediated via the directed release of perforin and granzymes or signaling via the death receptor pathway, which induces apoptosis in target cells. The second major function of NK cells is cytokine release, which occurs as the result of receptor engagement via cell-cell contact (for example between NK and B cells) or soluble factors (for

example IL-12 and IL-18). NK cells are capable of secreting IFN-γ, tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, IL-10, and IL-13 (9-13).

NK cells are not only important for the innate immune response, but influence the adaptive immune response. Crosstalk between NK cells and DCs is important in NK and T cell responses (reviewed in (14, 15). Freshly isolated, 'resting', NK cells are induced to proliferate, are capable of IFN-γ secretion, and acquire cytotoxic activity following co-culture with DCs; this DC mediated activation of NK cells requires cell-cell contact, although DC-derived cytokines do play a role (15). The converse is also true. Co-culture of immature DCs with NK cells results in DC maturation, production of TNF and IL-12, and up-regulation of costimulatory ligands. NK cell-matured DCs are more effective at inducing T cell and NK cell responses compared to immature DCs. The cytokines produced during NK-DC interactions, such as IFN-γ, influence further T cell responses by promoting antigen processing and T helper 1 cell polarization critical for a cell mediated immune response. Studies have shown that NK cells not only activate immature DCs, but also can kill them. Lysis of DCs is mediated through NKp30, a natural cytotoxicity receptor (NCR), discussed in detail in the following NCR receptor section. Cell culture experiments have shown that DC lysis is favored when NK cells out number immature DCs. In contrast, DC

activation is favored when immature DCs out number NK cells (16). The mechanisms that regulate DC lysis or activation may serve as a checkpoint to eliminate DCs that may be presenting antigens in a non-productive manner (15). NK cells may function as regulators of the immune response in this situation preventing potential autoimmune or non-productive immune responses.

B. Specificity of NK cell Receptors

It is now well known that many NK receptors recognize MHC molecules. Class I MHC molecules (H2-K, -D, and -L in rodents and HLA-A, -B, -C in humans) contain an α or heavy chain composed of three extracellular domains that associates with an invariant small polypeptide called β2-microglobulin (β2m). These structures are termed classical class I MHC molecules. β2m is required for MHC class I surface expression. The process of MHC class I formation occurs in the endoplasmic reticulum (ER). First, a partially folded α chain is bound by calnexin, a chaperone protein, to stabilize the protein structure until the α chain binds β2m. This MHC class I:β2m complex is released from calnexin and binds a complex of chaperone proteins including calreticulin, tapasin, and TAP-1. Transporters associated with Antigen Processing-1 and -2 (TAP-1 and TAP-2) are ATP-binding cassette proteins involved in transporting short peptides from the cytosol that have been degraded by the proteasome into the lumen of the ER. Once the TAP transporter delivers a peptide that binds to the

MHC class I molecule, MHC folding is completed and the MHC class I molecule/peptide complex is released from the TAP complex and exported to the cell surface (2). Mice devoid of TAP-1 or TAP-2 express very low levels of MHC class I.

In the 1960s, a phenomenon termed hybrid resistance was first described. It was observed that F1 hybrid progeny could reject bone marrow from either parent (17, 18), and this rejection was mediated by NK cells (19, 20). F1 hybrid progeny inherit "self" MHC from both parents. However, subsets of NK cells develop in F1 progeny that only express receptors that recognize MHC molecules from one parent. Thus, when these NK cells encounter bone marrow for the other parent, they fail to recognize the parental MHC as "self". These NK subsets lyse the parental bone marrow as it is identified as foreign. Later, Klas Kärre and colleagues proposed the "missing self" hypothesis, suggesting that NK cells lyse target cells with absent or altered self-MHC class I molecules (21, 22). However, this did not completely explain hybrid resistances. It was not until Yu *et al* showed the existence of overlapping subsets of NK cells that could react to parental BMC that the mechanism of hybrid resistance was explained (23).

Subsequent studies determined that NK cells often express multiple receptors simultaneously. This allows NK cells to be broken down into subset

populations expressing specific combinations of activating or inhibitory receptors. NK cell function is governed by a balance between inhibitory and activating receptors, where the inhibitory signal generally dominates over the activating signal (8). These receptors can be divided into two families based on structure: the C-type lectin superfamily and the Immunoglobulin superfamily (Ig-SF) (8, 24). Alternatively, NK receptors could be categorized based on ligand specificity or receptor function (i.e. activating vs inhibitory). However, for the purposes of this dissertation NK receptors will be divided based on structure. The C-type lectin family, which contains type II membrane glycoproteins with lectin like extracellular domains, includes the murine Ly49 family of receptors, and the NKG2/CD94 receptors found on human and murine NK cells (8). The Ig superfamily, which is characterized as type I membrane glycoproteins containing Ig extracellular domains, includes the killer immunoglobulin-like receptors (KIRs) and natural cytotoxicity receptors (NCR) found on human NK cells, and the CD2/SLAM family of receptors found on murine and human NK cells (8). These families contain both activating and inhibitory receptors that are discussed in detail below. Although, many of these receptors are characterized as NK receptors, several are expressed on terminally differentiated lymphocytes, such as NKG2D on T cells.

i. C-type Lectin Superfamily (CLR)

a. Ly49s

The murine Ly49 family of receptors are type II membrane glycoproteins that are expressed as disulfide-linked homodimers (25). The Ly49 receptor gene family is located on murine chromosome 6 in a region termed the NK gene complex (26). The Ly49 family of receptors appears to be highly polymorphic between mouse strains, where mouse strains express different combinations of *Ly49* genes (25). This family of receptors recognizes MHC class I molecules expressed on target cells. While engagement of Ly49 molecules requires peptide bound to the MHC ligand, most Ly49 binding is only minimally influenced by the actual peptide sequence, and is more influenced by how the peptide alters MHC structure (27-29). In fact, structural studies have identified consensus sequences for specific MHC class I/Ly49 binding, i.e. sequences conserved between Ly49 receptors that bind D^b or D^d independent of the binding groove (25).

Ly49 receptors can be broken down into two groups, inhibitory and activating, based on receptor structure and function. Based on cDNA sequences of Ly49 genes *Ly49a, b, c, e, f, g, i, j, o, q, s, t,* and *v* are predicted to code for inhibitory receptors. Similarly, Ly49*d, h, k, l, m, n, p, r, u*, and *w* are predicted to code for activating receptors (25). Inhibitory Ly49s contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) composed of the consensus sequence

I/VxYxxI/L/V in their cytoplasmic domain. Each monomer contains a single ITIM; therefore each homodimer contains two ITIMs. Following receptor engagement, the tyrosines within the ITIMs become phosphorylated which results in the recruitment of cytoplasmic tyrosine phosphatases (SHP-1, SHP-2, SHIP) via SH2 domains. This signaling cascade generates a negative signal preventing NK lysis (25, 30). In contrast, activating Ly49s do not contain ITIMs; rather they contain a positively charged residue within their transmembrane domains, which facilitates binding of the adaptor protein DNAX-activating protein of 12 kDa (DAP 12) (31). DAP 12 contains a CXC motif likely involved in its homodimerization and a negatively charged aspartic acid in the transmembrane domain which interacts with a positively charged amino acid within the transmembrane region in activating Ly49s. The key feature of DAP 12 linking it to activating function is the presence of an immunoreceptor tyrosine-based activation motif (ITAM), YxxL x6–8 YxxL, containing two phosphorylated tyrosine residues. Upon receptor engagement, the 2 tyrosines located in the ITAMs of DAP-12 become phosphorylated and subsequently recruit Syk or ZAP-70 initiating an activating signaling cascade resulting in NK cell activation and target cell lysis (25, 32). These inhibitory and activating receptors appear to have evolved as pairs with highly homologous recognition domains. Although, how and why this may have occurred remains unclear. One might predict that these receptors evolved as pairs where the inhibitory receptor prevents lysis of

autologous cells and the activating receptors recognize pathogen derived MHC like molecules. It is currently thought that duplication of an inhibitory receptor was probably followed by recombination with an activating receptor (25).

Expression of Ly49 receptors occurs gradually over the first few weeks of life, reaching a plateau by one month of age (33, 34). Different Ly49 genes are expressed in overlapping subsets within the total NK population (8). The percentage of NK cells expressing more than one Ly49 receptor can be determined by the product rule where the frequency of NK cells expressing two or more Ly49 receptors is approximately the same as the product of the individual frequencies of cells expressing each receptor (35). Ly49 genes are stochastically and monoallelically expressed (36). Recent studies have demonstrated that NK cells acquire functional competence through 'licensing' by self-MHC molecules (37). Kim *et al* showed that in order for NK cells to become fully lytic and produce cytokines, during development NK cell inhibitory receptors must exhibit a productive interaction between self specific Ly49s and MHC class I (37). For example, Ly49C must be in contact with its ligand D^b or K^b for Ly49C+ NK cells to develop full lytic and cytokine function. Engagement of an activating receptor, such as NK1.1, on Ly49C+ NK cells that have interacted with self-MHC results in full lytic function. However, engagement of NK1.1 on Ly49C+ NK cells from D^b/K^b double KO mice resulted in decreased NK cytotoxicity and cytokine

function in these NK cells because they have not been 'licensed' through productive interaction with self MHC. The mechanism of licensing explains how NK cells are tolerized, preventing lyses of normal autologous cells. Licensing requires the cytoplasmic inhibitory motifs of the inhibitory Ly49s, but does not require the inhibitory signaling molecule SHP-1, and preliminary studies also suggest no requirement for SHIP (37). Therefore, ITIMs may be required for recruiting signaling molecules needed for 'licensing' signals that are independent of SHP-1 and SHIP inhibitory effector signals required for cytotoxicity.

b. CD94/NKG2

Unlike Ly49s, CD94/NKG2 receptors are common to primates and rodents. The CD94/NKG2 receptors are type II transmembrane receptors also belonging to the C-type lectin family and located in the NK gene complex. The NK gene complex is located on chromosome 12 in humans and chromosome 6 in the mouse (8, 26). These receptors are found as disulfide-linked heterodimers of CD94 and one of several NKG2 family members, NKG2A, NKG2C, or NKG2E. NKG2 receptors recognize non-classical MHC class I molecules known as Qa-1 (in mice) and HLA-E (in humans), which present the signal peptides of classical class I MHC allowing NKG2 receptors to indirectly monitor MHC class I expression (38, 39). CD94/NKG2A is currently the only known inhibitory

receptor of this family. Similar to the Ly49 genes, the *Nkg2a* gene is stochastically and monoallelically expressed (40).

In contrast, CD94/NKG2C and CD94/NKG2E are thought to have activating function because they lack ITIMs and have charged transmembrane residues (41). Moreover, CD94/NKG2C associates with the ITAM containing DAP 12 adapter protein and has been shown to have activating function (42).

NKG2D is another member of this family. NKG2D is a type II lectin-like receptor, and unlike other NKG2 receptors, exists as a homodimeric receptor. The presence of a charged amino-acid residue in the transmembrane domain implicated a stimulatory role for NKG2D, a notion validated in functional studies (43). NKG2D associates with the adaptor molecule DAP 10, which lacks an ITAM but does contain tyrosine-based motifs that can mediate an activating signal by recruiting PI3K (44, 45). Long and short NKG2D isoforms have been identified in the mouse, where the long form associates with DAP 10 only, and the short form associates with both DAP 10 and DAP 12; both isoforms are present in NK cells (46, 47). Studies have shown that T cells and freshly isolated 'naïve' NK cells express only the long form. Engagement of NKG2D-L results in costimulatory signals culminating in NK and T cell effector function. However, upon activation NK cells up-regulate the short form of NKG2D, and NKG2D-S

engagement leads to a direct stimulatory signal in NK cells (46). Recently the ligands for NKG2D have been identified as several cell-surface molecules that are distantly related to MHC class I molecules: MHC class-I-chain-related protein A (MICA), MICB, the UL16-binding proteins (ULBPs) in humans, retinoic acid early transcript 1 (Rae1), histocompatibility 60 (H60), and mouse UL16-binding protein-like transcript 1 (Mult1) in mice (reviewed in (43)). These ligands have distinct expression patterns. In general, they are expressed at low to undetectable levels on normal cells; however, their expression is up-regulated in pathological states such a transformation or bacterial infection leading to the NKG2D-mediated clearance of these aberrant cells. Expression of these ligands is closely tied to activation of DNA repair mechanisms (48).

ii. Immunoglobulin Superfamily (Ig-SF)

a. Killer Cell Immunoglobulin-like Receptors (KIRs)

KIRs are found in human NK cells and are functionally equivalent to the murine Ly49s, in that they recognize classical MHC class I molecules. KIRs are type I transmembrane glycoproteins with two or three extracellular constant-2 (C2) Ig domains, a transmembrane domain, and a cytoplasmic tail. As a family, KIRs contain both activating and inhibitory receptors, and similar to Ly49s, inhibitory KIRs contain ITIMs in their cytoplasmic tails. The activating KIR receptors contain a positively charged residue in their transmembrane region that

associates with DAP 12 (8, 49). Approximately 12 genes located in the leukocyte receptor complex region on chromosome 19 encode human KIRs. Conserved residues within polymorphic HLA regions enables individual KIR receptors to recognize multiple class I HLA molecules while discriminating among various allotypes (50).

b. Natural Cytotoxicity Receptors (NCRs)

NCRs are a family of receptors found on human NK cells that include NKp46, NKp44, and NKp30 (24). These family members have a low degree of structural homology. General characteristic of NCRs are: they are non-polymorphic receptors, do not have subset distribution but are expressed on all NK cells, are responsible for the lysis of a wide range of targets and are implicated in xenogeneic killing. NKp46 is a type I transmembrane glycoprotein with two C2-Ig domains, and it contains a transmembrane domain containing a positively charged amino acid (Arg), which associates with CD3ζ and/or FcεRγ ITAM containing adaptor proteins. NKp46 has also been shown to have activating function and associate with CD3ζ in the rat (51). A murine homologue of NKp46, Mar-1, has been identified. NKp44 is characterized by a single extracellular variable (V) type domain and a transmembrane domain containing a positively charged amino acid (Lys) that may associate with DAP 12. Finally, NKp30 contains a single V-type domain and has a positively charged Arg in its

transmembrane that associates with the CD3ζ ITAM containing adaptor (24, 52). Recently, NKp30 has been shown to be the main receptor involved in NK killing of immature DCs, a possible mechanism for preventing non-productive antigen presentation (53, 54). To a lesser extent, NKp46 is also involved in NK killing of immature DCs. Ligands for these receptors have yet to be identified, however, it has been postulated that cellular activation, stress, or tumor transformation may alter ligand expression because NCR ligands are expressed on both normal and tumor cells. Evidence supporting this notion is that NCRs play a role in killing xenogenic tumor cells and EBV-transformed tumor cells (24). In addition to KIR and NCR receptors the Ig-SF include the SLAM family of receptors, which will be discussed in detail in section IV.

C. NK Effector Functions

The two major effector functions of NK cells are cytotoxicity and cytokine secretion. Although, these functions are not unique to NK cells, the receptors and means by which NK cells mediate these functions are specific to NK cells.

i. Cytotoxicity

NK cells and cytotoxic T lymphocytes (CTLs) are highly effective killers of infected and transformed cells. Both cell types utilize the same secretory machinery for killing described in detail below; however, the receptors governing

the mechanisms that induce cytotoxicity differ. In contrast to CTLs, NK cells do not require antigen-specific recognition to kill target cells or prior exposure to the antigen, allowing for the rapid elimination of target cells prior to an adaptive immune response (review in (55)).

a. Granule Exocytosis Pathway

The granule exocytosis pathway involves the calcium dependent release of cytotoxic granules containing perforin and granzymes (56, 57). The use of perforin and granzyme knock out mice have shown that the granule exocytosis pathway is the principal mechanism used by NK cells to eliminate virus infected and transformed cells. Although the exact mechanism of lysis is unknown, the previous model of lysis suggested that perforin facilitated granzyme entry into target cells by forming pores in the target cell membrane. However, studies have demonstrated that this is most likely not the mechanism because granzymes are rapidly endocytosed by target cells independent of perforin (58-60). Moreover, perforin pores seen on electron micrographs may be too small (≤ 50 nm diameter) to act as channels for globular molecules like granzymes (61, 62). The current model is that perforin creates pores in the target cell membrane, transiently allowing Ca^{2+} into the cell. This Ca^{2+} flux triggers a wounded membrane repair response in which internal vesicles donate their membranes to reseal the damaged membrane. This also triggers rapid endocytosis of granzymes, which then play a

critical role in initiating apoptotic cell death either directly via the mitochondria, via the activation of cellular caspases, or by uncharacterized caspase-independent pathways (56, 63). Wülfing *et al* have detailed CTL-target cell and NK-target cell interactions using live cell video fluorescence microscopy (64, 65). Upon CTL target cell recognition, CTLs rapidly establish and invariably maintain cell polarity. Target cell lysis is a gradual process, the first sign of which is membrane blebbing. Generally, this process takes longer than 15 minutes. In contrast, NK cells, upon target recognition, lyse targets within 20 seconds. Wulfing *et al* have also shown that NK cells contain 50-fold more perforin compared to CTLs (64). In vitro studies have shown that a "sublytic" concentration of perforin delivers granzymes to induce apoptosis, a process that occurs slowly over hours, but causes little cell death on its own. At higher "lytic" concentrations, perforin induces necrotic death, but not apoptosis, independently of granzymes (63). Necrotic death occurs within minutes. Thus suggesting that the rapid killing mediated by NK cells may actually be necrotic cell death rather than apoptosis.

b. Death Receptor Pathway

The death receptor pathway is another pathway utilized by NK cells to mediate cytotoxicity. The tumor necrosis factor receptor family (TNFR) is composed of receptors containing cytoplasmic death domains that once engaged by their ligands activate caspase-signaling pathways leading to apoptosis (66).

This family includes Fas, TNF receptor 1 (TNFR1), TNFR2, TNF-receptor-related apoptosis-mediated protein (TRAMP), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), and TRAIL-R2 (66, 67) (68). NK cells can express at least three death receptor ligands: Fas ligand (FasL), TNF-α, and TNF-related apoptosis-inducing ligand (TRAIL), all of which induce apoptosis in their targets (69-72). Because Fas was the first TNFR family member linked to apoptotic activity, it has been the most studied (73, 74). Engagement of Fas by FasL induces apoptosis via triggering the caspase cascade in the Fas expressing cell (75). NK cells express significant levels of intracellular FasL (75). Engagement of NK activating receptors up-regulates surface FasL; however, co-engagement of an inhibitory receptor and an activating receptor abrogates FasL up-regulation (76). The Fas-FasL pathway is the principal signaling pathway used in activation induced cell death (AICD) where excess activated lymphocytes are removed at the end of an immune response (77, 78). Abrogation of this pathway leads to lymphoproliferative disorders as seen in Fas and FasL deficient mice (79-81). There is some evidence that the FasL and granular exocytosis pathways may work together; NK cells store newly synthesized FasL on the inner surface of the lytic granules that contain perforin and granzymes (82, 83). Thus, FasL and perforin are simultaneously delivered to the area of cell membrane in contact with the target cell.

Cytotoxicity is a critical function of NK cells. Thus it is not surprising that viruses and tumors cells have developed mechanisms to try and evade or inhibit the granular exocytosis or death receptor pathways.

ii. Cytokine Secretion

In addition to their cytotoxic function, NK cells can secrete several cytokines and chemokines including IFN-γ, tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, regulated on activation-normal T cell expressed and secreted (RANTES), IL-3, IL-10, and IL-13 (9-13, 84, 85). These cytokines play diverse roles in immune function. They activate NK cells and recruit neighboring lymphocytes, such as macrophages, to help clear infected cells, they modulate infected or transformed cells making them more susceptible to clearance via increasing MHC expression, and finally they can shape the adaptive immune response. NK cells are the major source of IFN-γ during the innate immune response (86). NK cells secrete IFN-γ in response to IL-12 and IL-18 produced by macrophages and DCs (87, 88). Although NK cells are generally regarded as a major source of IFN-γ, studies using a Listeria infection model have shown that CD8 T cells can also be an early source of IFN-γ in response to IL-12 and IL-18 (89). IFN-γ has several different functions (90), including anti-viral properties

where IFN-γ directly inhibits viral replication or disrupts viral infectivity. IFN-γ also up-regulates transcription of MHC class I, class II, and peptide transporter molecules thus increasing antigen presentation. IFN-γ secreted by NK cells activates macrophages, the major producers of IL-12, and recruits them to the site of infection where they function as effector cells and antigen-presenting cells (APC). IFN-γ is a key cytokine that directs T helper cells to differentiate into Th1 cells that carry out a cell-mediated immune response required for intracellular pathogen clearance. In fact, recent studies have shown that immunization with specific adjuvants recruited NK cells to antigen-stimulated lymph nodes where they provided an early source of IFN-γ necessary for Th1 polarization (91). NK cell secreted cytokines not only influence macrophage and T cell functions but also influence B cell switch recombination and differentiation (92). Therefore, NK cells function as critical players in the innate immune response, but also bridge the gap between innate and adaptive immunity via the cytokines they secrete. Thus, a dysregulation of NK function may alter the adaptive immune response and help set the stage for developing autoimmunity or disease.

D. NKT cells

NKT cells are a unique population of cells that contain characteristics of both the innate and adaptive immune system. NKT cells express NK cell markers (such as NK1.1 in the mouse and CD161 in the human) in addition to a

somatically rearranged semi-invariant TCR (Vα14-Jα18/Vβ8.2 chains in mouse and Vα24-Jα18/Vβ11 chains in humans) (reviewed in (93, 94)). This TCR recognizes glycolipid antigens presented by CD1d, a non-polymorphic surface protein related to classical MHC class I. CD1d knockout mice are devoid of NKT cells because CD1d is required for development. NKT cells are activated early in the immune response like NK cells and are capable of activating other cell types but in contrast to T cells lack immunological memory. Upon engagement of the NKT-TCR, NKT cells secrete large amounts of IFN-γ and IL-4. Early studies found that α-galactosylceramide (αGalCer), a marine-sponge-derived glycosphingolipid, could activate NKT cells. Recent studies have determined that an endogenous lysosomal glycosphingolipid, isoglobotrihexosylceramide (iGb3), presented by double positive thymocytes is required for intrathymic development of NKT cells. Thus, similar to conventional T cells, NKT cells are positively selected via recognition of a self-ligand in the thymus. This NKT-TCR also has high affinity recognition of pathogen-encoded ligands. In addition to development, iGb3 plays a critical role in the activation of NKT cells during infections with bacteria that contain lipopolysaccharide (LPS) in their cell wall. NKT cells are activated through iGb3 presented by LPS-activated DCs (95). A second mechanism of NKT activation is via direct recognition of glycosphingolipids from the cell wall of Gram-negative bacteria that lack LPS, such as Sphingomonas. The fact that NKT cells recognize both endogenous and

exogenous glycolipids suggests a potential role for NKT cells in controlling microbial infections as well as a potential for regulating or participating in autoimmunity.

III. T CELLS

A. Background and Immunobiology

T cells are a critical component of the adaptive immune response. α/β T cells make up the largest population of T cells (95-99%). While the remaining T cell population includes γ/δ T cells and NKT cells (1-5%) that develop from different pathways (2). α/β T cells can be further divided into two groups based on the class of MHC molecule the T cell recognizes: CD8+ T cells interact with MHC class I molecules and possess cytotoxic effector mechanisms for clearing intracellular pathogens termed cell mediated immunity. The second group of T cells is CD4+ T helper cells (Th), which interact with MHC class II molecules and are responsible for mediating humoral immunity and function in the clearance of extracellular pathogens. CD4+ Th cells can be subdivided into Th1 and Th2 cells based on the cytokines they secrete. Th1 cells secrete cytokines (IFN-γ and IL-12) that help CD8+ T cells to elicit cellular mediated immunity. In contrast, Th2 helper cells mediate extracellular and humoral immunity by direct T cell-B cell contact and IL-4, IL-5, IL-13, and IL-10 cytokine secretion (2). Effector functions of T cells will be discussed in the following section.

T cells arise from a pluripotent hematopoietic stem cell that develops in the bone marrow (2). The earliest cell in the T-lymphocyte lineage migrates from the bone marrow to the thymus, where T cell development and selection is completed. Newly immigrated T cell precursors migrate to the thymic cortex. Thymic stroma provides a physical microenvironment and produces necessary factors, such as stem cell factor (SCF) and IL-7 for early CD4-CD8- (double negative, DN) proliferation. T cells in IL-7 KO mice do not develop past that DN stage demonstrating the requirement of IL-7 for complete T cell development (96). Following expansion of the DN population, β and δ chain T cell receptor (TCR) rearrangement begins. If the δ chain productively rearranges first, T cells proceed down the default γ/δ T cell development pathway. If the β chain is rearranged to generate a functional TCR β, it complexes with the invariant pre-T-α chain and the invariant CD3 chains and is expressed on the cell surface. Formation of the pre-TCR and signaling through this receptor stops β-chain rearrangement ensuring that a single T cell only expresses one β chain, known as allelic exclusion. T cells that fail to undergo rearrangement die via apoptosis. Signaling through the pre-TCR triggers α chain rearrangement and co expression of the CD4 and CD8 coreceptors on the cell surface. Once a productively rearranged α chain associates with the β chain, the TCR and CD3 are expressed on the cell surface along with CD4 and CD8 marking the transition to the double

positive (CD4+CD8+, DP) stage. DP cells then undergo positive or negative selection by interacting with moderate affinity to self-MHC molecules on thymic epithelial cells. If DP cells do not interact with self-MHC, they die via apoptosis. If DP cells interact too strongly with self-MHC they also die by apoptosis (2).

How DP cells are finally committed to the CD4 or CD8 lineage is currently unknown. However, recent studies have shown that increased signal strength and/or duration of contact between the TCR and MHC can influence lineage commitment. For example, increased Lck activity in developing thymocytes favors development to the CD4 lineage (97). In addition to signal strength, regulation of specific transcriptional factors, such a GATA-3, has also been shown to play a role in lineage commitment (97). Once DP cell are positively selected based on MHC specificity, they down regulate either CD4 or CD8 and transition to the single positive stage (CD4+CD8-, or CD4-CD8+, SP). SP cells then undergo negative selection, where they are selected based on their recognition of self-antigen bound by MHC on thymic DCs. If the TCR strongly recognizes self-peptide bound by MHC, these cells are deleted via apoptosis or undergo T cell anergy. Alternatively, if the TCR does not recognize self-peptide and MHC, it dies via neglect. Only TCR that weakly recognize self are positively selected and allowed to migrate out into the periphery as naive mature T cells (2,

98). Defects in T cell development allow auto-reactive T cells to enter the periphery, which can lead to autoimmune disease states.

Once in the periphery and in primary and secondary lymphoid organs, TCR engagement by peptide-MHC complexes and co-stimulatory molecules on the cell surface of an APC results in the recruitment of Src-family kinases, such as Lck, to the cytoplasmic domain of the TCR. Lck phosphorylates the tyrosines in ITAMs of the TCR ζ- and CD3γ,δ, and ε chains, which then recruit and activate ZAP-70 (ζ-chain-associated protein kinase of 70 kDa) and PI3K. ZAP-70 subsequently phosphorylates the adaptor proteins LAT (linker for activation of T cells) and SLP-76 (SH2-domain containing leukocyte protein of 76 kDa), which work together to initiate several signaling cascades through PLC-γ, GRB2, and VAV. TCR signaling ultimately leads to Ca^{2+} mobilization and MAPK activation, which in turn activates downstream transcription factors, actin cytoskeleton regulation, effector functions, and proliferation (99). Defects in TCR signaling can alter T cell development and T helper cell differentiation processes resulting in defective immune responses to pathogens and disease development.

B. T cell Effector Functions

i. Cytotoxic T Lymphocytes (CTLs)

Cytotoxic T lymphocytes like NK cells circulate through the blood and lymphoid tissues searching for virus infected or transformed cells that need to be eliminated. As mentioned above, CTLs are αβ T cells that primarily co-express CD8; although, a small subset does express CD4. CTLs are a critical component of the adaptive immune response, and are responsible for killing cells infected with viruses or intracellular bacteria. These cells also play a role in clearing tumor cells. CTLs mediate these cytotoxic effects using the same cytotoxic machinery as NK cells: perforin, granzymes and the death receptor pathway. CTLs also release cytokines, such as IFN-γ and IL-12, that aid in pathogen clearance, influence the development of other effector cells, and further shape the adaptive immune response (2, 100, 101). However, the CTL response is delayed taking several days to weeks to respond to pathogens compared to NK cells, which take hours to days. Upon initial infection, very few CTLs express TCRs that recognize specific pathogenic determinants. Specific signaling pathways triggered by APCs allow for the clonal expansion of antigen specific CTLs and pathogen clearance. This response usually occurs around 7 days after initial infection. Once the pathogen has been cleared, the majority of the expanded clonal CTL population dies via AICD mechanisms. CTLs not eliminated via AICD generate a small pool of antigen-specific memory T cells that can rapidly respond to a secondary challenge with the specific pathogen.

ii. T Helper 1 Cells (Th1)

Naive CD4+ Th0 cells from the thymus differentiate into Th1 or Th2 cells. How uncommitted Th0 cells develop into mature Th1 or Th2 cells is an area of intensive investigation (102). It is currently known that exposure of Th0 cells to IFN-γ during TCR engagement leads to signal transducer and activator of transcription 1 (STAT 1) activation, which up-regulates T-box transcription factor (T-bet). T-bet regulates chromatin remodeling at the IFN-γ locus and enhances expression of the interleukin-12 receptor β2-subunit (IL12Rβ2). These Th1 cells can then be stimulated to produce IFN-γ through the TCR or combined IL-12 and IL-18 receptor engagement. The exact signaling pathways required for Th1 differentiation are currently undefined, but studies have implicated the involvement of TEC family kinases in T helper cell differentiation. RLK, resting lymphocyte kinase, a TEC family member, is preferentially expressed in Th1 cells compared to Th2 cells. Further supporting a role for RLK in Th1 differentiation is the fact that nuclear translocation of RLK is required for the induction of IFN-γ production (103). Alternatively, a number of other models have been proposed to explain T helper differentiation, for example antigen dose, co-stimulators, genetic modifiers and other non-cytokine factors have all been shown to have crucial roles in determining the differentiation of T helper cells (102). In addition to IFN-γ, Th1 cells produce IL-2 and lymphotoxin. Th1 cells participate in cell-mediated immune responses producing cytokines similar to CD8 T cells. Th1

cells secrete cytokines that up-regulate MHC class I, which helps to activate further the CTL response. Aberrant Th1 function, resulting in altered cytokine production or defective B cell help, can lead to the development of autoimmunity by skewing cytokine profiles leading to increased inflammation and mediating tissue damage (102), such as that observed in lupus and multiple sclerosis.

iii. T Helper 2 Cells (Th2)

In contrast to Th1 cells, naive Th0 cells stimulated through the TCR in the presence of IL-4 activates STAT6 signaling, which then rapidly up-regulates GATA-3 resulting in Th2 differentiation (102). GATA-3 is a Th2-lineage-specific transcription factor that can transactivate the *IL-5* promoter, and is involved in chromatin remodeling at the IL-4, IL-5, and IL-13 loci, similar to T-bet and the IFN-γ locus in Th1 cells. During Th2 differentiation, the Th2 cytokine cluster is positively regulated by GATA-3 leading to IL-4, IL-5, and IL-13 production. GATA-3 appears to work upstream of other Th2 specific transcription factors like cMaf which is also required for Th2 differentiation. In addition to GATA-3, cMaf can also transactivate the *IL-4* promoter. Th2 cells also produce IL-9 and IL-10. Similar to Th1 cells, Th2 differentiation may depend on the activation of specific signaling molecules, antigen dose, costimulatory signals, genetic modifiers, and other non-cytokine factors. As described for Th1 cells, one potential mechanism for Th2 differentiation is altered signaling by TEC kinases. The promoter region

of ITK (interlekin-2-inducible T cell kinase) contains GATA-3 binding sites (104). Further supporting the role of ITK in Th2 differentiation, ITK-deficient CD4+ T cells have decreased IL-4 production (104). The main function of Th2 cells is to secrete cytokines and provide B cell help to clear extracellular pathogens such as parasites, fungi, and extracellular bacteria. Th2 cells also play a critical role in directing B cell responses, which leads to plasma cell development and long-term B cell antibody production (2). Aberrant immune responses such as allergy and hypersensitivity are often associated with altered Th2 function.

As described above, NK, NKT, and T cell functions are regulated via signaling through cell surface receptors. Emerging studies have provided strong evidence that, in addition to the antigen receptors and other co-stimulatory molecules such as CD2 and CD28, the CD2/SLAM family of receptors play a crucial role in regulating many of these NK, NKT, and T cell effector functions. The following sections will discuss the CD2/SLAM family in the context of normal and aberrant NK, NKT and T cell function.

IV. CD2/SLAM FAMILY OF RECEPTORS

A. General Characteristics

The CD2/Signaling lymphocytic activation molecule (CD2/SLAM) family includes 11 family members: CD2 (E-rosette receptor, sheep red blood cell receptor, LFA-2, T11), CD58 (LFA-3), BLAME (B lymphocyte activator macrophage expressed, BCM-like membrane protein), SF2001 (CD2F-10), Ly108 (NTB-A, SF2000), CD84, CD150 (SLAM), CD48 (BCM1, Blast-1, OX-45), CS1 (19A24, CRACC), CD229 (Ly9), and CD244 (2B4) (reviewed in (105)). These genes map to two gene clusters in both humans and rodents; CD2, CD58 (human only), and BLAME map to chromosome 1p13 in humans and chromosome 3 in rodents ((106, 107). The SLAM subset of receptors (CD150 (108), CD244 (109), CD84 (110), CD299 (111), Ly108 (112), CS1 (113), and CD48 (114)) map to 1q21-24 in humans and are present in a syntenic region on chromosome 1 in rodents. This family of receptors is expressed on cells of hematopoietic origin.

All CD2/SF family members have a common protein structure. They contain an extracellular signal sequence followed by a variable-Ig (V-Ig) domain, a constant-2-Ig (C2-Ig) domain, a transmembrane domain, and most SF family members contain an intracellular signaling domain. The N–terminal V-Ig domain lacks disulfide bonds and serves as the ligand-binding domain. The C-terminal C2-Ig domain contains at least two intra-disulfide bonds and is truncated. These domains are duplicated in CD299 (111)(See figure 1(115)). SF family members are highly N-linked and O-linked glycosylated proteins, which may play a role in

ligand binding (reviewed in (105, 116)). The CD2/SLAM family of receptors exhibit either homotypic binding or SLAM/CD2 intrafamilial receptor-ligand binding. SLAM (117), CD299 (118), Ly108 (119, 120), CD84 (121), and CS1 (122) bind homotypically; while CD244-CD48 and CD2-CD48 exhibit intrafamilial binding (123, 124). Several of the CD2/SF receptors also recognize pathogens. For instance, SLAM is a receptor for measles virus (125), and CD48 recognizes FimH, a bacterial lectin (126). SLAM family expression, ligand binding, and function are summarized in table 1(127).

Most CD2/SLAM family members contain cytoplasmic signaling domains, with the exception of CD48 and CD58, which are GPI linked, and BLAME and CD84H1 which have a short cytoplasmic domain (106, 114, 115, 128) The remaining CD2/SF family members all contain one or more immunoreceptor tyrosine-based switch motifs (ITSM) that are composed of the consensus sequence TxYxxV/I. These ITSM motifs bind SH2 domain containing proteins, such as SLAM-associated protein (SAP), Eat-2a, Eat-2b, SHP-1, SHP-2, and SHIP. Eat-2a/b, SHP-1/2, and SHIP can mediate inhibitory signals, while SAP mediates an activating signal through its recruitment of FynT (reviewed in (115, 127, 129, 130)). Several of the SF members are found in signaling molecule-rich GEM (glycolipid enriched microdomains) domains, further supporting their role as important immune signaling receptors ((118, 131) and Schatzle unpublished

observation). It has been shown that SAP, Eat-2a, and Eat-2b association with SF receptors requires ITSM phosphorylation, with the exception of SAP association with SLAM (132, 133). Recently a novel mechanism of SAP adaptor function has been identified where the SAP SH2 domain binds the SH3 domain of FynT, which is then recruited to the SF receptor cytoplasmic domain to initiate signaling through SAP (134, 135). A critical argine residue at position 78 in SAP (134) mediates this SAP-FynT association. In contrast, SAP family members, Eat-2a and Eat-2b do not recruit FynT, nor do they contain a similar FynT binding motif; however, they may recruit an unidentified kinase (Calpe et al. in press). Recently Eat-2a and Eat-2b KO mice have revealed a negative role for Eat-2a/b in NK cytotoxicity. Eat-2a KO mice showed increased cytotoxicity and IFN-γ production; Eat-2b KO mice have a similar but less pronounced phenotype (136). Signaling though the SF receptors results in a variety of cellular functions such as cytokine secretion, apoptosis, cellular activation, proliferation, antibody production, and cytotoxicity. Thus, it is not surprising that dysregulation of this family of receptors is associated with several disease states which will be discussed below.

B. CD2/SLAM Family Association with Disease States

i. X-linked lymphoproliferative disease

X-linked lymphoproliferative disease (XLP) was originally called Duncan's disease after the family it was first identified in, where six male members of this family died of a progressive combined variable immunodeficiency associated with the aberrant proliferation of lymphocytes ((137) reviewed in (115)). Later, the disease was identified as an inherited X-linked disorder, which mapped to a single locus on the X-chromosome at band Xq25 and was renamed XLP (138, 139). XLP affects one to three out of every million male individuals and is fatal with 70% of patients dying by the age of 10 (140-142).

Although, there is great variability in XLP disease, there are three common clinical features: fulminant infectious mononucleosis (FIM), lymphoproliferative disorders, and dysgammaglobulinemia (142). These disease states are often triggered following Epstein-Barr virus (EBV) infection. FIM is the most common clinical manifestation of XLP affecting approximately 60% of patients, where patients die from end organ failure due to uncontrolled EBV reactive T cell and EBV infected B cell proliferation and defective NK clearance of infected cells. (115). Interestingly, recent studies have shown that 40% of XLP patients never develop symptoms of FIM, despite evidence of EBV infection (141). Lymphoproliferative disorders, both malignant and non-malignant, affect 20-30% of XLP patients and occur at rates approximately 200 times greater than

that in the general population (115, 143). These disorders are mostly lymphomas of B-cell immunophenotype, usually of the Burkitt type (144). XLP patients positive for EBV infection have a poorer prognosis and median survival rate versus EBV negative patients (141). Currently, bone marrow transplantation is the only known treatment for these clinical manifestations of XLP. Finally, dysgammaglobulinemia affects 30% of XLP patients. Patients exhibit global reductions in the levels of serum Igs; the levels of IgG1 and IgG3 are reduced with increased IgA and IgM (145). Patients with dysgammaglobulinemia have the most favorable prognosis especially if treated with regular Ig infusions.

The gene defective in XLP was identified by three different groups as SAP (a.k.a. SH2D1A and DSHP) using positional cloning and functional/biochemical approaches (133, 146, 147). In addition to XLP, mutations in SAP have also been found in some common variable immunodeficiency disease (CVID) patients (148). SAP consists of four exons spanning approximately 25 kb and encodes a 15 kDa cytoplasmic protein that contains a single SH2 domain with a 28 amino acid tail (147, 149). As described above, the SH2 domain of SAP binds the phosphorylated ITSMs of SF receptors to transduce cellular phospho-dependent signals. SAP is expressed in NK cells, CD4+ and CD8+ T cells, thymocytes and NKT cells (150, 151). It is also expressed in some germinal center and memory B cells (152, 153). A variety of point mutations and/or truncations in SAP have

been found in XLP (154) that result in a complete lack of SAP or non-functional SAP expression. Thus, defective SLAM family receptor function has been associated with XLP, specifically defects in CD244 and Ly108 function (155-159). The role of CD244 in XLP will be discussed in detail in section VI. To study further the role of defective SF receptor signaling in XLP, SAP KO mice have been generated. SAP KO mice show phenotypes similar to that observed in XLP and will be discussed in greater detail in the following section on mouse models of human disease.

ii. Systemic Lupus Erythematosus (SLE)

SF receptor signaling plays a role in several lymphocyte functions such a cytokine secretion, Ig production, proliferation, apoptosis, and cytotoxicity. Defects in all of these functions have been associated with SLE disease. SLE is a chronic autoimmune disorder caused by the production of pathogenic autoantibodies to a spectrum of nuclear antigens that leads to end organ failure. SLE is a polygenic disease that can be influenced by environmental factors (reviewed in (160)). The clinical manifestations of SLE are varied and generally a patient is diagnosed with SLE if they present with multiple symptoms from the classification criteria for both SLE and antiphospholipid syndrome (APS) (161). It is estimated that at least five million people worldwide have SLE. SLE shows a strong female:male gender bias of 9:1 (160). Although there are a variety of

murine models of lupus, studies using the spontaneous NZM2410 model have identified several causative disease loci that are syntenic to human SLE disease loci. The genetic dissection and fine mapping of the NZM2410 mouse model of lupus has identified the Sle1b locus as being responsible for autoantibody production (162, 163). The Sle1b locus is syntenic with genomic intervals associated with susceptibility to SLE in human linkage studies (164, 165). Interestingly, the SF gene cluster maps to the center of the Sle1b locus (166). Wandstrat et al show that extensive polymorphic differences and expression level differences in SF receptors exist between non-SLE prone and SLE prone mice implicating SF receptor involvement in the development of autoantibodies (166). The use of lupus mouse models to study SF receptor function in the context of SLE will be discussed in section V.

iii. Multiple Sclerosis (MS)

Multiple sclerosis is a chronic inflammatory, demyelinating disease of the central nervous system with varied clinical presentations that range from clearly defined relapses and remissions to slow accumulation of disability with or without exacerbations (167). Similar to SLE, MS is a polygenic disease also influenced by environmental factors (167). Studies have linked SLAM to MS immune dysregulation showing that increased IFN-γ production and skewed Th1 and Th2 cytokine production correlated with increased numbers of SLAM+ CD4+ T cells

(168) in both acute and stable MS patients compared to healthy controls. In addition, Valdez et al identified Ly108 as playing a critical role in MS disease progression using the experimental autoimmune encephalomyelitis (EAE) mouse model of MS (169). These studies showed that EAE disease progression was delayed and disease severity dampened in mice transgenic for Myelin Basic Protein (MBP) following treatment with Ly108-Fc fusion protein, whereas control fusion protein had no effect of EAE (169). All together, these studies indicate further that SF receptor signaling is critical for proper immune function.

V. MOUSE MODELS OF HUMAN DISEASE RELATED TO THE CD2/SLAM FAMILY

A. Systemic Lupus Erythematosus and B6.*Sle1b*

The CD2/SLAM family has been tightly linked to human SLE by genetic susceptibility linkages studies (LOD≥ 3.3), but studies of human SLE are very difficult due to the genetic and environmental complexities of human SLE (165, 170). There are several spontaneous mouse models of murine lupus, which include MRL.lpr, BXSB.yaa, [NZB x NZW]F_1 (BWF_1), and NZM2410. The MRL.lpr and BXSB.yaa mouse models of lupus stem from mutations in single genes. In contrast the [NZB x NZW]F_1 (BWF_1), and NZM2410 mouse models of lupus are complex models involving multiple genetic loci. [NZB x NZW]F_1 (BWF_1), and NZM2410 more closely resemble what is observed in human SLE.

A portion of this dissertation will be focused on the NZM2410 model because genetic dissection of NZM2410 disease susceptibility has directly implicated the SLAM family in disease pathology.

Unlike lpr and *yaa,* no single gene mutation is responsible for full disease progression in the [NZB x NZW]F_1 (BWF_1) mouse model. BWF_1 mice develop glomerulonephritis, and increased anti-nuclear antigen specific IgG autoantibodies. This mouse model most closely resembles human lupus in both disease phenotype and genetic complexity. Neither parental strain develops lupus but each does get late-onset mild autoimmunity demonstrating that full disease requires genetic interactions of both genomes (171). Backcrossing BWF_1 mice onto both autoimmune and non-autoimmune strains (Balb.$H2^z$ and B6.$H2^z$) have identified loci of susceptibility on chromosomes 1 and 4 (172). Chromosome 1, containing the SLAM family locus, contributes to nephritis, hypergammaglobulinema, autoantibodies, and polyclonal B cell activation phenotypes (172-174).

The NZM mouse strain was derived from [NZB X NZW]F_1 X NZW backcross progeny and was termed the New Zealand Mixed (NZM) strain. This mouse strain was developed because the heterogenic genomic complexity of the BWF_1 lupus model made it difficult to study disease susceptibility. Thus a series

of 27 backcross NZM mouse strains were made; the NZM2410 strain developed lupus like phenotypes very similar to BWF_1 mice including glomerulonephritis and anti-dsDNA autoantibodies (175). Backcrossing the NZM2410 mouse onto the C57BL/6 background identified three chromosomal intervals containing strong recessive GN susceptibility alleles on chromosomes 1, 4, and 7 termed *Sle1*, *Sle2* and *Sle3* respectively (175). In addition, heterozygosity at the H-2 locus was also found to correlate strongly with GN susceptibility, and this locus was termed *Sle4* (175). To study further the contribution of each of these loci in SLE autoimmune phenotypes, individual loci were intercrossed onto the B6 background and studied as B6.*Sle* strains.

B6.*Sle1* mice exhibit a loss of tolerance to chromatin in both T and B cell compartments. In addition, these mice spontaneously produce IgG autoantibodies specific for subnucleosome components of chromatin and develop spontaneous autoreactive T cells responding to histone epitopes (176-178). Adoptive transfer experiments showed that *Sle1* is functionally expressed in B cells, and that expression in B cells is essential to break tolerance to nuclear autoantigens and develop humoral autoimmunity (178). Further experiments using bone marrow chimeras identified that both B and T cells of the *Sle1*genotype have intrinsic defects. B cells have a loss of tolerance to ssDNA and chromatin resulting in increased IgM ssDNA antibodies and anti-chromatin antibodies in addition to

increased activation marker expression and total IgM serum levels, all of which are independent of T cells (179). *Sle1*-specific defects in T cells include increased activation marker expression and cytokine production, both of which are independent of B cell interactions (179).

Fine mapping of the *Sle1* locus identified four loci, termed *Sle1a, Sle1b, Sle1c*, and *Sle1d* (162). All of these loci, with the exception of *Sle1d*, are associated with varying degrees of anti-chromatin autoantibody production. However, each loci contributes to different serological and lymphocyte distribution and activation phenotypes. Although each locus plays a role in disease, the contribution of *Sle1b* is the strongest with its penetrance being even greater than that of the whole *Sle1* interval (57% in females and 34% in males) (162).

Subsequent fine mapping and sequencing of the *Sle1b* locus has associated extensive polymorphisms in the CD2/SLAM family of genes with autoimmunity in B6.*Sle1b* mice (166). As described above, the CD2/SLAM family is expressed in various immune cell lineages and plays an important role in regulating many lymphocyte functions. Phenotypic analysis has revealed variations in the structure and expression of several members of the SLAM family in T and B lymphocytes from B6.*Sle1b* mice compared to B6 mice (166).

Sequence analysis of the extracellular Ig regions and SNPs flanking the SLAM family of 34 inbred mouse strains identified two SLAM family haplotypes (*b* and *z*). The most prevalent haplotype, the *z* haplotype, is found in both non-autoimmune and autoimmune laboratory mouse strains. Whereas, the less common haplotype, the *b* haplotype, is found in C57-derived mouse strains (166). The presence of the *z* haplotype in the context of the C57BL/6 genetic background results in the development of autoimmunity, specifically ANA production. However, studies by Subramanian *et al* have shown that *Sles 1*, an epistatic suppressive modifier, prevents T and B cell activation phenotypes and autoimmunity in B6.*Sle1Sles1* mice (180). These studies suggest that epistatic modifiers can alter SF function thus preventing autoimmunity. Because the *z* haplotype appears to be the common murine allele, it would be advantageous to also inherit these modifiers preventing autoimmunity in a specific genetic context. Further work has revealed that both haplotypes are being maintained in natural, outbred mouse populations and appear to be under balancing selection, supporting the idea that common variants can mediate autoimmunity in specific genomic contexts (N. Limaye et al., unpublished observations). Interestingly, several of the immune phenotypes observed in B6.*Sle1b* mice are similar to those of XLP patients and are recapitulated in SAP KO mice, discussed below.

B. X-linked lymphoproliferative disease and SAP KO mice

XLP, as described in section IV B, is a genetic disease characterized by defects in the SAP gene. Three groups have generated SAP KO mice by targeted disruption of the SAP gene (181-183). Upon initial examination, T, NK and B cell development appeared normal in SAP KO mice. Defects in EBV clearance are often associated with XLP; however, EBV does not infect mice. Therefore, two different virus models, murine gammaherpesvirus-68 (MγHV-68) and lymphocyte choriomeningitis virus (LCMV), with some similar pathological features to EBV have been used to challenge these SAP KO mice. LCMV elicits a strong CD8+ CTL response (182). Two models of LCMV infection have been characterized. An acute infection mediated by the Armstrong strain of LCMV and a chronic infection mediated by the clone 13 and WE strains of LCMV. MγHV-68 is a naturally occurring herpes virus of rodents and is genetically related to EBV. Both EBV and MγHV-68 are characterized by the establishment of life-long latency in B lymphocytes, and primary infection of MγHV-68 results in lymphoproliferation, similar to infectious mononucleosis (183).

Infection with the acute Armstrong strain of LCMV has shown that SAP KO mice clear the virus similar to wild type mice, but have elevated numbers of CD4 and CD8 virus specific T cells. In addition to elevated T cell numbers, these T cells produce increased amounts of IFN-γ and TNF-α compared to wild type

mice (181, 182). This increase in T cell numbers is similar to phenotypes observed in XLP. XLP is also associated with end organ failure due to uncontrolled T cell proliferation. This phenotype is also observed in SAP KO mice infected with the chronic LCMV strains, Clone 13 and WE, where nearly 100% of SAP KO mice die from uncontrolled proliferation of LCMV responsive T and infected B cells compared to 30% mortality in wild type mice (181, 182). SAP KO mice infected with MγHV-68 clear infection similar to wild type mice, but show increased numbers of CD8 virus-specific T cells and increased T cell tissue infiltration and damage (183). Additionally, Chen et al have shown that SAP is a negative regulator of CD8+ T cell MγHV-68 specific responses (184). Thus, chronic viral infection in SAP KO mice is the result of defective CD8+ T cell responses, T helper cytokine production, and defects in antibody production, similar to XLP patients. These SAP KO studies of virus infection mimic what has been shown in XLP, where patients appear healthy until infected with EBV.

Two of the groups that generated SAP KO mice have shown that SAP KO T cells intrinsically have the ability to differentiate into Th1 and Th2 cells under strong polarizing conditions (182, 185). However upon stimulation by anti-CD3 and CD28 mAb, SAP KO CD4+ T cells produced increased amounts of IFN-γ and had profound defects in IL-4 production, showing a Th1 bias. To determine if this Th1 biased cytokine profile altered the ability of these mice to clear

intracellular parasites, Leishmania major was used to challenge SAP KO mice. The susceptibility of Balb/c mice to this parasite is due to a rapid response by CD4+ T cells and NKT cells to produce large amounts of IL-4 (181). SAP KO mice on the Balb/c background challenged with L. *major* were more resistant then wild type Balb/c mice, showing lower parasite burdens. In addition, lymph nodes from L. *major*-infected SAP KO mice produced less IL-4, 10, and 13. These cytokine defects correlate with decreased levels of IgG subclasses associated with IL-4 mediated switch, and decreased IgE levels (181-183). These SAP KO phenotypes of decreased IL-4, 10, 13 and decreased IgE and IgG subclasses mimic those observed in XLP patients. T cell studies in XLP patients have shown the CD4+ T cells are unable to differentiate into IL-10+ effector cells and thus are defective in their ability to provide B cell help (186).

The generation of SAP KO mice have allowed for detailed T cell signaling and functional studies to determine the mechanisms underlying XLP disease. Such studies using SAP KO mice have revealed that SAP is required for the phosphorylation of ITSMs in the cytoplasmic signaling domain of SLAM (134, 135). These studies detailed the specific recruitment of FynT by SAP through an argine residue at position 78 in the SH2 domain of SAP interacting with the SH3 domain of FynT. Although SAP recruits FynT through its SH2 domain, this interaction has no effect on SAP-SH2 binding to the ITSMs of SLAM.

Mutational analysis of SAP in XLP patients has shown that most mutations disrupt the ability of SAP to bind SLAM or alter protein stability reducing the half-life of SAP. No known mutations altering the SAP-FynT interaction have been found in XLP patients (135). SAP binding of the FynT-SH3 domain competes with the FynT-SH3 and FynT-kinase domain interaction responsible for auto-inhibition of FynT activity (135). Transgenic mice over expressing a R78A SAP mutant show no decrease in IFN-γ and proliferation compared to SAP KO mice, but show a decrease in IL-4 and IL-13 production (134, 187). Thus, FynT is also required for Th2 cytokines and not IFN-γ secretion. In contrast, over expression of SAP results in increased IL-4 and decreased IFN-γ production (opposite of what is observed in SAP KO mice) (134). This implicates further a role for SAP signaling in T cell cytokine production.

Studies have also focused on the role of SAP as a regulator of T cell differentiation to Th1 and Th2 subsets. Cannons et al showed that the defect in IL-4 production of SAP KO T cells is not a result of the increased IFN-γ production observed in these mice (185). However, TCR engagement of CD4+ T cells from SAP KO mice showed a marked defect in the induction of GATA-3; GATA-3 is a transcription factor considered to be the master regulator of Th2 differentiation (102, 185, 187). CD4+ T cells of SAP KO mice cultured in Th2 polarizing conditions up regulated GATA-3 similar to wild type T cells,

suggesting that the defect in GATA-3 up regulation is specific to TCR-mediated signaling (185, 187). Furthermore, retroviral expression of GATA-3 in SAP KO T cells restores IL-4 production but does not alter the increase in IFN-γ production from these T cells, demonstrating that the SAP KO T cells retain the Th1 bias. Based on the observation that altered TCR-mediated signaling resulted in decreased GATA-3 activation, a more detailed examination of T cell signaling was done. A major outcome of TCR signaling is actin cytoskeleton polarization to the site of TCR stimulation; PKC-θ is know to localize to the T cell synapse and has a role in IL-4 production (188). Examination of the TCR synapse in SAP KO and wild type mice indicated that SAP KO T cells have decreased PKC-θ recruitment to the TCR synapse and decreased lipid raft localization. PKC-θ is required for Bcl-10 activation and recruitment. Thus, it is not surprising that decreased PKC-θ localization at the synapse leads to impaired Bcl-10 recruitment and ultimately impaired IKBα degradation in SAP KO CD4+ T cells (185). Similar defects in T cell signaling are observed in Fyn KO mice. Thus suggesting that the SAP-FynT signaling pathway regulates Th2 differentiation in CD4+ T cells (185, 187). These data examined in the context of what is known about Th1 and Th2 differentiation indicate that the SF plays a role in T helper cell differentiation.

The SAP KO mice also presented altered B cell functions when challenged with pathogens or immunized. Characterization of short term B cell function following LCMV infection in SAP KO mice showed a similar number of antibody secreting cells (ASCs) during the acute viral response compared to wild type mice (189). T-independent immune responses in SAP KO mice were also normal as determined by immunization with TNP-Ficoll (190). In contrast, immunization of SAP KO mice with T-dependent antigens, TNP-CGG and KLH, identified a defect in T cell help resulting in decreased T-dependent antibody responses and germinal center formation (190, 191). Following KLH immunization, SAP KO mice have decreased IgM and IgG responses compared to wild type mice and impaired secondary IgG responses (191). Characterization of long term B cell function following LCMV infection showed a near complete absence of virus-specific long-lived plasma cells (189). Adoptive transfer experiments determined that SAP KO B cells are normal and that the inability of SAP KO mice to establish long-term humoral responses is due to defects in CD4+ T cells (189). Interestingly, this same defect in CD4+ T cell help protects SAP KO animals from developing lupus in the pristane-induced lupus model (190). Similarly, in vitro stimulation of B cells from XLP patients showed similar proliferation and activation phenotypes to that of B cells from healthy individuals. However, in vivo studies have shown that the number of memory B cells in XLP

patients is significantly reduced and the memory B cells XLP patients do have are IgM+ showing a defect in isotype switching (186).

Subsequent B cell studies showed that SAP is expressed in some B cell populations, and similar to T cells SAP expression in B cells decreases following B cell stimulation (192). SAP KO B cells have decreased IgG1, IgG2a, IgG2b, and IgG3 levels compared to wild type B cells, which corresponds with decreased Iα, Iγ2a, Iγ2b, and Iγ3 transcript levels and impaired class switch recombination. These decreases in IgG cannot be explained by differential activation-induced cytidine deaminase (AID) or T-bet expression (192). Morra *et al* performed a detailed characterization of the B cell compartment in the lymphoid organs of SAP KO mice (191) and showed that SAP KO mice have decreased numbers of T2, follicular B cells, that are found in germinal centers, giving insight into why SAP KO mice have decreased germinal center formation. The presence of wild type T cells can not correct SAP KO B cell defects in this model (191). The defects in B cell antibody production observed in SAP KO mice give insight into the mechanisms involved in dysgammaglobulenemia development in XLP patients.

Interestingly, a previously underappreciated role for SAP in NKT development has been observed in SAP KO mice, which are devoid of NKT cells.

Injection of the NKT cell-specific agonist α-galactosyl ceramide (αGC) into SAP KO mice induced no IL-4 or IFN-γ secretion compared to wild type mice (193, 194). This observation revealed that SAP KO mice have no NKT cells, while Fyn KO mice show a decrease in NKT numbers. As a result of the lack of NKT cells, NKT dependent antigen-specific CTL responses are impaired or absent in SAP KO mice (194). A lack of CD1d expression, the ligand for the invariant chain of NKT cells that presents glycolipid antigens required for NKT development, does not explain the SAP KO NKT defect, because SAP and WT mice have equivalent CD1d expression (193). However, NKT development requires cell-cell contact between DP thymocytes. Therefore, SF receptors may play a critical role in these interactions, so altered SF signaling in SAP KO mice could ablate NKT development. Thus, SAP expression and potentially SF signals are critical for NKT development. As expected, XLP patients have a similar NKT cell deficiency (193, 194).

SAP KO mice have provided important insight into the role of SAP in normal and aberrant immune function. The generation of specific SLAM family member knockout mice has expanded our understanding of the role each SLAM family member plays in normal and altered immune function. The following SLAM family member knockout mice have been generated and their phenotypes

will be discussed briefly: CD48 (195), CD244 (196), SLAM (197), and Ly108 (198).

CD48 KO mice have a slight increase in CD4+CD8- thymocytes and increased CD2 expression in thymocytes and spleen cells. CD48 KO CD4+ T cells have severely impaired activation. T cell activation is diminished in response to lectins, anti-CD3 mAb, and alloantigens in CD48 KO mice (195). CD48 KO T cells have decreased IL-2 production and proliferation responses in MLR experiments compared to wild type mice. APCs from CD48 KO mice also have a decreased ability to mediate allotypic T cell proliferation (195). These defects may be important in the context of CD48-CD244 interactions between lymphocytes, an interaction that is defective in XLP.

CD244 KO mice have normal gross lymphocyte development. However, infection of CD244 KO mice with the chronic LCMV strain, clone 13, results in an increased number of CD8+ T cells, increased numbers of IFN-γ producing cells, and splenomegaly (S. Stepp personal communication, abstract #T46.86 FOCIS 2004). Furthermore, B16 tumor challenge of these mice revealed a gender specific and CD48-independent defect in melanoma clearance, where female mice poorly reject tumors compared to males (196). In contrast, male CD244 KO mice exhibited an enhanced ability to reject CD48+ tumors compared to wild type

mice. CD244 KO mice also exhibit altered responses in bone marrow transplants, where CD244 KO mice show increased rejection of β2m KO BM compared to B6 mice, indicating that the CD244-CD48 interaction protects autologous cells from NK-cell rejection in the absence of self-MHC class I (199). In addition to altered responses to LCMV infection and B16 tumor challenge, NK cells from CD244 KO mice are defective in B cell stimulated IL-13 production by NK cells (D. Yuan unpublished observation).

SLAM KO mice have no defects in NK, T, and B cell development. However, SLAM KO CD8+ T cells have increased IFN-γ secretion, and CD4+ T cells from SLAM KO mice stimulated via the TCR are reported to have decreased IL-4 and increased IFN-γ production (197). However, this CD4+ T cell phenotype may be the result of the genetic background used to generate these mice. As discussed in chapter five, the presence of the *z* haplotype of the SLAM family on a B6 genetic background results in decreased IL-4 production from CD4+ T cells suggesting that SLAM KO mice have decreased IL-4 due to the genetic background the mice are derived from and not due to the lack of SLAM. Macrophages from SLAM KO mice are defective in IL-12, TNF-α, and nitric oxide production following LPS stimulation. In addition, SLAM KO macrophages have increased IL-6 production, implicating SLAM as a positive regulator of IL-12 production and a negative regulator of IL-6. As a result of

defective macrophage function, SLAM KO mice have impaired clearance of Leishmania major (197).

Ly108 KO mice have normal lymphocyte development. CD4+ T cells from Ly108 KO mice stimulated in vitro by anti-CD3 and CD28 mAb or PMA and Ionomycin have decreased IL-4 production. Similarly, in vivo challenge with Leishmania *mexicana* also showed decreased IL-4 production in Ly108 KO mice. As described for SLAM KO mice, Ly108 mice were generated in the 129 background and backcrossed to B6 mice, therefore this decrease in IL-4 production could be due to the genetic background and not the absence of Ly108. In addition to T cell defects, Ly108 KO mice have impaired neutrophil functions, thus increasing their susceptibility to Salmonella *typhimurium*. The aberrant bactericidal activity of Ly108 KO neutrophils is a consequence of severely reduced production of reactive oxygen species following phagocytosis of bacteria (198). Ly108 KO mice infected with S. *typhimurium* have enhanced serum levels of IL-6, IL-12, and TNF-α and mortality compared to wild type mice (198).

As can be seen from the diverse phenotypes of SLAM family knockout mice, the global disruption of this family by altering SF signaling interferes with a plethora of immune functions resulting in devastating disease states like those observed in XLP and SLE. As expected, most SF KO mice have phenotypes of

altered cytokine profiles and defective effector functions resulting in impaired pathogen clearance, very similar to what has been described in disease states associated with the SAP deficiencies.

VI. CD244 (2B4)

Early XLP studies implicated defective CD244 function as the potential cause of disease. These observations lead to an extensive study of CD244 in immune function, which is the focus of the following section.

A. Overview

As described in section IV, CD244 is a member of the CD2/SLAM family found on chromosome 1 in both humans and rodents (116, 200). The counter-receptor for CD244 was identified as CD48, which is expressed on all hematopoietic cells (123, 124). Human CD244 is expressed on NK cells, γδ T cells, ~50% of CD8+ T cells, and on subsets of basophils, monocytes, and eosinophils (201-205). In mice, CD244 is expressed on all NK cells and subsets of CD8+ αβ T cells, γδ T cells, monocytes, and mast cells (109, 200, 206-208). CD244 expression on CD8+ T cells is restricted to memory and activated populations and can be acquired following cytokine stimulation in vitro or during viral infection in vivo (208, 209). As described previously, the SF of receptors

exists as one of two stable haplotypes in common lab strains of mice (*b* or *z* haplotype). The *b* allele of CD244, found in all C57 derived lab strains of mice, exists as a single gene, which can be differentially spliced to encode either a long or short isoform that differ only in their cytoplasmic domains (see figure 1.2) (166, 210). The CD244 gene locus of the *z* haplotype is expanded compared to the b haplotype and contains 4 genes (166). Sequence comparison of the first exon of the extracellular domains of these genes suggests that 3 genes encode functional products, while one contains a premature stop codon in the V-Ig domain and thus a truncated or nonfunctional product (166). In humans, CD244 is found as a single gene and a single isoform with a molecular weight of 63-70 kDa similar to the C57BL/6 CD244L isoform (202, 203, 211). A current understanding of CD244 function in humans and rodents will be discussed below.

B. CD244 Function in mice

CD244 was first identified as a marker for non-MHC-restricted cytotoxic NK and T cell populations in C57BL/6 mice (*b* allele). NK and T cell populations were shown to readily kill hybridoma cells making anti-CD244 mAb (109). Subsequent studies using redirected lysis assays showed that in the presence of anti-CD244 mAb, cultured NK cells and non-MHC restricted T cells had increased lytic activity. The addition of anti-CD244 mAb to IL-2 activated NK cells induced IFN-γ secretion and granule exocytosis, suggesting that CD244

functions as an activating receptor (109). Kubota *et al* also described an activating function for CD244 in NK cells generated from C3H/He mice (*z* allele) (212). As mentioned above, the *b* allele of CD244 can be expressed as a long isoform, containing four ITSMs, or a short isoform containing one ITSM (210). Exogenous expression of these isoforms in RNK-16 cells defined an activating function for CD244 short and an inhibitory function for CD244 long (213). The long form is the predominant isoform found in IL-2 C57BL/6 LAK cultures, which will be discussed in chapter three. Although the early studies by Garni-Wagner *et al* and Kubota *et al* described an activating role for CD244, at that time the ligand for CD244, CD48, was not known. Recent studies taking into account CD48 expression on target cells have described an inhibitory role for murine CD244 (214, 215) showing that CD244 engagement via CD48 expression on target cells inhibits NK cytotoxicity and IFN-γ. This inhibition can be alleviated by the addition of blocking antibodies, which will be discussed in detail in chapter three. Interestingly, the CD244-CD48 interaction may play a role in NK cell self-tolerance. CD244 is expressed on NK cells early in development and prior to expression of inhibitory MHC receptors (3, 216). A population of NK cells exist that does not express self inhibitory MHC receptors, yet they still remain self-tolerant. McNerney et al have shown that NK cells from β2m-deficient mice and NK cells that lack expression of self-MHC binding inhibitory receptors are inhibited by CD244 (199). In addition, CD244 KO mice showed increased lysis of

CD48+ targets compared to wild type mice further suggesting an inhibitory role for the *b* allele of CD244 (196, 214, 215). Although blocking CD244-CD48 interactions may explain the earlier reports of activating function for CD244, a complete explanation for these discrepancies remains to be determined.

Resent studies have shown that SAP recruits FynT to the cytoplasmic domain of CD244 similar to what is observed for SLAM (217). SAP KO mice have decreased CD244-mediated cytotoxicity and IFN-γ production, suggesting that SAP mediates an activating signal. Similarly, FynT KO mice also have defective CD244 cytotoxicity and IFN-γ production (218). In contrast, mice deficient in Eat-2a or Eat-2b expression have increased CD244 mediated cytotoxicity and IFN-γ production, suggesting that Eat-2a and Eat-2b are negative regulators of CD244 function (136). These data suggest that CD244 function is regulated by signaling through adaptor molecules, where SAP leads to an activating signal and Eat2a/b leads to an inhibitory signal.

The CD244-CD48 interaction is not only critical for NK cell effector functions on mature NK cells, but also is important for NK cell development. NK-NK cell CD244-CD48 interactions are necessary for the development of full lytic function and cytokine production (219). CD48 binds CD2 as well as CD244, yet only the CD244 interaction is required for full lytic function. Blocking

CD244-CD48, but not CD2-CD48 interactions, among NK cells impaired NK cytotoxicity and IFN-γ secretion. Moreover, CD244 KO and CD48 KO mice have reduced cytotoxicity compared to wild type mice (219). Thus, the CD244-CD48 interaction acts as a co-stimulatory signal between NK-NK cell interactions in addition to mediating NK cell effector function.

The co-stimulatory function of CD244-CD48 interactions is not limited to interactions between NK cells but also affects NK interactions with T and B cells, as well as interactions between T cells (reviewed in (220)). Engagement of CD48 on T cells by CD244 on NK cells enhances T cell proliferation, an increase that can be seen with as few as one NK cell per 16 T cells (221). Co-culture of NK cells with T cells also increases T cell activation characterized by increased CD69 expression (221). It has also been shown that CD244 ligation on CD8+ T cells by CD48 on target cells does not result in non-MHC-restricted lysis (222). Rather, CD244-CD48 interactions among T cells leads to increased antigen specific cytotoxicity (222). Interestingly, CD244+ T cells both proliferate faster in response to cytokines and exhibit higher levels of baseline apoptosis compared to CD244 negative T cells (J. Klem unpublished observations). Thus, NK-T cell CD244-CD48 interactions augment T cell effector functions, as well as CD244-CD48 T-T interactions.

NK cells can affect B cell differentiation by their ability to secrete IFN-γ. Recently, direct NK-B cell interactions have been shown to induce switch recombination to IgG2a (223). CD2, and to a lesser extent CD244, are the receptors responsible for initiating this switch. Collectively, these studies demonstrate a critical role for CD244 not only in carrying out effector functions but also as a co-stimulatory receptor between lymphocyte populations. The majority of the studies presented above looked at *b* allele CD244 function, while currently little is known about *z* allele function. The focus of chapter four is to detail *z* allele function in NK cells

C. CD244 Function in Humans

Human CD244 has a broad expression distribution compared to murine CD244. Human CD244 is expressed on NK cells, γδ T cells, ~50% of CD8+ T cells, and on subsets of basophils, monocytes, and eosinophils (201-205). Human eosinophils express CD244 (205), and engagement of CD244 on eosinophils results in increased IL-4 and IFN-γ production, eosinophil peroxidase release and cytotoxicity. Eosinophils express SAP, a signaling molecule critical to CD244-mediated signaling, thus XLP patients may suffer from eosinophil defects along with NK and T cell defective function.

CD244 is also expressed on ~50% of CD8+ T cells. Conflicting evidence for the role of human CD244 on CD8+ T cells exists, where several groups have described increased cytotoxicity and an activating role for CD244 following engagement on CD8+ T cells (201, 224). In contrast, other reports fail to show CTL cytotoxicity following CD244 engagement (158, 202). These differences may be explained by culture conditions, where prolonged culture in IL-2 leads to non-MHC restricted killing. Alternatively, SAP expression is known to decrease with T cell activation, thus differences in SAP expression could explain altered CD244 function. Detailed studies examining the role of CD244 in CD8+ T cell function will reconcile these conflicting observations.

CD244 is also expressed on all human NK cells. Anti-CD244 mAb engagement or engagement by the natural ligand, CD48, of human CD244 on NK cells activates cytotoxicity and cytokine production similar to what was first observed for murine CD244 (201). Since then, several investigators have demonstrated that CD244 engagement leads to increased cytotoxicity in healthy patients but XLP patients, which lack functional SAP, have impaired CD244-mediated cytotoxicity (156-159, 202, 225). Demonstrating further that SAP expression is required for CD244-mediated cytotoxicity.

In addition to CD244 acting directly as an activating receptor, a role for CD244 as a co-receptor has been described in human NK cells. Sivori *et al* have shown that engagement of CD244 and NCR receptors results in increased cytotoxicity compared to engagement of each receptor alone (226). Resent studies by Bryceson *et al* have also described CD244 as a co-receptor for another NK cell activating receptor, CD16, in freshly isolated "resting" NK cells (227). However, direct engagement of CD244 or CD16 in IL-2 activated NK cells resulted in increased cytotoxicity. These studies by Bryceson *et al* suggest that the activation state of the NK cell expressing CD244 may dictate whether CD244 functions as a co-receptor or directly as an activating receptor.

A general paradigm among NK receptors is that inhibitory receptors dominate over activating receptors. Tangye *et al* has shown that engagement of the CD94/NKG2A inhibitory complex suppresses CD244-mediated NK cytotoxicity (158). Furthermore, Watzl *et al* has shown that co-engagement of inhibitory receptors and CD244 blocks CD244 phosphorylation and localization to detergent-resistant membrane fractions that contain lipid rafts (228). Recent studies have established a hierarchy among NK cell activating receptors on fresh and IL-2 cultured NK cells where CD16>NKp46>CD244>NKG2D and DNAM-1> CD2 using cytotoxicity, cytokine production, and calcium flux as functional readouts. These studies showed that freshly isolated NK cells require engagement

of multiple receptors for effector function, whereas engagement of individual receptors on IL-2 cultured NK cells was sufficient for effector function (229). The decrease in CD244 mediated cytotoxicity observed in freshly isolated NK cells may be the result of decreased CD244 expression on freshly isolated NK cells versus IL-2 activated NK cells or the result of decreased SAP expression. SAP expression is up-regulated following NK cell activation (230). Therefore, the activating function of CD244 can be regulated by multiple mechanisms, co-engagement of inhibitory receptors, CD244 expression level, or by SAP expression.

Previous reports had shown that CD244 is expressed early in NK development, before NK cells acquire lytic activity concomitant with NCR expression. CD244 and NCR expression also occur before the acquisition of MHC inhibitory receptors (231). Sivori *et al* also reported that CD244 inhibits NK cytotoxicity in immature NK cells that do not express SAP, but upon NK maturation when SAP is expressed, CD244 functions as an activating receptor (231). These observations suggest that CD244-CD48 interactions may provide a fail-safe mechanism preventing killing of normal autologous cells in early NK development (231). Although, CD244 engagement in immature NK cells results in inhibitory function; engagement of CD244 on mature NK cell results in

increased cytotoxicity, suggesting that the dominant role of CD244 in human peripheral blood NK cells is as an activating or stimulatory receptor.

As described in the NK immunobiology section, NK-DC crosstalk is an important mechanism in activating the early phases of the innate immune response, and the outcome of NK-DC interactions can then direct the adaptive immune response. The CD244-CD48 interaction plays a role in NK-DC crosstalk. CD48 expression on DCs is regulated by DC maturation and anatomic location (232). CD48 is expressed on myeloid DCs in the blood, bone marrow, and thymus. However, CD48 is down-regulated on monocytes differentiating into DCs, and CD48 is not expressed on DCs from inflamed lymph nodes, or plasmacytoid DCs (232). Morandi *et al* demonstrated that increased CD48 expression on DCs can induce CD244 mediated NK cytotoxicity. Conversely, down modulating CD48 expression on DCs found in the lymph node prevents or inhibits CD244-mediated-IFN-γ-production from lymph node NK cells (232). Their observations suggest that NK-DC CD244-CD48 interactions may play a critical role in the early stages of the innate immune response by altering cytokine production and cellular activation states of NK cells and DCs, thus influencing the subsequent adaptive immune response. The studies presented above illustrate that the CD244-CD48 interaction is involved in many aspects of the human immune response.

D. CD244 Signaling

Several groups have focused on studying the molecular signaling mechanisms of CD244, yet many aspects of the CD244 signaling pathway remain unresolved. This section will discuss what is collectively known about human and murine CD244 signaling. Engagement of CD244 leads to phosphorylation of the tyrosines within the cytoplasmic ITSMs which is required for CD244 signaling and receptor co-localization to lipid-rafts (228). Once localized to lipid rafts, CD244 associates with LAT which is required for CD244-mediated cytotoxicity (131, 233). LAT KO mice have defective CD244-mediated cytotoxicity (131, 233). LAT can then recruit PLCγ and Grb2 in a phosphorylation dependent manner initiating a signaling cascade that results in cytotoxicity and cytokine production, as shown in figure 1.3a (234). As described in previous sections, SAP is another signaling molecule required for CD244 signaling. How and if SAP relates to CD244 signaling through LAT remains to be determined.

As mentioned previously, CD244 signaling is dependent on the phosphorylation of the tyrosine residues in the cytoplasmic ITSM motifs to facilitate adaptor molecule binding. These ITSM motifs can bind SAP, Eat-2a, Eat-2b, SHP-1, SHP-2, and SHIP. Binding of SAP results in recruitment of FynT

kinase which propagates an activating signal leading to increased cytotoxicity and IFN-γ secretion, see figure 1.3b (149, 217, 218). Human CD244 signaling studies have shown that all ITSMs bind SAP in a phosphorylation dependent manner; however, the third ITSM also binds SHIP, SHP-1, SHP-2, and CSK and may participate in the negative signaling of CD244 (235). XLP patients that lack functional SAP have an early block in CD244 mediated signaling. Alternatively if Eat-2a or Eat-2b associate with these ITSM motifs, CD244 signaling is inhibited. The details of how Eat-2a or Eat-2b inhibits CD244 signaling are currently under investigation. However, it is known that tyrosine phosphorylation of the C-terminus of Eat-2a is required for inhibition, and Eat-2a and Eat-2b do not associate with Fyn-T (136). Signaling studies using Eat-2a and Eat-2b KO mice suggest that the C-terminus of these adaptor molecules could associate with CSK, SHP-1, SHP-2, or SHIP resulting in propagation of an inhibitory signal blocking cytotoxicity and IFN-γ secretion. These studies demonstrated that differential adaptor molecule association can regulate CD244 function.

Human CD244 studies have identified another signaling molecule in NK cells that can associate with CD244, 3BP2 (c-Abl-Src homology3 domain binding protein-2). The 3BP2 binding motif found in human CD244 is not found in murine CD244, suggesting that 3BP2 mediated signaling is specific to human CD244 (Schatzle unpublished observation). Binding of 3BP2 to the cytoplasmic

domain of CD244 results in Vav-1 recruitment, as shown in figure 1.3c. Previously, 3BP2 has been shown to recruit Vav-1 and PLC-γ (236). Over expression of 3BP2 in NK cells increases baseline Erk activation, a down stream signaling target of Vav-1, and cytotoxicity, while IFN-γ production is unaffected (237). This observation suggested that CD244 signaling through 3BP2 and Vav-1 may be required for cytotoxicity but not IFN-γ production. Chuang *et al* have previously shown divergent CD244 signaling between cytotoxicity and IFN-γ production (238). Studies using Vav-1 KO mice have also shown divergent signaling requirements for cytotoxicity and IFN-γ production, where NK cells from Vav-1 KO mice have defective cytotoxicity but normal IFN-γ production (239). Further implicating a requirement for Vav in CD244-mediated cytotoxicity, a study of an XLP patient has shown a defect in Vav and glycogen synthase kinase-3 (GSK-3) activation, and thus defects in activation of Vav downstream signaling molecules Rac-1, Raf-1, Mek-2, and Erk 1/2 (240). These observations suggest that CD244 mediated cytotoxicity may require signaling through 3BP2 and Vav-1, whereas CD244 mediated IFN-γ secretion pathways may diverge from cytotoxicity pathways in human NK cells.

Although key signaling molecules in the CD244 signaling pathway have been identified, the exact mechanism of CD244 signaling remains an active area

of research, especially the nature of SAP independent inhibition of murine CD244.

E. Topics Addressed in Dissertation Research

Currently, it remains to be determined if differences in SLAM family haplotype mediate differences in lymphocyte function, specifically skewing Th1 vs Th2 responses, leading to disease states like SLE. This dissertation will discuss potential mechanisms describing how differences in SLAM haplotype expression leads to autoimmunity. Furthermore, little is know about the function of the *z* allele of CD244 in murine NK cells, or why CD244 mediates opposing functions in human and murine NK cells considering the structural homology between species. Chapters two through four will present potential explanations to questions concerning CD244. Chapter five will present data on how polymorphisms at the SLAM family locus can alter CD4+ T cell function, while chapter six will discuss the role of CD244 in NK cell function and how polymorphisms at the SF locus in the appropriate genetic context could lead to a break in immune tolerance.

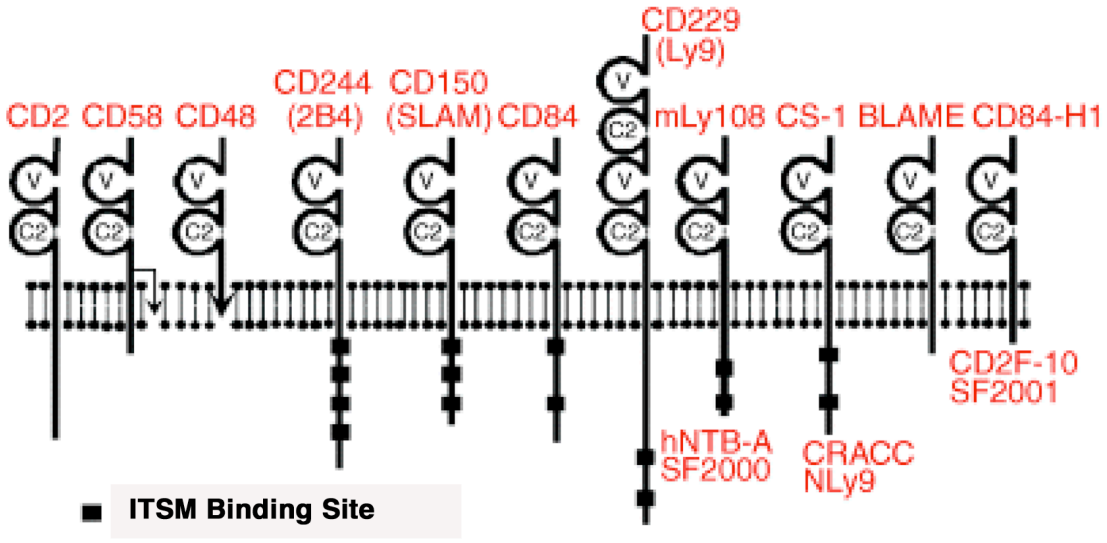


Figure 1.1 The CD2/SLAM subset of the immunoglobulin (Ig) superfamily of cell-surface receptors

There are presently 11 members of the CD2/SLAM family. The names originally given to these receptors are indicated at the top of each receptor; alternative names are listed below. The SLAM family of receptors contain tyrosine-based motifs, TxYxxV/I, ITSM within their cytoplasmic domains. This figure was adapted from Nichols *et al*, Immunological Reviews (2005).

Receptor	Ligand	Expression	Function
CD84	CD84	B and T cells, $CD45RO^{Hi}$ memory/effector T cells, monocytes and platelets	Control of TCR-induced IFN-γ production
CD48	CD244	Hematopoietic cells	CD4+ T-cell activation, lipid-raft associated
CD150 (SLAM)	CD150	$CD45RO^{Hi}$ memory/effector T cells, rapidly upregulated by B and T cells, activated monocytes and DCs	Regulation of cytokine production, T_H1/T_H2 cell polarization, B and T cell proliferation, MΦ cytokine production
CD229	CD229	B and T cells	Unknown
CD244	CD48	NK cells, CD8+ T cells, monocytes, basophils, and eosinophils	NK and T cell cytotoxicity, NK-cell co-stimulatory molecule, activating receptor on eosinophils
CS1	CS1	NK, B, T, and DC cells	SAP-independent NK-cell cytotoxicity
Ly108	Ly108	NK, B, and T cells	NK-cell cytotoxicity T cell cytokine production, neutrophil function

Table 1: SLAM Family Receptors
Adapted from Engel et al, Nature Reviews (2003)

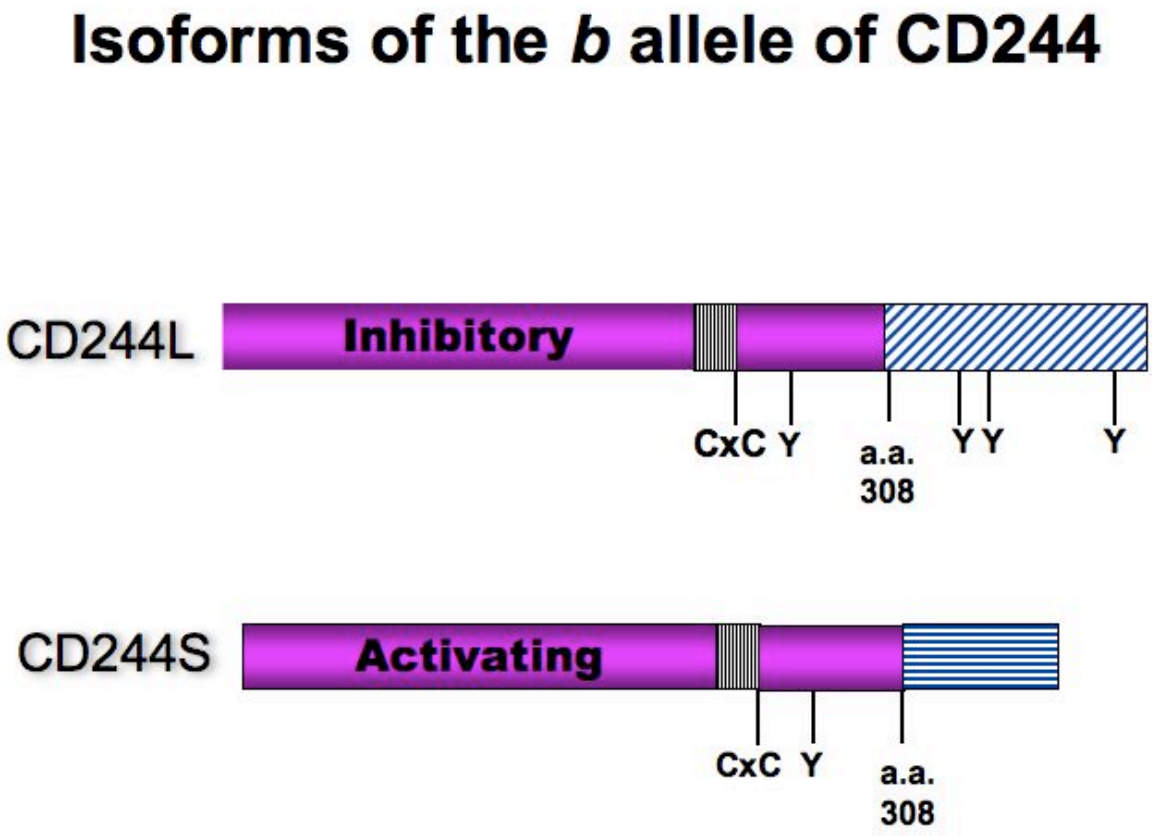


Figure 1.2: Isoforms of the *b* allele of CD244

CD244L and CD244S isoforms are identical through amino acid 308. The transmembrane region is indicated by the vertical striped boxes. The blue striped boxes denote the distinct cytoplasmic regions of each isoform. Ys denote ITSM motifs.

(Figure adapted from J Klem 2003)

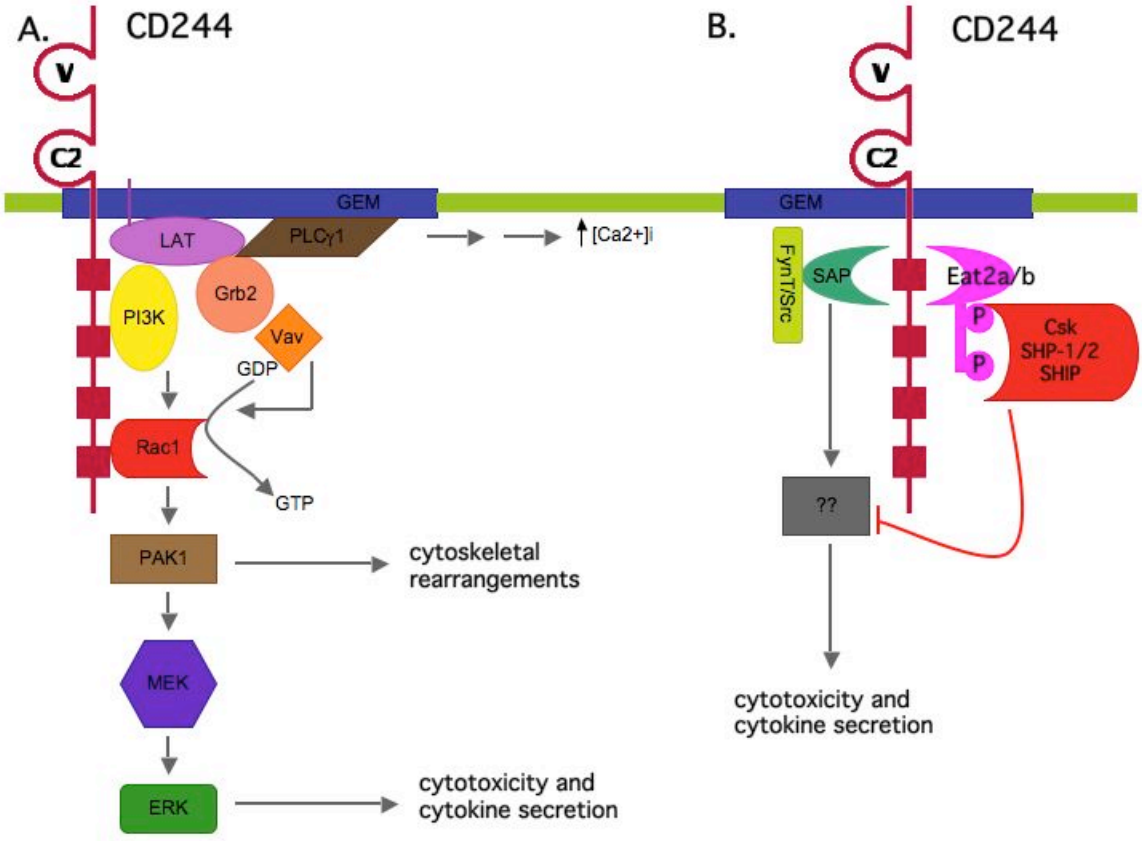


Figure 1.3 Model of CD244 Signaling

Panel A: CD244 signaling through LAT

Panel B: CD244 signaling through SAP or Eat-2a/b

Figure Adapted from J. Klem 2003

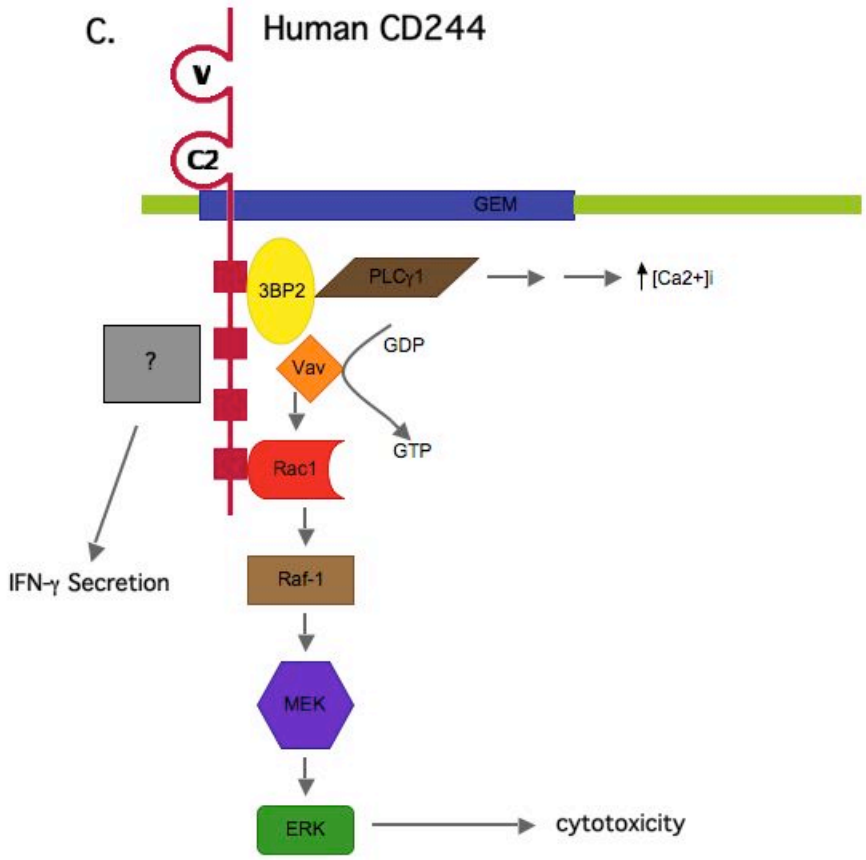


Figure 1.3 (cont) Model of CD244 Signaling

Panel C: Model of human CD244 signaling through 3BP2

CHAPTER II

MATERIALS AND METHODS

I. Mice

C57BL/6 and 129/Sv mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained at the University of Texas Southwestern Medical Center (Dallas, TX). CD244 -/- mice were generated by disruption of exon 2 and part of 3 with the neo^r gene. ES cells derived from C57BL/6 mice were used for generation of the CD244-/- mice and a full description of these mice is provided elsewhere (215). SAP -/- mice were generated by introducing a site mutation that truncated the SAP protein at the beginning of the third exon. The targeting vector was introduced into TC1 ES cells and then chimeric mice were bred to C57BL/6 mice (182). B6.*Sle1b* mice were generated as previously described (162). B6.*Sle1b* mice were crossed to homozygosity with SAP -/- mice to generate B6.*Sle1b*/SAP -/- mice. The B6.*Castc1* mouse strain has a congenic interval similar in size to B6.*Sle1b* as described by Wandstrat et al (166). Crossing B6 mice to B6.*Sle1b* generated B6 x B6.*Sle1b* F1 mice, which are heterozygous for the *b* and *z* alleles at the SLAM family locus. All mice used were 2-4 months of age. All experiments were performed in compliance with the relevant laws and institutional guidelines of the University of Texas Southwestern Medical Center.

II. Cells and tissue culture

A P815 CD48- FcR+ variant cell line was transfected with CD48 and cultured in complete RPMI 1640 medium (10% FCS, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES) supplemented with 1 mg/ml G418. The variant cell line was also transfected with empty pcDNA 3.1 vector and cultured under selection. Lymphokine-activated killer (LAK) cells were made from a single cell suspension of splenocytes devoid of red blood cells and cultured at 3 x 10^6 cells/ml for 5 days in DMEM complete media supplemented with 0.25 μM β-mercapto-ethanol and a total of 1000 U/ml IL-2 as described (23). 300ng/ml human rIL-15, or 1ng/ml IL-12 and 100 ng/ml IL-18 were provided for IL-15 or IL-12/18 LAK cultures respectively. Purified T cells were culture in DMEM complete media supplemented with 50 μM β-mercapto-ethanol.

III. Antibodies

Purified CD244 B6 alloantigen mAb (mouse IgG2b$^{\kappa}$), CD244 Balb/c alloantigen mAb (rat IgG2b$^{\kappa}$), PE-conjugated CD244 B6 alloantigen mAb, FITC-conjugated CD3ε (Hamster IgG), purified NK1.1 (Mouse IgG2a$^{\kappa}$), Biotin, FITC and PE-conjugated NK1.1, purified and APC-conjugated CD3ε (Hamster IgG2b$^{\lambda}$), purified CD28 (Hamster IgG2), biotin-conjugate CD154 (Hamster IgG3$^{\kappa}$), PE-conjugated CD4 (rat IgG2b$^{\kappa}$), streptavidin-APC, and streptavidin-

PerCp were obtained from BD PharMingen (San Diego, CA). FITC-conjugated goat anti-rat secondary was obtained from Jackson Immuno Research (West Grove, PA). Undiluted cell free 2.4G2 hybridoma supernatant and FITC-conjugated anti-rat secondary antibodies were used to stain for FcR expression. CD48 and CD244 Fab fragments were prepared using the Papain enzyme as described by (241).

IV. Flow Cytometry

Flow cytometry was conducted following standard flow cytometry procedures. Briefly, cell were washed and resuspended in PBS supplemented with 2% FCS. Cells were incubated in the dark at 4 °C for 15 minutes with antibody diluted in PBS with 2% FCS. At least 10,000 events were collected using a FACScan or FACSCalibur, flow cytometer (Becton Dickinson, San Jose, CA). Cell Quest analysis and acquisition software was used to analyze data collected. A FACSVantage (Becton Dickinson, San Jose CA) or MoFlow (Cytomation, Fort Collins, CO) flow cytometer was used for sorting.

V. Cytotoxicity assays

Specific lysis of targets was determined by using a standard 4-hour ^{51}Cr release assay in 96 well flat bottom plates as previously described (23). Redirected lysis assays using P815 CD48- and P815 CD48+ targets were

performed as previously described (213). Briefly, chromium labeled P815 CD48- and P815 CD48+ target cells were preincubated with mAb for 20 minutes at room temperature and then incubated with effector cells at 37°C. One tailed student T-tests were used to determine statistical significance between experimental groups.

VI. T cell Purification and Stimulation

A single cell suspension of splenocytes devoid of red blood cells were incubate with the following antibody cocktail for 15 min at 4 °C; biotinylated-B220, Mac1α, Gr1, NK1.1, DX5, CD8α, HSA, and Ter119 (BD PharMingen, San Diego, CA). Cells were incubated with magnetic streptavidin beads and negatively selected following the manufactures protocol (BD PharMingen, San Diego, CA). Purified CD4+ T cells were resuspended in complete DMEM supplemented with 50μM β-mercapto-ethanol. T cells were stimulated by plate bound mAb. Plates were coated with CD3 (1 μg/ml) and/or CD28 mAb (10μg/ml) overnight at 4 °C or for 1 hour at 37 °C. Following stimulation, cells and supernatants were harvested and used for cytokine and expression analysis.

VII. Cytokine Bead Assays

Supernatant from T cell stimulations at 72 hours were analyzed for TNF-α, IFN-γ, IL-12, IL-10, IL-6, IL-5, and IL-4. Cytokine bead assays were

performed following the manufactures instructions and analyzed on a FACSAria (Becton Dickinson, San Jose, CA).

VIII. Immunoprecipitation and Western blot analysis

Approximately $5X10^7$ cells (RNK-16/CD244L, RNK-16/CD244S or IL-2 generated LAKs) were surface labeled with biotin using the EZ-link sulfo-NHS-Biotin reagent from Pierce (Rockford, IL). Cells were lysed and CD244 was immunoprecipitated as previously described (131). Immunoprecipitates were treated with 1u of N-glycosidase F from Roche laboratories (Indianapolis, IN) overnight at 37^0C according to manufacturer's instructions to remove N-linked carbohydrates. Samples were then resolved by 10% non-reducing SDS-PAGE analysis and subjected to western blot analysis using streptavidin-HRP, obtained from Pierce (Rockford, IL), to detect biotinylated, immunoprecipitated proteins by enhanced chemiluminescence.

IX. Isolation of CD244 transcripts

RNA isolated from sorted, B6, B6.*Sle1b*, and B6.Castc1, NK populations obtained from day 5, IL-2 LAK cultures was used to isolate CD244 transcripts. Using a common 5' primer for CD244 (AGAATGTTGGGGCAAGCTGTCCTGTTC) in conjunction with a 3' RACE system (Invitrogen, Life Technologies, Carlsbad, CA) cDNAs for CD244 were

isolated. These cDNA products were cloned into the pGEM-T vector (Promega Life Sciences, Madison, WI) for sequence analysis. Approximately five to ten independent clones were sequenced for each representative cDNA product. The GenBank accession numbers for CD244S Cast and CD244L Cast are DQ167570 and DQ167571 respectively.

X. RT- PCR and Real-Time PCR

B6 CD244L, CD244S, and actin transcripts were detected using total RNA isolated from B6 NK cells cultured for 5 days with IL-2 and sorted for the NK1.1+ CD3- population. RNA was converted to cDNA using SuperScript First-Strand Synthesis System for RT-PCR with Taq following the manufactures instructions (Invitrogen, Carlsbad, CA). The following actin and isoform specific primers were used B6 CD244L (nucleotide 1080) 5':GTTGCCACAGCAGACTTTC; B6 CD244L (nt1250) 3':TTCCAACCTCCTCGTACACGGTAC; B6 CD244S (nt1072) 5':ATGTTCAGCTCCCTTCTAG; CD244S (nt1200) 3':GAGTTCCTCCCTGGCAGATC; Actin 5': GAGGCCCAGAGCAAGAGAG; Actin 3': GTCATCTTTTCACGGTTGG. B6.Castc1 CD244L and CD244S isoforms and actin transcripts were detected using total RNA isolated from B6.Castc1 NK cells as described for B6 isoforms. The following isoform-specific primers were used: B6.Castc1 CD244L and CD244S (nt 852) 5'-

GGCCTCTGGATCTCCCTCAGATGT; B6.Castc1 CD244L (nt 1038) 3'-CTGAATATACTGTACATTTTTCTT; B6.Castc1 CD244S (nt1035) 3'-ACTAGTGATTCTATTTCCCATTTT. Real time PCR was then performed on the Perkin-Elmer GeneAmp 5700 sequence detection system using their SYBR green PCR assay. Standard curves for all primer pairs showed similar slopes indicative of equivalent annealing efficiencies. In addition, cDNAs of each CD244 isoform cloned into expression vectors were amplified with equal efficiency by their respective primers.

Eat-2a, Eat-2b, and actin transcripts were detected using total RNA isolated from NK cells cultured for 5 days with IL-2 and sorted for the NK1.1+ CD3- population. cDNA was synthesized using SuperScript First-Strand Synthesis System for RT-PCR using *Taq* following the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA). The Eat-2a, Eat-2b, and actin primers have been described previously (214, 242). SAP and actin transcripts were detected using Superscript III One-Step RT-PCR system with platinum Taq following the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA). The following SAP primers were used: 5'-TCTCATCTCAGCGTTTCAGAAG; 3'-CTCTTCTGCCTGTGGGAGCTT. Control reactions confirming the absence of genomic DNA contaminant was preformed by excluding the

RT-Platinum Taq mix and including only Platinum Taq in the amplification reaction, which detected no transcripts (data not shown).

RNA isolated from purified CD4+ T cells was converted to cDNA using SuperScript First-Strand Synthesis System for RT-PCR with Taq following the manufactures instructions (Invitrogen, Carlsbad, CA). Actin and SAP expression were determined using the previously described actin primer and the following SAP primers: 5'- TCTCATCTCAGCGTTTCAGAAG; 3'- CTCTTCTGCCTGTGGGAGCTT. SAP expression was then normalized to actin. SLAM family member expression in purified CD4+ T cells stimulated via CD3 only or CD3 and CD28 were analyzed using the real time primers previously described (166).

XI. PCR

EAT-2, SAP, and actin transcripts were amplified from cDNA generated from RNA isolated as described above from B6 NK cells housed in SPF and conventional colonies. The actin and SAP primers are described in section X. Eat-2 primers have previously been described (215).

XII. Video Microscopy

The green fluorescent protein was fused to the C-terminus of murine CD244 (the CD244 long isoform, as it is the major isoform present in LAK cells) with an 18 amino acid linker (AAAGGGGSGGGGSGGGGS). Primers for amplification of CD244 were GGATCCGTGGTGATGTTGGGGCAAGC for the 5' primer (start codon underlined) and CCTGCGGCCGCGGAGTAGACATCAAAGTTC (codon preceding original stop codon underlined) for the 3' primer (bold indicates added restriction sites for cloning, BamHI for the 5'primer and NotI for the 3' primer). The PCR product for CD244 was then cloned in frame (via the NotI site) with a GFP-18 amino acid linker construct obtained from the laboratory of Christoph Wulfing. The resulting fusion construct was confirmed by sequencing analysis. To confirm that the fusion protein retained structure of CD244 and GFP, 293 cells were transfected with the expression construct and surface CD244 expression and GFP fluorescence were detected by FACs analysis. The fusion protein was then expressed in WT IL-12/18 LAK cells using a Moloney murine leukemia virus-based retroviral system as previously described (64, 65). FACS analysis of transduced and non-transduced LAKs demonstrated that the total surface expression of CD244 was doubled by transduction. Video fluorescence microscopy systems and procedures, including the identification of productive cell couples, have been described previously (64, 65). Briefly, CD244-GFP

accumulation was classified as follows. Average fluorescence intensity of the area of accumulation at a cellular interface was measured using Metamorph Software, Universal Imaging, Downington, PA. Strong accumulation at an interface was defined as >40% intensity over anywhere else in the cell. Partial accumulation was defined as >40% intensity of the background but less than 40% greater than other areas of the cell. Minimal accumulation was defined as >40% intensity of background but less than other areas of accumulation within the cell. No accumulation was defined as <40% intensity of the background. Incubation with Jasplakinolide (0.5uM pre-treatment of NK cells and 0.1uM in assay) was performed as described previously (243).

CHAPTER III

THE MURINE NK RECEPTOR CD244 (2B4) EXHIBITS INHIBITORY FUNCTION INDEPENDENT OF SAP EXPRESSION

OBJECTIVE

Previously, two murine CD244 isoforms had been identified in our laboratory. Exogenous expression of these isoforms in a rat NK cell line (RNK-16) characterized an inhibitory function for the long form and an activating function for the short form. Current work in the field had described an activating function for murine CD244. However, these studies were done before the identification of the ligand, CD48, for CD244 and exclusively relied on antibody mediated engagement of CD244. Therefore, we wanted to study the function of murine CD244 using a controlled system where target cells only differed in CD48 expression to assess the role of CD244 in murine NK cell function when engaged by its natural ligand. In addition, we sought to determine if CD244 function correlated with isoform expression and SAP levels in murine NK cells.

INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes that mediate killing of tumors and virally infected cells (6, 7). They are also responsible for the

acute rejection of MHC mismatched bone marrow transplants (20). In addition to their cytotoxic effector function, NK cells modulate the immune system via secreted cytokines, such as interferon-γ (IFN-γ) (87, 88). NK cell function is governed by a balance between inhibitory and activating receptors, where the inhibitory signal generally dominates the activating signal (244). These receptors can be divided into two families based on structure: the C-type lectin superfamily and the Ig-superfamily (Ig-SF)(24, 244). The C-type lectin family, which contains type II membrane glycoproteins, includes the murine Ly49 family of receptors, and the NKG2/CD94 receptors found on human and murine NK cells (244). MHC class I molecules represent the majority of known ligands for the C-type lectin family. The Ig-SF includes the Killer Cell Inhibitory receptors (KIR) and Natural Cytotoxicity receptors (NCR) found on human NK cells, and the CD2/SLAM family of receptors found on murine and human NK cells. Members of the Ig-SF are type I membrane glycoproteins that contain two or more Ig domains (244). The known ligands for the KIRs include mostly MHC class I molecules; however, the ligands for the NCRs are still relatively undefined. The CD2/SLAM family of receptors exhibit homotypic binding or bind other family members and several members also serve as receptors for pathogens such as the case with SLAM and the Measles virus (125). The CD2/SLAM family includes CD2, CD48, CD2F-10, CD58, CD244 (2B4), Ly9 (CD229), Ly108, CD84, CS1, SLAM (CD150) and BLAME (106, 108-114, 245-247).

Murine CD244 is expressed on subsets of γ/δ T cells, mast cells, α/β T cells, and all NK cells (109, 200, 206). In C57BL/6 mice the CD244 gene encodes two different isoforms that arise by alternative splicing and differ only in their cytoplasmic tails (210). These isoforms have been shown to have opposing functions in transfected RNK-16 cells where the long form has an inhibitory function and the short form has an activating function (213). In contrast, the human CD244 gene encodes a single transcript that is closely related to the long form of murine CD244 and is expressed on all NK cells, γ/δ T cells, monocytes, and 50% of peripheral CD8+ T cells (202, 203). A role for human CD244 in mediating cytotoxicity and IFN-γ secretion has been described by engagement with monoclonal antibodies and the ligand for CD244, CD48 (109, 123, 124, 158, 201, 202). Studies have also shown that CD244 may act as a co-receptor to the NCR NKp46 (226). However, an inhibitory role for human CD244 has also been described in NK cells isolated from patients with X-linked lymphoproliferative disease (XLP) (156-159). XLP is an inherited disease characterized by an inability to control Epstein-Barr virus (EBV) infection, which results in acute infectious mononucleosis or lymphoma. XLP is the result of mutations in the SLAM-associated protein (SAP) gene; SAP associates with the immunoreceptor tyrosine-based switch motifs (ITSMs) in the cytoplasmic tail of CD244 and other SLAM family members (127). In addition, immature human NK cells that also lack SAP

expression demonstrate an inhibitory function of CD244 (231). Thus lack of SAP correlates with inhibition of NK cytotoxicity after CD244 engagement in human NK cells.

Our studies focus on examining the role of CD244:CD48 interactions in murine NK cell cytotoxicity. We show that CD48 expression on target cells inhibits NK cytotoxicity in a CD244-dependent manner. Surprisingly, unlike in human NK cells this inhibition is independent of SAP expression. This inhibitory phenotype correlates with the preferential expression of the CD244L isoform, which has been shown to have inhibitory function in transfected cell lines.

RESULTS

The CD244:CD48 interaction inhibits target cell lysis

The studies defining a role for murine CD244 as an NK activating receptor have relied upon engagement of the receptor by monoclonal antibody (109). To study CD244 function due to engagement by its ligand, CD48, we took advantage of a naturally occurring CD48 negative variant of the Fc receptor (FcR) positive P815 tumor cell line. This variant was isolated by flow cytometric screening for CD48 expression on P815 cells. This variant was confirmed to be CD48 negative by flow cytometry (Figure 3.1a and b), western blot analysis and RT-PCR (data not shown). To establish cohort cell lines that differ only in CD48 expression

(both cell lines express similar levels of FcR, Figure 3.1c), this variant was transfected with a CD48 expression construct. Multiple clonal isolates exhibiting CD48 expression profiles similar to the parental CD48 positive cell line were used in chromium release assays and showed the same susceptibility to lysis as the parental cell line (data not shown). As a control, an empty expression vector was also transfected into the CD48 negative variant and put under selection. The CD48 transfected cell line was used in all the following studies and is denoted P815 CD48+, while the empty vector transfected cell line is denoted P815 CD48-. These tranfectants were then used in chromium release assays to determine the effect of CD48 expression on target cell lysis. If CD244 were acting as an activating receptor as described in the human system, then CD48 positive targets should be more susceptible to lysis. However, as shown in figure 3.2a, P815 CD48+ targets were less susceptible to lysis than their CD48- cohorts (this was true of the original CD48+/- variants isolated as well as the CD48+/- transfected cells). To determine if the differential lysis of these two targets was due to CD244:CD48 interaction, we added anti-CD244 mAb to the chromium release assays. As shown in figure 3.2a, the addition of anti-CD244 mAb only increased lysis of the P815 CD48+ targets. If CD244 were mediating redirected lysis, both targets should have shown increased killing in the presence of the anti-CD244 mAb since their FcR levels were similar.

Similar results were obtained with LAKs prepared from C57BL/6 SCID mice (90% NK cells), IL-12/18 LAKs (90% NK cells), and IL-15 LAKs (60% NK cells) (data not shown). However, this effect is not restricted to NK cells alone. IL-2 LAKs are composed of three CD244 positive populations NK1.1+ T cells (40%), NK cells (15%), and NK1.1- CD3+ T cells (35%) (data not shown). These populations were sorted and used as effectors in chromium release assays. Only the NK1.1+ T cells and NK cells showed decreased baseline lysis of the P815 CD48+ targets that was abrogated in the presence of CD244 mAb (Figure 3.2b and c). Decreased lysis of CD48+ targets is not restricted to P815 cell lines as similar results were observed with CD48hi/lo expressing variants of the FcR negative tumor line, RMA/S (Figure 3.3a,c versus b,d). Addition of anti-CD244 mAb only increased the lysis of the CD48 hi expressing variant.

There are two possible explanations for the results we observed: one, the CD244:CD48 interaction actually inhibits target cell lysis, or two; the two targets are inherently different in their ability to be killed by NK cells. Each of these is addressed below.

Blocking the CD244:CD48 interaction relieves inhibition of NK cytotoxicity

As further confirmation that the anti-CD244 mAb blocked the CD244:CD48 interaction and relieved inhibition of target cell lysis, chromium

release assays were done in the presence of anti-CD244 or anti-CD48 Fab fragments which lack the ability to induce redirected lysis. Thus, any difference in cytotoxicity observed would be due to an interruption of the CD244:CD48 interaction. It has been shown that the anti-CD48 and anti-CD244 monoclonal antibodies either completely or partially block the CD244:CD48 interaction (124) (Wulfing and Schatzle, unpublished observation). As shown in figure 3a and c, the addition of anti-CD244 or anti-CD48 Fabs increased lysis of only the P815 CD48+ targets. Furthermore, lysis of CD48 high expressing, FcR negative RMA/S cells was increased in the presence of anti-CD244 or anti-CD48 Fabs, while CD48 low expressing RMA/S cells showed no difference (Figure 3.3b and d). These data support an inhibitory role for the CD244:CD48 interaction.

To confirm further that CD244 is solely responsible for the decreased lysis of CD48 positive targets, that there are no inherent differences between these two P815 target cells and, that anti-CD48 antibody directed cell cytotoxicity (ADCC) is not taking place, CD244 knockout IL-2 LAK effector cells were generated and used in chromium release assays against both P815 tumor targets (Figure 3.4a and b). CD244 knockout mice have normal NK cell numbers and receptor repertoires as described in detail by Lee et al ((215) and Kumar personal communication). Unlike what we observed for wild type LAKs (Figure 3.2a), CD48 expression on target cells did not affect baseline lysis by CD244 knockout IL-2 LAKs. The

addition of anti-CD48 Fab fragments also had no effect on lysis. The addition of anti-NK1.1 mAb resulted in both P815 targets being killed equally well indicating their equivalent susceptibility to lysis by engagement of a known activating receptor (Figure 3.4a and b). Taken together, these results confirm that the CD244:CD48 interaction inhibits lysis of CD48 expressing target cells and that addition of blocking reagents that disrupt this interaction restores the susceptibility of these targets to NK lysis.

CD244 preferentially accumulates between NK and target cell interfaces of non-lytic interactions

Taking advantage of a retroviral transduction approach coupled with live cell imaging that we have recently described, we were able to define the pattern of CD244 accumulation at interfaces between NK and target cells (64, 65). This system affords us the opportunity to observe accumulation of fluorescently tagged proteins and assign accumulation patterns to both lytic and non-lytic events. Based on observations with fixed cell couples examining MHC-inhibitory receptors, one would predict that as an inhibitory receptor CD244 accumulation would preferentially occur at those NK/target cell interactions that represent non-lytic cell couples (248). To visualize the pattern of CD244 accumulation in NK cell/target cell interactions, we fused GFP to the C-terminus of the murine CD244 receptor and this fusion construct was expressed by retroviral transduction of

primary IL-12/18 cultured NK cells. The interaction between transduced NK cells (expressing CD244:GFP) and P815 CD48+ targets (labeled with the vital dye, SNARF-1) was then observed using video fluorescence microscopy as previously described (64). Using this approach, we observed 44 total productive cell couples of which 32 constituted non-lytic interactions and only 12 led to target cell lysis. Using a scoring system as defined in the materials and methods section, we determined that 56% of non-lytic events exhibited strong accumulation of CD244:GFP at the NK/target cell interface compared to only 8% of lytic events (Figure 3.5). In addition, 75% of lytic events showed no accumulation of CD244:GFP compared to only 21% of non-lytic events. Addition of anti-CD48 or anti-CD244 Fab fragments prevented the accumulation of CD244:GFP at target cell interfaces further indicating that these are blocking reagents (data not shown). These data indicate that accumulation of CD244 at target cell interfaces inversely correlates with the ability of NK cells to lyse that target, a feature that would be expected of an inhibitory receptor.

A trivial explanation for these observations may be that CD244 accumulation at non-lytic interfaces is merely a passive diffusion process that occurs due to the prolonged interaction of non-lytic couples. To address this, NK cells were treated with a concentration of the inhibitor of actin depolymerization, Jasplakinolide, which slows but does not completely block actin dynamics. We

have previously determined that this treatment inhibits NK lysis but still allows for effective cell couple formation (64). We observed that Jasplakinolide treatment inhibited CD244:GFP accumulation at non-lytic interfaces (Figure 3.5). This shows that CD244 accumulation at the non-lytic interfaces is an active process and not due to passive diffusion. The fact that strong CD244 accumulation only occurs during non-lytic NK/target cell interactions, and CD244 accumulation is minimal or absent during lytic NK/target cell interactions is consistent with an inhibitory role of CD244:CD48 engagement.

The CD244:CD48 interaction inhibits target cell lysis independent of SAP expression

Human and murine CD244 have been shown to associate with SAP through their cytoplasmic ITSM motifs (149, 156, 157). In XLP patients who lack functional SAP, CD244 is unable to promote cytotoxicity of NK cells when engaged by either the natural ligand for CD244, CD48, or by antibodies to CD244 (156-159). The LAK cultures used in our studies all express SAP as determined by RT-PCR and western blot analysis (data not shown). To assess if SAP levels can modulate the cytotoxic function of CD244 in murine NK cells, IL-2 LAKs were generated from SAP knockout mice and used in chromium release assays with P815 CD48- and P815 CD48+ targets in the presence and absence of anti-CD244 mAb (Figure 3.6). SAP knockout NK cells have no gross NK defects and

no change in NK function (P. Schwartzberg unpublished data). To assess if SAP knockout IL-2 LAKs have aberrant CD244 expression, flow cytometry staining for NK1.1, CD244, and CD3 expression after culture for 5 days was done. The NK1.1+ and NK1.1+ T cell populations, which composes 17-40% of LAK cultures, were all positive for CD244 with expression levels similar to that of wild LAKs (data not shown). Figure 3.6 shows that SAP knockout LAKs behave like wildtype LAKs in that a decrease in lysis is observed for only P815 CD48+ targets which is abrogated by the addition of anti-CD244 mAb. The addition of anti-NK1.1 mAb showed increased killing of both CD48- and CD48+ targets similar to wild type (data not shown). These results suggest that blocking the CD244:CD48 interaction with anti-CD244 mAb relieves NK cytotoxicity, and unlike for human NK cells, CD244 inhibition is independent of SAP expression.

Increased CD244L over CD244S isoform expression correlates with inhibition of cytotoxicity

The C57BL/6 gene of CD244 is known to encode two isoforms (210), a long isoform, which contains four ITSM motifs, and a short isoform that contains one ITSM. These isoforms have been shown to have opposing functions, the long inhibitory and the short activating when expressed in RNK-16 cells (213). Northern analysis using 3' and 5' specific probes for hybridization showed multiple CD244 transcripts in C57BL/6 LAKs (200). The most prominent

transcript corresponds to the CD244L isoform (200). Thus, skewed isoform expression favoring CD244L could explain the observation of inhibited cytotoxicity when CD244 is engaged by CD48 on target cells. To determine the relative levels of CD244 isoform expression in C57BL/6 IL-2 LAK cultures, RNA was isolated from sorted NK cells and converted to cDNA, which was used in PCR and real-time PCR analysis. CD244 isoform and actin specific primers showed an amplification of CD244L and actin transcripts while CD244S transcripts are undetectable at 30 cycles (Figure 3.7a). A representative amplification plot of real time PCR analysis confirms this observation as shown in figure 3.7b. CD244L transcripts are amplified in earlier cycles compared to CD244S indicating increased amounts of CD244L over CD244S. In fact, CD244S transcripts only become detectable after CD244L amplification is out of the range of linear amplification. Differences in primer efficiency do not account for this skewed isoform expression because both primer pairs amplified CD244 isoform cDNA in expression vectors equally well and have similar standard curve slopes. Both freshly isolated and cultured NK cells showed increased CD244L levels over CD244S (data not shown).

To confirm that these differences in mRNA expression levels were also reflected by differential protein expression, surface proteins were biotinylated and cell lysates were immunoprecipitated with anti-CD244 mAb. Lysates were

prepared from C57BL/6 IL-2 cultured LAK cells and lysates from RNK-16 cells transfected with each CD244 isoform served as controls. To control for differential glycosylation patterns between RNK-16 cell lines and primary, murine NK cells, the immunoprecipitates were subjected to treatment with N-glycosidase F to remove N-linked carbohydrates and resolved by non-reducing SDS-PAGE analysis followed by western blotting with straptavidin-HRP. As seen in figure 3.7c, immunoprecipitation and blotting of lysates from RNK-16/CD244L and RNK-16/CD244S detected biotinylated proteins of the predicted size (43 and 37kD respectively). The predominant surface protein immunoprecipitated by the anti-CD244 mAb from LAK cells co-migrated with the deglycosylated CD244L isoform from RNK-16/CD244L. Immunoprecipitation with isotype control antibodies failed to result in any detectable biotinylated proteins (data not shown). Therefore, it appears that the major isoform expressed on the surface of these C57BL/6 IL-2 LAKs is the CD244L isoform consistent with the RT-PCR data described above. The preferential expression of this isoform may account for the inhibitory role of CD48:CD244 engagement in LAK cells. Recent studies also support the predominant role of the CD244L isoform in inhibition of murine NK cell function (215).

DISCUSSION

These studies show that CD244 engagement, by its counter-receptor CD48, inhibits NK cytotoxicity independent of SAP expression. The original work describing murine CD244 function suggested that CD244 was an activating receptor found on NK cells; however, in light of our studies the original work may need to be re-interpreted (109, 131, 249). At the time of this characterization, the ligand for CD244, CD48, was not known. The original chromium release assays were done with CD48 expressing target cells making it difficult to determine if the anti-CD244 mAb was engaging the CD244 receptor or blocking the CD244:CD48 interaction leading to increased target cell lysis. Interestingly, many of these targets were FcR negative yet addition of anti-CD244 mAb resulted in their increased lysis consistent with a blocking role of the anti-CD244 mAb. Subsequent studies failed to exhibit direct signaling either biochemically or through calcium flux by anti-CD244 mAb engagement arguing against a cross-linking role for this antibody (Schatzle unpublished data and Garni-Wagner personal communication). Similarly, comparing human and murine CD244 studies is complicated by the variability between human NK clones, antibodies used, and target cell lines (156-159, 201, 202).

To address some of these concerns, our studies utilize a defined system with CD48+/ – targets derived from the same parental cell line as well as CD244

knockout and SAP knockout mice to determine the role of CD244:CD48 engagement on NK cytotoxicity. We show that this engagement inhibits NK cytotoxicity independent of SAP expression and this inhibition can be blocked by the addition of anti-CD244 or anti-CD48 antibodies. During the completion of these studies a similar conclusion regarding the inhibitory role of CD244 in NK function was published. In this study, CD244 engagement also inhibited cytokine production (IFN-γ) as well as cytotoxicity (215). There is precedence for an inhibitory role of CD244 in human NK cells as well. Expression of CD244 in human NK cells before the acquisition of KIR and NCR receptors prevents killing of normal autologous cells (231). Additionally, NK cells from XLP patients exhibit an inhibitory phenotype where blocking the CD244:CD48 interaction between NK and target cells increases cytotoxicity (156-159). A common theme in both these systems is the absence of SAP expression correlating with the inhibitory function of CD244. Previously, studies of the CD244-signaling pathway have shown that SAP binds the ITSMs in the cytoplasmic domain of CD244. It has been hypothesized that SAP and SHP-1/SHP-2 compete for mutually exclusive binding to these ITSMs, which dictates CD244 function; where binding of SAP leads to an activating signal possibly through Fyn recruitment, and binding of SHP-1/SHP-2 leads to an inhibitory function (127, 129, 134, 135, 250). Surprisingly, the cytotoxic inhibition by CD244 engagement

observed in the murine system works independently of SAP expression (compare Figure 3.2a and 6).

Although we have characterized CD244 inhibition of NK cytotoxicity, the mechanism by which CD244 inhibits cytotoxicity still remains largely undefined. If SAP levels do not dictate CD244 function in the murine system, how might CD244 inhibition be explained? Studies with RNK-16, a rat NK cell line, have shown that over expression of the CD244L isoform inhibits cytotoxicity despite SAP being expressed in these cells and co-immunoprecipitating with CD244 (149). When looking at isoform expression via RT-PCR and western blot analysis in C57BL/6 IL-2 LAKs, the CD244L isoform is the major expressed version of this receptor and this could account for the predominant inhibitory function of CD244 (Figure 3.7). In support of this hypothesis, a recent study has shown that reconstitution of CD244L but not CD244S expression in CD244 knock-out NK cells is able to restore the inhibitory phenotype (215). Interestingly, these studies revealed some differences in the function of each isoform of CD244 when exogenously expressed in murine NK cells compared to results obtained from RNK-16 transfectants. While the inhibitory function of CD244L was observed in both cell types, the activating role of CD244S was only observed in RNK-16 transfectants. We do not know the reason for this discrepancy but murine CD244 function may be due to differential recruitment of

downstream signaling molecules to the cytoplasmic ITSMs of these CD244 isoforms. Alternatively, the signaling milieu inherent to each cell type may dictate receptor function. This could explain differential receptor function in human and murine NK cells despite the fact that both human and murine CD244 proteins are similar structurally in their cytoplasmic domains. Future studies should be aimed at defining the mechanisms to account for this difference and determine if this is due to structural differences between the murine and human CD244 receptors or differences in the signal transduction pathways predominant in murine vs human NK cells.

The ability of CD244 to inhibit NK cytotoxicity may have important biological consequences by preventing lysis of hematopoietic cells in the absence of MHC inhibitory receptors. As mentioned previously, studies of human NK development have shown that CD244 is expressed before the NCRs and MHC restricted inhibitory receptors (231). Interestingly, NK cells acquired lytic activity with NCR expression, which occurs before inhibitory receptors are expressed. Blocking the CD244:CD48 interaction between these NK cells and autologous myelomonocytic precursors led to autologous cell lysis (231). Thus, CD244 expression may be responsible for preventing lysis of normal autologous cells by immature but lytic NK cells. Similarly, using an in vitro murine NK cell differentiation system we observed that CD244 is expressed prior to the NKp46

murine homologue Mar-1, a NCR receptor, as determined by RT-PCR; (Schatzle unpublished results). As both of these receptors are expressed prior to Ly49 inhibitory receptors, this may be a mechanism to prevent NK cell autoreactivity. In addition, a recent study in the murine system has shown that the CD244:CD48 interaction among NK cells is critical for the generation of maximal NK effector function (Lee and Kumar manuscript in preparation). In this context, CD244 serves as a ligand for CD48 on neighboring cells, which is required for the generation of fully lytic NK cells. Thus, it is beneficial for CD244 to have an inhibitory function so that it is able to serve as a ligand for CD48 on adjacent NK cells, but prevent fratricide. CD244 may have multiple functions that are regulated by the context of CD244 engagement. When CD244 serves as a ligand for CD48 it may increase signaling thresholds required for development of full NK function but engagement of CD48 alone is not sufficient to mediate lytic granule release. Instead CD244 inhibition is either overcome by engagement of bona fide activating receptors or by lack or reduced expression of CD48 on target cells.

In summary, we show that CD244 engagement on NK cells by CD48 expressing target cells inhibits NK cytotoxicity independent of the presence of SAP, and that CD244 isoform expression may dictate CD244 function in the murine system. It remains to be determined how isoform expression is regulated.

And more importantly, defining the molecular mechanisms of CD244 mediated inhibition of murine NK function is warranted.

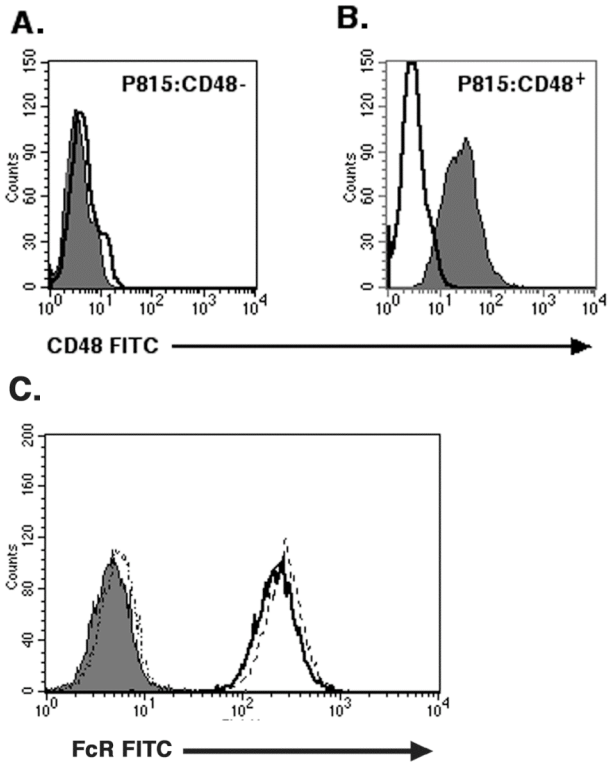


Figure 3.1: Isolation and characterization of CD48+/- variants of the P815 cell line

Panel A and B: The CD48- variant of P815 cells transfected with empty vector (P815) or P815:CD48+ transfectants were stained with FITC-conjugated anti-CD48 mAb (shaded histogram) or FITC-conjugated isotype-matched control Ab (open histogram) and analyzed by flow cytometry. **Panel C**: Empty vector P815 cells or P815:CD48+ transfectants were stained with anti-FcR and anti-rat FITC-conjugated secondary Ab (open solid and dashed histograms respectively) or anti-rat FITC-conjugated Ab alone (shaded and open dotted histograms respectively) and analyzed by flow cytometry.

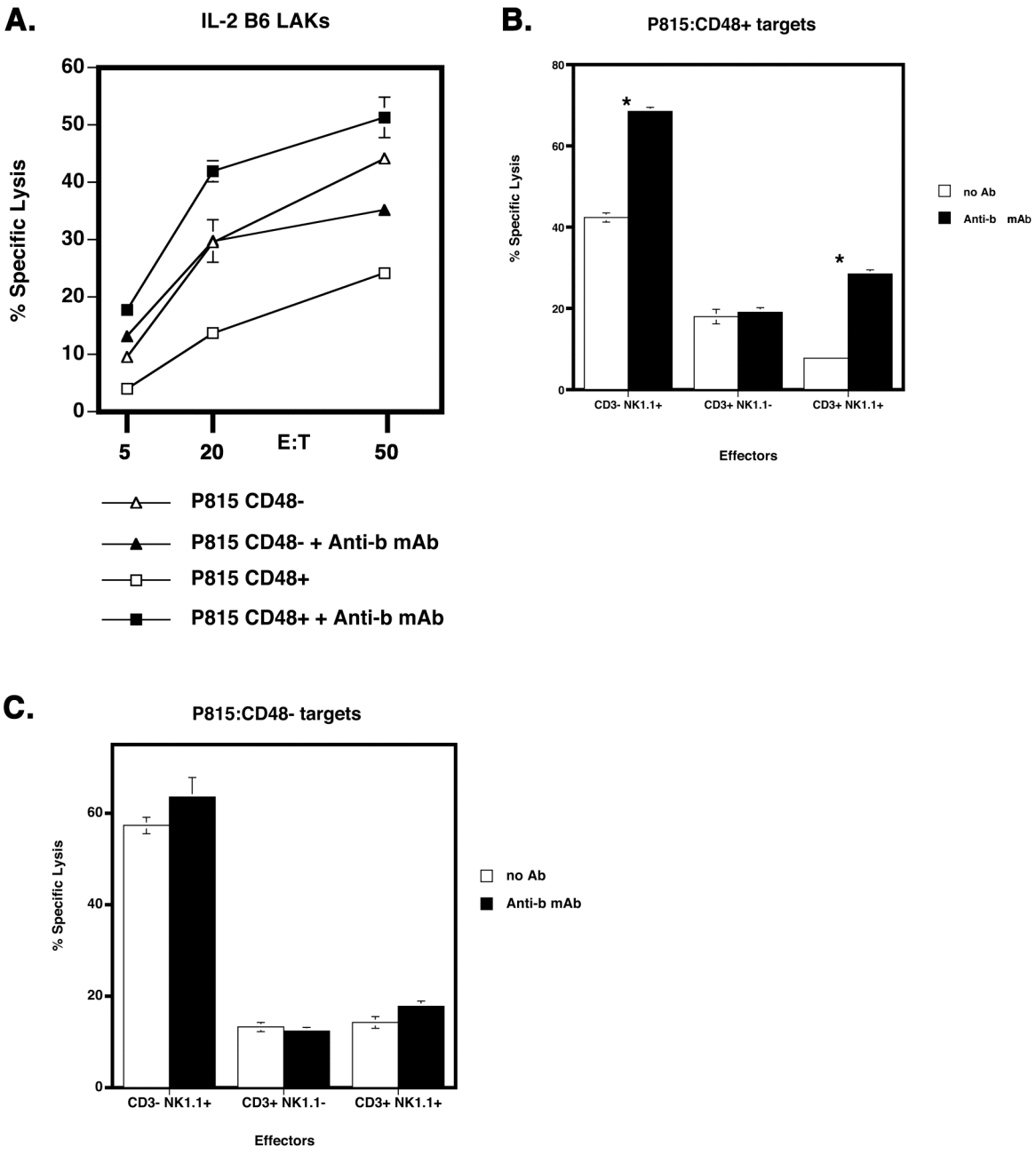

A.
IL-2 B6 LAKs
% Specific Lysis
60
50
40
30
20
10
0
5
20
E:T
50
P815 CD48-
P815 CD48- + Anti-b mAb
P815 CD48+
P815 CD48+ + Anti-b mAb
B.
P815:CD48+ targets
% Specific Lysis
80
60
40
20
0
*
*
CD3- NK1.1+
CD3+ NK1.1-
CD3+ NK1.1+
Effectors
no Ab
Anti-b mAb
C.
P815:CD48- targets
% Specific Lysis
60
40
20
0
CD3- NK1.1+
CD3+ NK1.1-
CD3+ NK1.1+
Effectors
no Ab
Anti-b mAb

Figure 3.2: NK cytotoxicity is inhibited by CD48 expression on targets cells

IL-2 stimulated C57BL/6 LAK cells were used as effectors in standard 4-hour chromium release assays with CD48- and CD48+ P815 (FcR+) target cells at the indicated E:T ratios. Results are representative of three independent experiments. **Panel A**: Anti-CD244 mAb was added at 10 μg/ml where indicated. **Panel B and C**: Sorted IL-2 LAK subsets were used as effectors in a chromium release assay against P815 CD48+ and P815 CD48- targets at a 25:1 E:T ratio. 10 μg/ml anti-CD244 mAb was added to effectors where indicated.

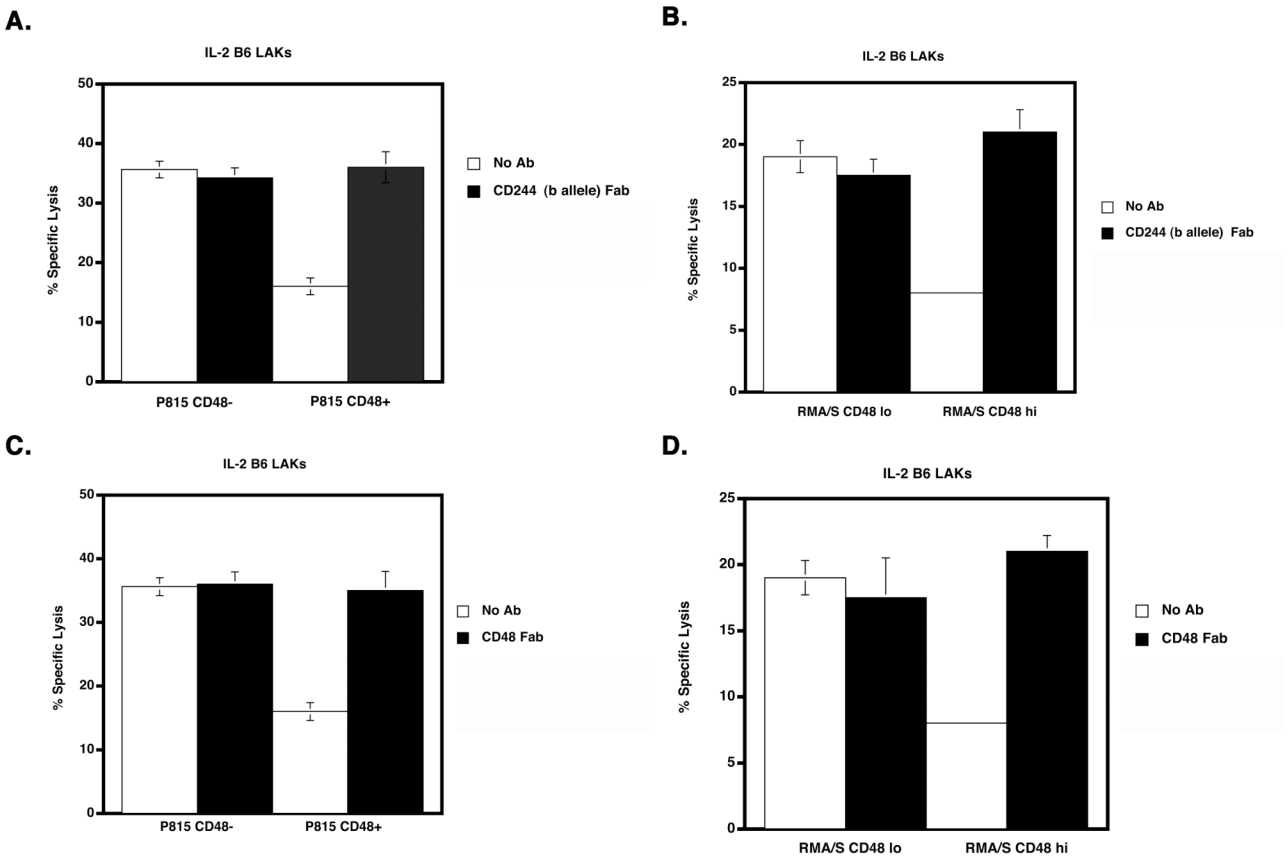


Figure 3.3: Blocking the CD244/CD48 interaction results in enhanced target cell lysis

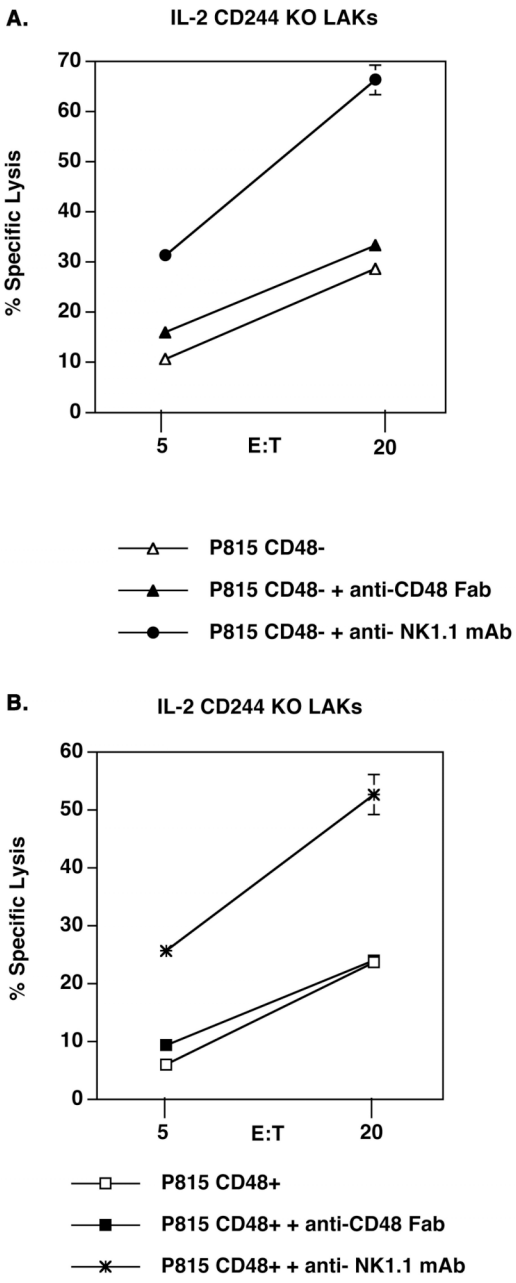
IL-2 stimulated C57BL/6 LAK cells were used in a standard 4 hour chromium release assay as described for figure 1. Results are representative of three independent experiments.

Panel A: P815 CD48- and P815 CD48+ target cells at an E:T of 20:1 with the addition of anti-CD244 Fab at 10 μg/ml.

Panel B: RMA/S CD48 low and RMA/S CD48 high target cells at an E:T of 100:1 with the addition of anti-CD244 Fab at 10 μg/ml.

Panel C: P815 CD48- and P815 CD48+ target cells at an E:T of 20:1 with the addition of anti-CD48 Fab at 10 μg/ml.

Panel D: RMA/S CD48 low and RMA/S CD48 high target cells at an E:T of 100:1 with the addition of anti-CD48 Fab at 10 μg/ml.


A.
IL-2 CD244 KO LAKs
% Specific Lysis
70
60
50
40
30
20
10
0
5
E:T
20
P815 CD48-
P815 CD48- + anti-CD48 Fab
P815 CD48- + anti- NK1.1 mAb

B.
IL-2 CD244 KO LAKs
% Specific Lysis
60
50
40
30
20
10
0
5
E:T
20
P815 CD48+
P815 CD48+ + anti-CD48 Fab
P815 CD48+ + anti- NK1.1 mAb

Figure 3.4: CD244 is required for the decreased lysis of CD48 expressing targets cells

IL-2 stimulated CD244 KO LAK cells were used in a chromium release assay as described for figure 3.2. Results are representative of three independent experiments.
Panel A: P815 CD48- targets with anti-CD48 Fab or anti-NK1.1 mAb at 10 μg/ml included where indicated.
Panel B: P815 CD48+ targets with anti-CD48 Fab or anti-NK1.1 mAb at 10 μg/ml included where indicated.

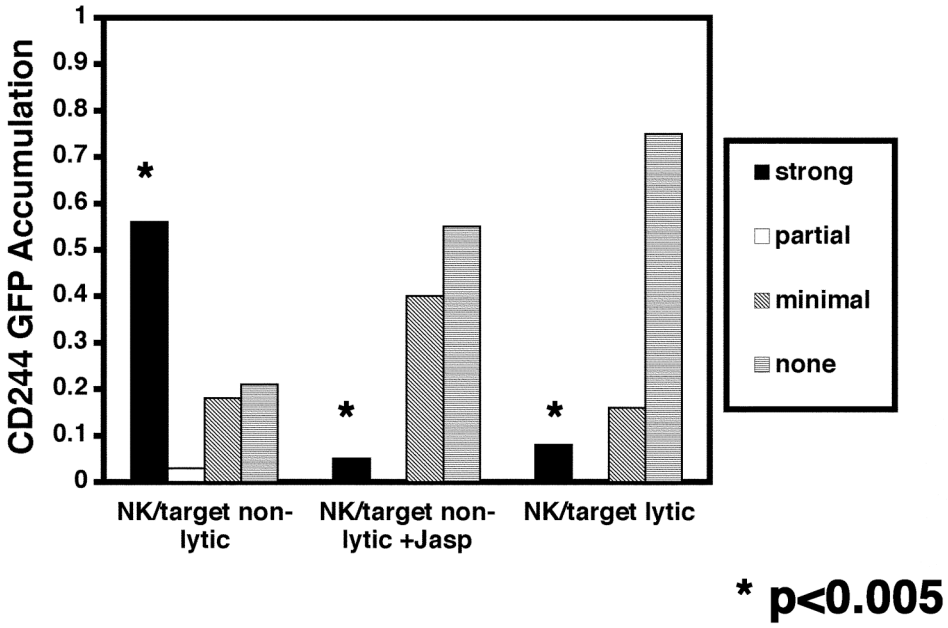


Figure 3.5: CD244 preferentially accumulates at the interface of NK/Target cell non-lytic interactions

A CD244-GFP construct was transduced into primary NK cells and GFP+ cells were sorted by flow cytometry and mixed with P815 CD48+ target cells. CD244-GFP accumulation was classified as follows. Strong accumulation at an interface was defined as >40% intensity over anywhere else in the cell. Partial accumulation was defined as >40% intensity of the background but less than 40% greater than other areas of the cell. Minimal accumulation was defined as >40% intensity of background but less than other areas of accumulation within the cell. No accumulation is less than 40% intensity of background. Where indicated, NK cells were pretreated for 30 minutes with 0.5um Jasplakinolide and 0.1uM Jasplakinolide was included for the duration of the assay. *$p<0.005$ statistically significant difference between strong accumulation of non-lytic and lytic target cell interactions.

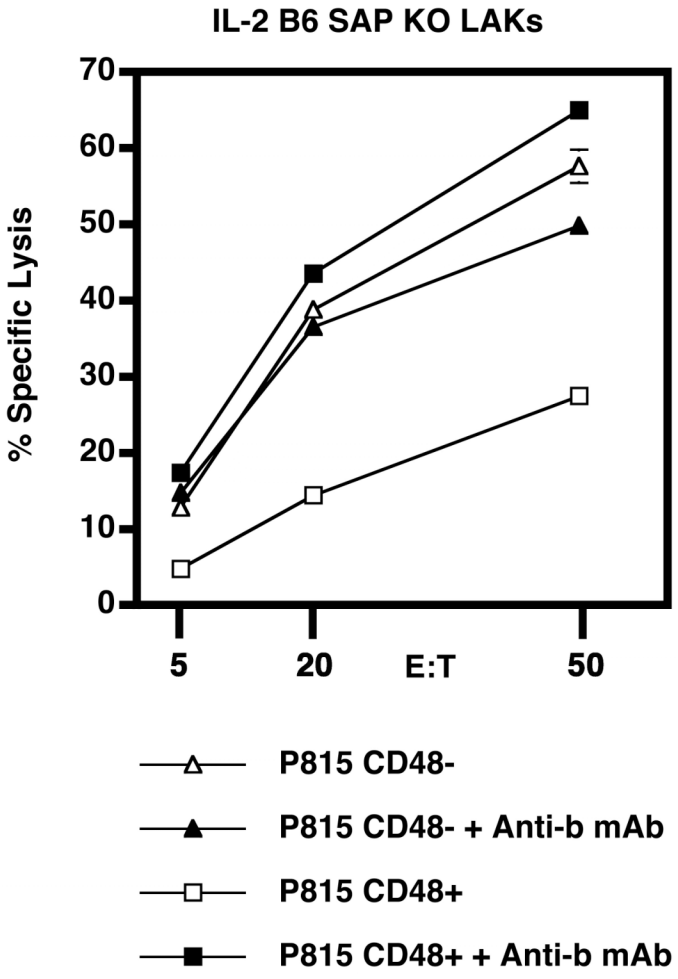


Figure 3.6: CD244-mediated inhibition of NK cytotoxicity is independent of SAP expression

IL-2 stimulated B6 SAP KO LAK cells were used as effectors in a standard 4-hour chromium release assay with CD48- and CD48+ P815 (FcR+) targets cells at the indicated E:T ratios. Results are representative of two independent experiments. Anti-CD244 mAb was added at 10 μg/ml where indicated.

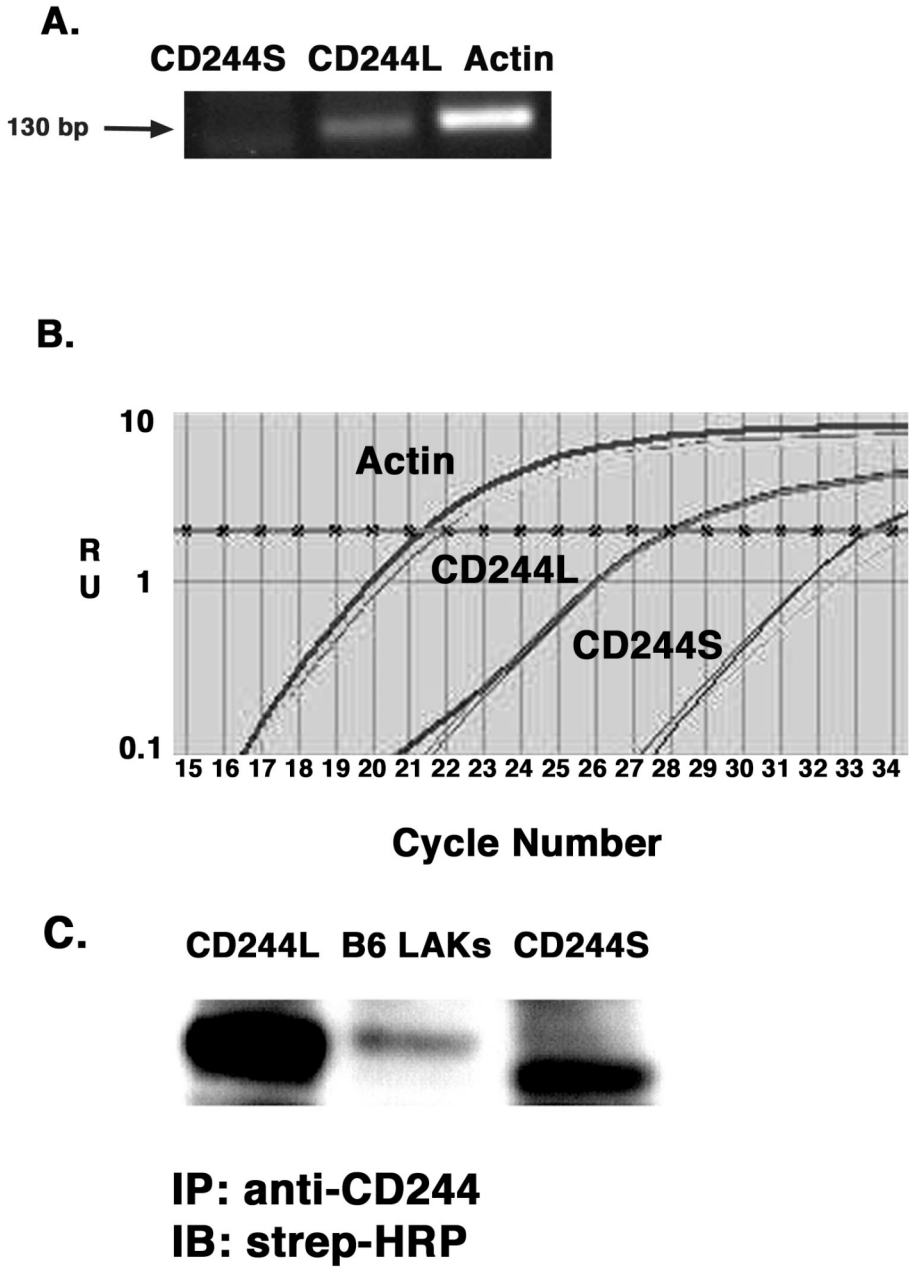

A.
CD244S CD244L Actin
130 bp
B.
10
1
0.1
RU
Actin
CD244L
CD244S
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Cycle Number
C.
CD244L B6 LAKs CD244S
IP: anti-CD244
IB: strep-HRP

Figure 3.7: Relative expression of CD244L and CD244S isoforms in IL-2 C57BL/6 LAKs

RNA was isolated from sorted NK1.1+ CD3- IL-2 C57BL/6 LAKs and used to synthesize cDNA. Panel A: Standard PCR was run at 30 cycles with isoform specific and actin primers where indicated. CD244S migrates at 170bps; CD244L migrates at 128 bps. Panel B: An amplification plot representative of three real-time PCR experiments with isoform specific primers or actin primers is shown. All samples were run in triplicate.
Panel C: Approximately $5x10^7$ cells (RNK-16/CD244L-lane 1, IL-2 murine LAKs-lane 2 or RNK-16/CD244S-lane 3) were incubated with Sulfo-NHS-Biotin to biotinylate surface proteins, and cell lysates were subjected to immunoprecipitation with anti-CD244 mAb or isotype control antibody. The resulting immunoprecipitates were treated with N-glycosidase F and resolved by 10% SDS-PAGE followed by western blotting with streptavidin-HRP. This resulted in the detection of biotinylated proteins of the correct size 43kD for the CD244L isoform and 37kD for the CD244S isoform. The predominant species of biotinylated surface protein immunoprecipitated from LAK cultures co-migrates with the CD244L isoform. Immunoprecipitation with isotype control antibody failed to generate any detectable bands following blotting with streptavidin-HRP.

CHAPTER IV

POLYMORPHIC ALLELES OF CD244 EXHIBIT DIVERGENT FUNCTION IN MURINE NK CELLS

OBJECTIVE

Prior to the following studies, there were several conflicting reports characterizing murine CD244 function (109, 212, 214, 215, 218). Thus the question remained, is murine CD244 activating or is it inhibitory? Recent studies have identified the existence of two stable haplotypes for the SLAM family in common laboratory strains of mice. One haplotype, *b*, found in C57-derived mouse strains. The second haplotype, *z*, found in all non-C57-derived mouse strains. This observation raised the following questions: 1) what is the role of CD244 in murine NK function? 2) is the more commonly occurring *z* allele of murine CD244 activating or inhibitory? The studies described in this chapter utilize congenic mouse strains containing the *b* or *z* haplotype of the SLAM family to define the role of CD244 function in murine NK cells. These mouse strains share the same NK gene complex and genetic backgrounds except for the 900 kb region surrounding the SLAM family, making direct comparisons of NK function possible. In the following studies, we characterize the function of the *b* and *z* alleles of CD244 in NK cell cytotoxicity.

INTRODUCTION

Natural killer (NK) cells are potent cytotoxic effector cells important in the innate immune response to virus infected cells and tumors (6, 7). In addition to their effector function, they secrete cytokines (such as, interferon-γ, GM-CSF, and TNF-α) that can regulate innate and adaptive immune responses, priming the adaptive immune response toward a Th1 response (87, 88, 91). NK cell function is governed by positive and negative signaling receptors, where the negative signal generally dominates (244). These receptors can be grouped into two categories based on structure. The first category is the C-type lectin family, which includes the NKG2/CD94 family of receptors found in humans and rodents and the Ly49 receptors found in rodents(244). The second category includes the Immunoglobulin-Superfamily, which includes the human Killer-cell Immunoglobulin-like Receptor and Natural Cytotoxicity Receptor families of receptors and the SLAM family found in rodents and humans(24, 244).

The SLAM family (SF) of receptors includes CD229 (Ly9), Ly108 (NTB-A), CD84, CS1 (CRACC), SLAM (CD150), and CD244 (2B4), which are all expressed on various subsets of hematopoietic cells(108-113). All family members share a similar extracellular domain structure characterized by a C-terminal Variable-Ig (V-Ig) domain followed by a membrane proximal Constant

2-Ig (C2-Ig) domain, with the exception of Ly9, which has duplicated V-Ig and C2-Ig domains(105, 116). This family of receptors exhibits homotypic binding or SLAM/CD2 intrafamilial receptor-ligand binding for example CD244 and CD48(116, 123, 124, 127, 129). Most SLAM family members contain cytoplasmic immuno-receptor tyrosine-based switch motifs (ITSMs). These ITSMs are known to associate with adaptor molecules such as SHP-1, SHP-2, SHIP, Eat-2 and SLAM-associated protein (SAP)(127, 129). SAP recruits FynT to these ITSMs via the SH2 domain of SAP interacting with the SH3 domain of FynT; this recruitment is essential for SF function(134, 135). Disruption of adaptor molecule association with these receptors results in altered SF receptor function and immune dysregulation such as in X-linked lymphoproliferative disease (XLP), where the SAP gene is defective(156-159). XLP is characterized by an inability to clear Epstein-Barr virus infection which leads to fatal infectious mononucleosis or lymphoma(127). Studies have shown that CD244 and NTBA NK mediated cytotoxicity is impaired in XLP; and therefore they have been implicated in the disease pathology(155-159, 226).

Murine CD244 is expressed on subsets of γδ T cells, mast cells, αβ T cells, eosinophils, and all NK cells(109, 200, 206). Recently, the SLAM family of receptors has been identified as having two possible haplotypes in common laboratory strains of mice (166). A *b* haplotype found in all C57-derived mouse

strains, and a *z* haplotype found in most other inbred laboratory mouse strains. In B6 mice, CD244 is found as a single gene, which can be differentially spliced to encode a long and short isoform(210). These isoforms are identical in the extracellular region but differ in their cytoplasmic tails. The long isoform is the predominant form found in NK cells (214). In mouse strains not of C57 origin (that express the *z* haplotype) the CD244 gene locus is expanded to contain four CD244 genes (166). Only the extracellular domain of these genes has been sequenced and three of these genes may encode functional products, while one appears to contain a premature stop codon in the V-Ig domain(166).

Currently, studies defining the function of CD244 in the murine system describe both an inhibitory and activating receptor function. Previously, an inhibitory function has been shown for the b allele of CD244 following receptor engagement via its ligand, CD48, expressed on target cells resulting in inhibition of cytotoxicity independent of SAP expression (199, 214, 215). In contrast, Bloch-Queyrat et al have recently described an activating function for CD244 which is dependent on SAP expression (218). Studies presented here may explain these conflicting results. We define the function of the *b* and *z* alleles of CD244 using congenic mouse strains that differ genetically only at the 900 kb region surrounding the SLAM family locus. We show that the *z* allele of CD244 has activating function dependent on SAP expression similar to results observed for

human CD244 and by Bloch-Queyrat. In addition we show that the *b* allele can have activating and inhibitory function dependent on environmental conditions which could influence studies defining CD244 function in B6 mice. This difference in function is inherent to each CD244 haplotype and may be dictated by structural polymorphisms located in the extracellular and cytoplasmic domains that could affect ligand binding or signaling of the various alleles of CD244.

RESULTS

The z allele of CD244 has activating function dependent on SAP expression

CD244 exists as one of two haplotypes, a *b* or *z*, in common lab strains of mice (166). Previously, we characterized the inhibitory function of the *b* allele of CD244 using a CD48-negative variant of the FcR-positive P815 tumor cell line (denoted P815 CD48-) and a CD48 transfectant of this parental P815 cell line (denoted P815 CD48+) (214). We showed that the *b* allele of CD244 acts as an inhibitory receptor independent of SAP expression. Previous studies of the *z* allele of CD244 described an activating function for CD244; these studies used mouse strains from different genetic backgrounds containing different NK gene complexes, which could influence their results (212). To compare the function of the *z* and *b* alleles of CD244 in a common genetic background, we used IL-2 lymphokine-activated killer cells (LAKs) derived from B6.*Sle1b* congenic mice that are of the B6 background except at a 900 kb interval flanked by *B4galt3* and

Copa on chromosome 1 containing the SF locus (166). Since B6 and B6.*Sle1b* mouse strains share the NK gene complex, NK cells from these mice are indistinguishable with regards to NK receptor expression profiles and overall NK function as determined by YAC-1 killing (data not shown).

B6.*Sle1b* LAKs were used as effectors in chromium release assays with P815 CD48- and CD48+ target cells. If the *z* allele functions as an activating receptor, then target cells expressing the ligand for CD244, CD48, should have an increased susceptibility to lysis compared to CD48- targets. As shown in figure 1a, P815 CD48+ targets were more susceptible to lysis than P815 CD48- targets indicative of an activating function for the *z* allele of CD244. We also engaged the receptor with allele specific CD244 mAb in a redirected lysis assay. Lysis of FcR+ P815 CD48- targets was increased by the addition of *z* allele specific mAb (Fig 4.1a). Activating function of the *z* allele of CD244 was also observed with IL-2 LAKs derived from 129/SvJ and B6.129c1 mice congenic for 129 at the SLAM family locus (Fig 4.1b). Therefore, the z allele of CD244 appears to activate NK cell cytotoxicity whether engaged by its ligand or by mAb. T cell depleted IL-2 LAKs showed similar activating function for the *z* allele of CD244 indicating CD244 function is restricted to the NK compartment at previously described (data not shown, and(214)). These studies were done with young mice (2-4 months of age) prior to systemic lupus erythematosus (SLE) disease onset to

avoid any complication due to SLE. Furthermore, the use of LAKs from non-autoimmune 129 mice gave the same results as LAKs from B6.*Sle1b* mice showing no influence of SLE on the function of CD244.

As described by Wandstrat et al, some outbred strains of mice, such as *Mus m. castaneus,* have undergone recombination between the *b* and *z* haplotypes at the SF locus (166). The *castaneus* SLAM locus contains unique alleles for CD229, Cs1, and CD244; the remaining SLAM family members are of the *z* haplotype (166). The CD244 allele found in B6.Castc1, a congenic mouse strain containing the SF locus derived from *Mus m. castaneus*, most closely resembles the *b* allele, having only one CD244 gene, in contrast to the *z* haplotype which has a gene duplication at this region. In addition, the Cast CD244 receptor is recognized by the *b* allele specific mAb. To determine the function of the Cast CD244 receptor, IL-2 LAKs generated from these mice were used in chromium release assays with both P815 CD48- and P815 CD48+ target cells in the presence or absence of *b* allele specific mAb. As shown in figure 4.1c, these results showed redirected lysis of both FcR+ P815 CD48- and P815 CD48+ target cells indicating an activating function for the recombinant Cast CD244 receptor when engaged by mAb. However, unlike what was observed for the *z* allele, both target cells were killed equally in the absence of mAb (Fig 4.1c). These data indicate that the B6.Castc1 CD244 receptor also has activating function when engaged by

mAb in redirected lysis assays but unlike what was observed for the *z* allele of CD244, engagement by the natural ligand shows no preferential lysis.

To determine if the activating function of the *z* allele of CD244 requires SAP, B6.*Sle1b* mice were crossed with SAP knockout mice (also of the B6 background) to homozygosity. IL-2 LAKs derived from these mice were used in chromium release assays with both P815 CD48-/+ targets and in redirect lysis assays with FcR+ P815 CD48- targets in the presence of *z* allele specific antibody. If SAP is required for the activating function of the *z* allele, then the absence of SAP would result in no increased lysis of P815 CD48+ targets compared to P815 CD48- and the absence of redirected lysis with the addition of CD244 allele specific mAb. As shown in figure 4.2, the activating function of the *z* allele of CD244 requires SAP expression. As predicted, P815 CD48+ targets show no increased susceptibility to lysis and in fact now show decreased lysis compared to P815 CD48- targets (compare figure 4.1a and 2). The addition of *z* allele specific mAb does not result in redirected lysis of P815 CD48- targets, further supporting the requirement of SAP expression for activating function. This is similar to what has been observed for XLP patients (156-159, 225).

The b allele of CD244 has both activating and inhibitory function

Our previous studies describing inhibitory function for the *b* allele of CD244 used B6 mice housed in a conventional colony. Our current studies characterizing the function of the *z* and Cast alleles of CD244 use B6.*Sle1b* and B6.Castc1 mice, which were housed in a specific pathogen free (SPF) facility under sterile housing conditions. Including the appropriate controls for these experiments required that IL-2 LAKs generated from B6 mice housed in the SPF facility be used to compare with B6.*Sle1b* and B6.*Castc1* LAKs. To our surprise, we observed an activating function for the *b* allele of CD244 (Fig 4.3a) (when engaged by mAb) in B6 NK cells from mice housed in the SPF colony. The addition of *b* allele specific mAb resulted in redirected lysis of both P815 CD48+ and P815 CD48- target cells, similar to the B6.Castc1 activating receptor. However, B6 LAKs showed no preferential lysis of P815 CD48+ targets similar to B6.Cast LAKs. When mice from this same cohort were housed in a conventional facility for at least five weeks and used to generate LAK cultures, an inhibitory function for CD244 was observed (Fig 4.3b). We now observed a preferential lysis of P815 CD48+ targets and addition of the *b* allele specific mAb resulted in increased lysis of only the P815 CD48+ targets. These data show that animal housing conditions can influence function of the *b* allele of CD244. Since adaptor proteins such as SAP and Eat-2 have been shown to play a role in CD244 function, it is possible that differences in housing environments may alter their

expression. To determine if differences in SAP and Eat-2a expression levels could explain these differences in *b* allele function, RNA was isolated from IL-2 expanded NK cells from mice housed in both facilities and used to generate cDNA. Semi-quantitative PCR showed no gross differences in SAP and Eat-2a expression levels between NK cells cultured from B6 mice housed in the SPF or conventional colony (Fig 4.3c). In contrast to the *b* allele, the *z* and Cast alleles of CD244 functions as an activating receptor regardless of housing condition, since LAKs generated from B6.*Sle1b*, B6.*Castc1*, B6.129c1, and 129 mice housed in a conventional facility exhibit the same phenotype as those from mice housed in the SPF facility (data not shown), indicating a unique environmental dependence on CD244 function in B6 mice.

CD244 functions independently of the cellular signaling milieu and other SLAM family members

These studies indicate that B6 and B6.*Sle1b* NK cells show different functions for CD244. These differences in CD244 function must be confined to the only genetic locus at which these mouse strains differ, the 900 kb region surrounding the SLAM family locus. There are several possible explanations to account for the differences in CD244 allele function. One explanation is that differences in the signaling milieu or adaptor molecule expression levels (i.e. SAP, SHP-1, SHP-2, and EAT-2a/b) vary between mouse strains leading to

opposing functions. However, this explanation is unlikely the answer because that would indicate that the *b* and *z* haplotypes of the SLAM family locus differentially regulate adaptor molecule expression levels, which is currently unprecedented for this locus. As shown in figure 4.4, no gross differences in mRNA expression levels of SAP, Eat-2a, and Eat-2b are observed in IL-2 propagated NK cells from B6 and B6.Sle1b mouse strains. A second explanation is that polymorphisms of other SLAM family members may influence or regulate CD244 function. A third possibility is that the primary structure of each CD244 allele may dictate its function.

To address if each allele of CD244 functions independently of other family members or adaptor molecule expression, [B6 X B6.*Sle1b*]F1 mice were used to generate IL-2 LAKs. [B6 X B6.*Sle1b*]F1 mice are of the B6 background except at the SLAM family locus where they are heterozygous for both the *b* and *z* alleles; both alleles were co-expressed on NK cells as determined by flow cytometry (data not shown). The use of allele specific antibodies allows for the independent engagement of each allelic version of CD244. These mice were housed in a conventional colony to ensure that the *b* allele of CD244 developed in an environment conducive to inhibitory function. As shown in figure 4.5a, the addition of the *b* allele specific CD244 mAb does not increase lysis of P815 CD48- targets, similar to results observed for B6 LAKs. In contrast, the addition

of the *z* allele specific CD244 mAb shows redirected lysis of P815 CD48- targets, like that of B6.*Sle1b* LAKs. Since both *b* and *z* alleles showed different cytotoxic outcomes when engaged by CD244 allele specific mAbs even though both receptors are expressed in the same F1 NK cell, this would suggest that the signaling milieu cannot account for divergent function.

As shown in figure 4.5b, the addition of the *z* allele specific mAb to P815 CD48+ targets does not result in increased target cell lysis. However, the addition of *b* allele specific mAb to P815 CD48+ targets does result in increased target cell lysis. A common theme in NK receptor signaling is that inhibitory signals dominate over activating signals. Therefore, the addition of the *z* allele specific mAb to P815 CD48+ targets may not result in increased cytotoxicity because the inhibitory signal of the *b* allele of CD244 interacting with CD48 on the target cell dominates over the activating *z* allele. The addition of the *b* allele specific mAb to P815 CD48+ targets blocks this inhibitory interaction leading to increased target cell lysis. Alternatively, the addition of the *b* allele mAb may allow full engagement of the *z* allele of CD244 by CD48 on the target cell resulting in increased cytotoxicity. To confirm that the *z* allele functions as an activating receptor independent of other SF receptors and to avoid potential inhibitory effects of the *b* allele, CD244 KO mice, generated in the C57BL/6 background, were crossed with B6.*Sle1b* mice, generating NK cells that only contain the *z*

allele of CD244 and are heterozygous for the *b* and *z* alleles for the remaining SF receptors. As shown in figure 4.5c, these [CD244KO x B6.*Sle1b*] F1 NK cells showed increased lysis of P815 CD48+ targets compared to P815 CD48- targets confirming that the *z* allele of CD244 functions as an activating receptor independent of other SF receptors.

Interestingly, we consistently observed an increase in the inhibitory affect of the *b* allele of CD244 in [B6 x B6.*Sle1b*] F1 NK cells following prolonged conventional colony housing (Figure 4.6a, b, and c), where P815 CD48+ targets become less susceptible to lysis compared to P815 CD48- targets. These data show that each CD244 allele functions independently of polymorphisms of other SLAM family members and the cellular signaling environment functioning as expected for B6 and B6.*Sle1b* NK cells, where the *b* allele is inhibitory and the *z* allele is activating within the same F1 NK cell.

Comparison of primary sequence of CD244 transcripts in IL-2 cultured NK cells

The data presented above indicate that neither the signaling environment nor polymorphisms at other SF receptors dictates CD244 allele specific function. Therefore, differences in primary receptor structure could explain the differences in function of each CD244 allele. To compare primary structure of each allele of CD244, the predominant transcripts encoding the *b*, *z*, and *castaneus* CD244

receptors were isolated from B6, B6.*Sle1b*, and B6.*Castc1* IL-2 LAKs. A primer common to all versions of CD244 anchored in exon 1 was used in combination with 3'RACE to isolate cDNAs. Sequence comparisons showed that the isolated *b* allele sequence was the same as that previously published for CD244 from B6 mice (200). cDNAs encoding both CD244S and CD244L isoforms were isolated for B6.*Castc1*. Similar to B6, the predominant CD244 isoform expressed in IL-2 LAK cultures from B6.*Castc1* mice is the long form as determined by standard PCR with isoform specific primers (Fig 4.7a) and by real time PCR (Fig 4.7b). Despite CD244 gene duplication at the *z* locus, one predominant transcript was isolated from B6.*Sle1b* LAKs, which corresponded with the sequence previously published from 129 mice (251). Interestingly, transcripts from the three other CD244 genes were not isolated from NK cell cultures even though RT-PCR with exon specific primers did isolate these cDNAs from total spleen RNA (166).

Alignment of the open reading frames encoded by the predominantly expressed CD244 transcripts identified two regions in the cytoplasmic domain at amino acids 293 and 318 that differ between the activating (*z* and *castaneus*) forms and the inhibitory (*b)* form of CD244 (Fig 4.8). The first single polymorphism at amino acid 293 is an Asp in the activating forms and an Ala in the inhibitory form. The second double polymorphism at amino acids 318 and 319 is an Asn and Ser or a Thr and Phe for the activating and inhibitory forms

respectively. While these two regions do not contain any known signaling motifs, they may alter the interaction of the receptor with important adaptor molecules, thus affecting CD244 signaling. Interestingly, all four ITSM motifs are conserved between the various CD244 receptors.

The crystal structure of CD244 has recently been published, implicating the CC' and FG-loops of the V-Ig domain as regions critical for CD48 binding (252). The region of greatest difference between murine CD244 receptors is in the CD48 binding domain found in the extracellular V-Ig as shown in figure 4.8. The Cast and *b* allele receptors have similar CC' and FG-loop sequences, while the *z* allele differs greatly from the other two receptors in these regions. There are 2 amino acid differences in the CC' loop between B6 and Cast and six residues different between these 2 alleles and the predominantly expressed *z* allele of CD244. Similarly, the Cast and *b* alleles differ only at one amino acid in the FG-loop while the *z* allele differs from the other 2 alleles at 5 amino acids in this region. These differences may affect CD244:CD48 binding affinities via altering CD244 conformation in the CD48 binding pocket and are likely the epitopes for the allele specific antibodies.

DISCUSSION

In the present study, we have characterized the function of both the *b* and *z* alleles of CD244, where the more commonly occurring *z* allele consistently exhibits activating function. As shown in figure 4.3, the *b* allele of CD244 can exhibit activating and inhibitory function dependent on environmental influences. Previously, housing environment has been shown to influence NK cell function in bone marrow transplantation where B6: 129 perforin KO mice housed in an SPF facility readily reject TAP-1 KO BMC but mice housed in a conventional colony are unable to mediate rejection (253). There is also evidence in human populations of environmental influences on SLAM family receptor function; for example, disease severity in XLP and Common Variable Immunodeficiency Disease varies between patients with the same SAP mutations and even within members of the same family (reviewed in (127)). Our studies further show that environmental influences can modulate SLAM family function, specifically CD244 function.

Several studies have characterized the activating function of the *b* allele of CD244 (109, 131, 218). Studies have also shown this activating function is dependent on SAP expression (218). Whereas, studies detailing the inhibitory function of the *b* allele of CD244 show inhibition is independent of SAP expression (214, 215). Although, semi-quantitative PCR showed no gross

differences in SAP expression levels between mice housed in the conventional and SPF colonies, subtle differences in SAP expression may still influence CD244 function (Fig 4.3c). Alternatively, changes in other signaling molecules may influence the function of the *b* allele of CD244. Interestingly, these same changes do not seem to affect the *z* allele of CD244. Our studies detailing differences in the function of the *b* allele of CD244 due to housing environment may explain the conflicting results of previously published studies describing the function of the *b* allele of CD244 (214, 215, 218).

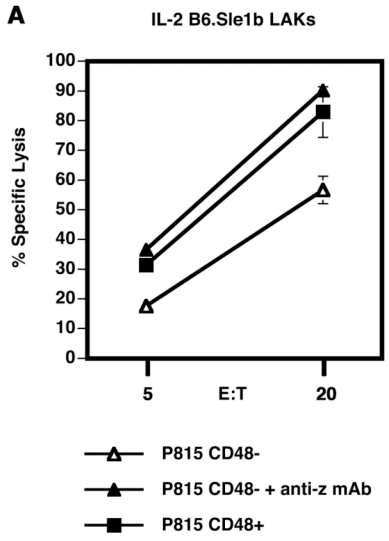
Previously, Wandstrat et al have shown that the *z* allele of CD244 is the common haplotype and that the *b* allele is the less common form of CD244 in the murine immune system(166). Our studies show that the *z* haplotype of CD244 functions as an activating receptor dependent on SAP expression regardless of housing environment (Fig 4.1a and b, Fig 4.2, and data not shown). These results are very similar to what has been observed in the human system (109, 202, 225). Interestingly, the *z* allele, which is the more commonly found haplotype, can only be found as one predominant transcript in B6.*Sle1b* NK cultures also similar to human CD244 (202, 203). Thus, the common function of murine CD244 may be that of an activating receptor; whereas, the inhibitory function of the *b* allele of CD244 may be a unique function of that receptor in C57 derived mouse strains.

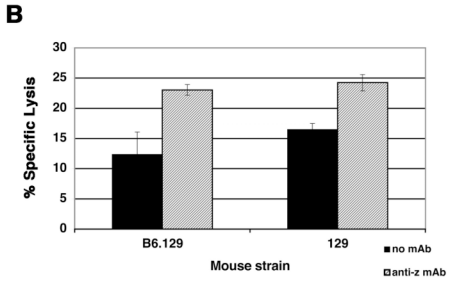
Sequence analysis between *b*, *z*, and Cast CD244 receptors shows that the region of greatest divergence is located in the extracellular CD48 binding domain. Recent structural studies, which modeled rat CD244 binding CD48, have shown that the CC' and FG loops of the V-Ig domain are important for ligand binding (252). Human CD244 studies have also shown two residues (Lys^{68} and Glu^{70}) located in the C'_2 region in the V-Ig domain to be critical for CD48 binding (254). The CC' and FG loops of the *b* and Cast CD244 receptors differ only at one or two amino acids, respectively. This suggests that they may have similar binding affinities for the CD48-ligand. As shown in figure 4.8, the CC' and FG loop regions of the *z* allele differ from the other two receptors at multiple sites. These polymorphisms may influence CD244 receptor function by altering CD48 binding affinities, where the strength and duration of binding dictates receptor signaling and function. The idea that signal strength can alter function in not novel. Signal strength has been shown to alter TLR function in macrophages, where lower doses of ligand engagement inhibits and higher doses stimulate inflammatory cytokine production (255).

The activating *z* and Cast CD244 receptors also differ from the inhibitory *b* CD244 receptor at two regions in the cytoplasmic domain as shown in figure 4.8. Intriguingly, while all four ITSMs are conserved between the allelic versions of CD244, the polymorphisms are located between the first and second ITSM.

These polymorphisms may influence adaptor molecule binding to the surrounding ITSMs thus altering CD244 function. In human studies, the third ITSM of CD244 has been shown to bind multiple adaptor molecules and can negatively influence CD244 signaling indicating ITSM specific effects (235). It is possible that these polymorphisms at residues 293 and 318 change the ability of the nearby ITSMs to bind potential negative signaling molecules (SHP-1, SHP-2, or SHIP) thereby altering the function of CD244. Future experiments reconstituting CD244 function in CD244 KO mice with each allele and various substitution mutants are needed to determine the mechanism of divergent CD244 function.

In summary, these data show that the *z* allele of CD244 functions as an activating receptor dependent on SAP expression similar to human CD244. We also show that the *b* allele of CD244 can function as an activating or inhibitory receptor dependent on environmental conditions. Further studies addressing how environmental influences may alter SLAM family receptor signaling are necessary. Finally, CD244 primary structure polymorphisms may dictate CD244 function and future studies will focus on defining the mechanism by which these polymorphisms may regulate CD244 function.


A
IL-2 B6.Sle1b LAKs
% Specific Lysis
100
90
80
70
60
50
40
30
20
10
0
5
E:T
20
P815 CD48-
P815 CD48- + anti-z mAb
P815 CD48+


B
% Specific Lysis
30
25
20
15
10
5
0
B6.129
129
Mouse strain
no mAb
anti-z mAb

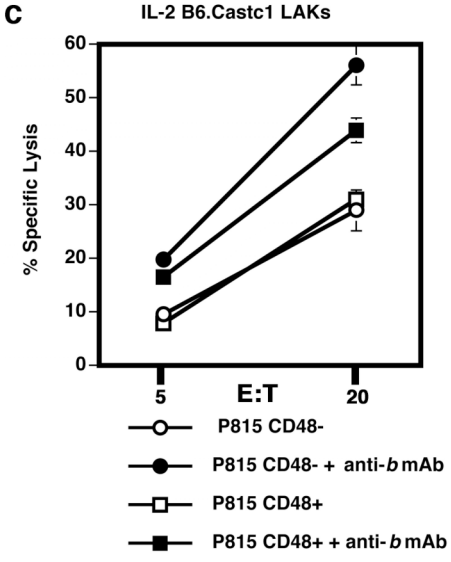

C
IL-2 B6.Castc1 LAKs
% Specific Lysis
60
50
40
30
20
10
0
5
E:T
20
P815 CD48-
P815 CD48- + anti-b mAb
P815 CD48+
P815 CD48+ + anti-b mAb

Figure 4.1: The z and Cast alleles of CD244 exhibit activating function

IL-2 stimulated B6.*Sle1b* LAK (**Panel A**) cells were used as effectors in standard 4-hour chromium release assays with FcR+ P815 CD48- and P815 CD48+ target cells at the indicated ratios. Anti-*z* allele specific mAb was added at 10μg/ml where indicated. ($P<0.01$ CD48- vs CD48- with mAb; $P<0.05$ CD48- vs CD48+) **Panel B**: IL-2 stimulated B6.129c1 and 129/SvJ LAK cells were used as effectors in a standard 4-hour chromium release assay with P815 CD48- target cells at an E:T ratio of 20:1. Anti-*z* allele specific mAb was added at 10μg/ml where indicated. ($P<0.01$ CD48- vs CD48- with mAb for B6.129; $P<.05$ for CD48- vs CD48- with mAb for 129) **Panel C**: B6.*Castc1* cells were used as effectors as described in panel A. Anti-*b* allele specific mAb was added at 10μg/ml where indicated. ($P<0.01$ for CD48- vs CD48- with mAb; $P<0.01$ for CD48+ vs CD48+ with mAb) Results are representative of three independent experiments.

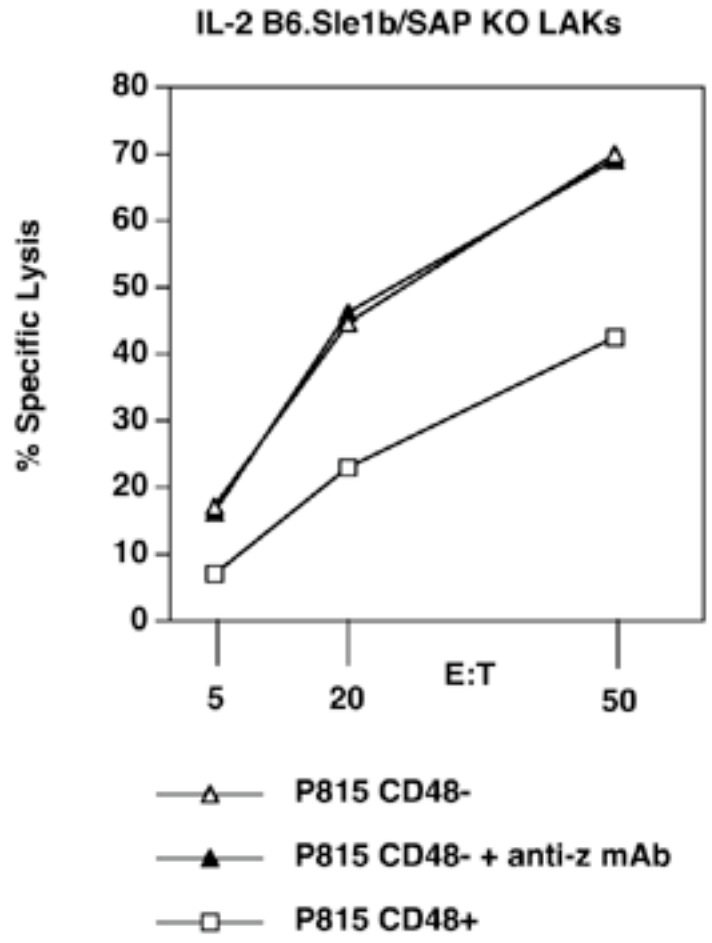
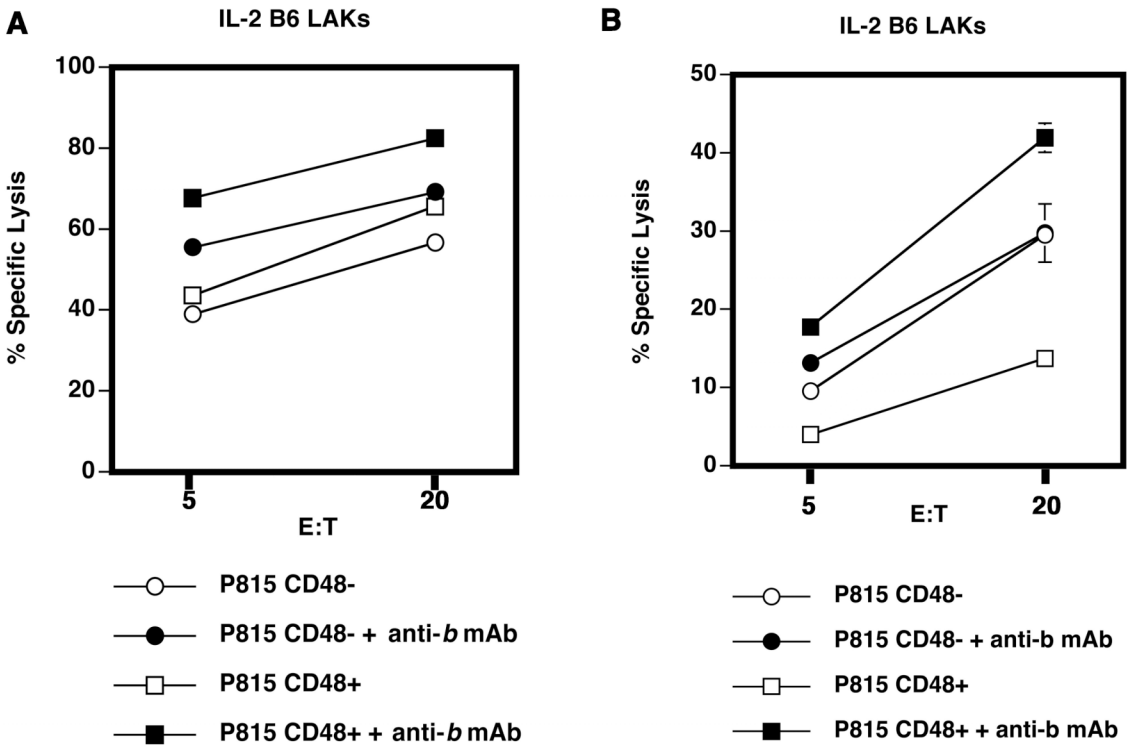


Figure 4.2: The activating function of the *z* allele of CD244 is dependent on SAP expression

IL-2 stimulated B6.*Sle1b*/SAP KO LAK cells were used as effectors in standard 4-hour chromium release assays with P815 CD48- and P815 CD48+ (FcR+) target cells at the indicated ratios. Anti-*z* allele specific mAb was added at 10μg/ml where indicated. Results are representative of three independent experiments. ($P<0.01$ for CD48- vs CD48+; $P<0.001$ for CD48- with mAb)


A
IL-2 B6 LAKs
% Specific Lysis
100
80
60
40
20
0
5
20
E:T
P815 CD48-
P815 CD48- + anti-b mAb
P815 CD48+
P815 CD48+ + anti-b mAb
B
IL-2 B6 LAKs
% Specific Lysis
50
40
30
20
10
0
5
E:T
20
P815 CD48-
P815 CD48- + anti-b mAb
P815 CD48+
P815 CD48+ + anti-b mAb

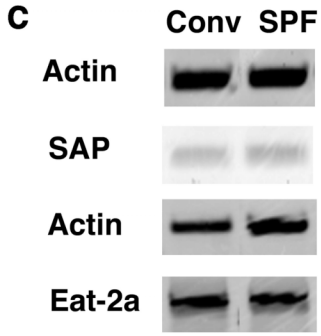

C
Conv SPF
Actin
SAP
Actin
Eat-2a

Figure 4.3: The *b* allele of CD244 has inhibitory and activating function depending on housing conditions

Cohort B6 mice housed in SPF facility (**Panel A**) or conventional facility (**Panel B**) were used to generate IL-2 LAK effectors for 4-hour chromium release assays as described in figure 1. Anti-*b* allele specific mAb was added at 10μg/ml where indicated. ($P<0.001$ for CD48- vs CD48- with mAb SPF facility) **Panel C:** RNA was isolated from sorted IL-2 stimulated NK cells (NK1.1+, CD3-) from B6 mice housed in conventional (Conv) and SPF facilities and used to generate cDNA. SAP and EAT-2a expression was determined by PCR. Actin expression was used to standardize results. Results are representative of three independent experiments.

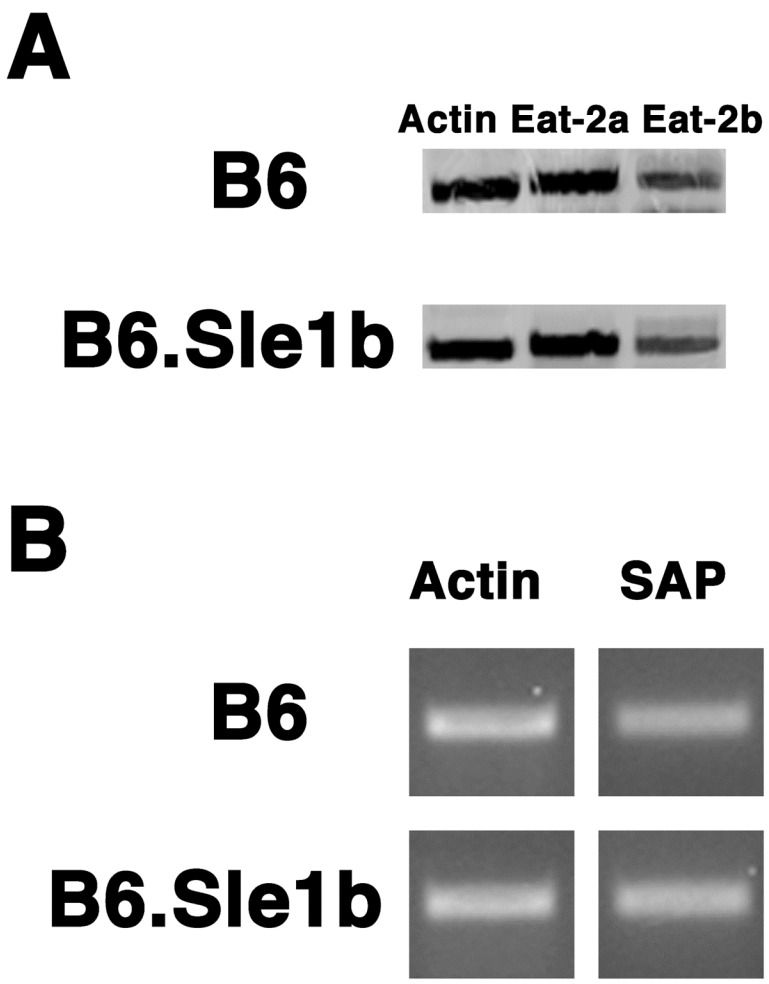
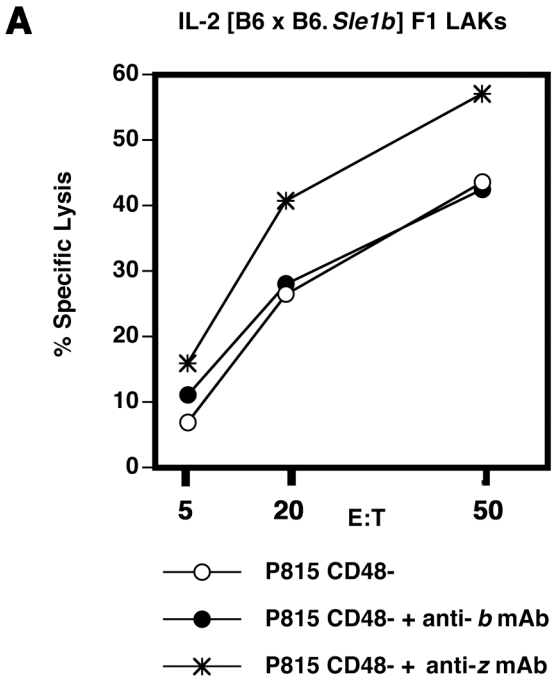
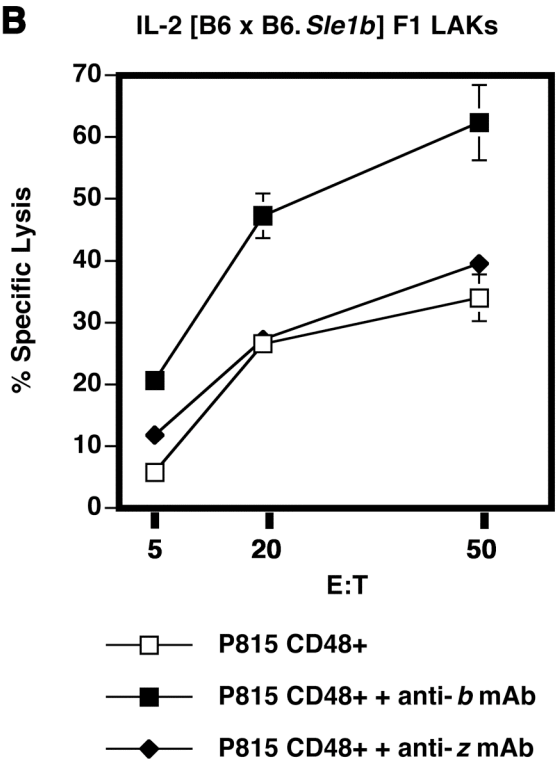


Figure 4.4: B6 and B6.*Sle1b* NK cells have similar Eat-2a, Eat-2b, and SAP expression

RNA was isolated from sorted day 5 IL-2 stimulated NK cells (NK1.1+, CD3-) from B6 and B6.*Sle1b* mice and used to generate cDNA. **Panel A**: Eat-2a, Eat-2b, and actin expression was determined by PCR. **Panel B**: SAP and actin expression was determined by one-step RT-PCR using SAP and Actin specific primers. Actin expression was used to standardize results. Results are representative of three independent experiments.


A
IL-2 [B6 x B6. Sle1b] F1 LAKs
% Specific Lysis
60
50
40
30
20
10
0
5
20
50
E:T
P815 CD48-
P815 CD48- + anti- b mAb
P815 CD48- + anti-z mAb


B
IL-2 [B6 x B6. Sle1b] F1 LAKs
% Specific Lysis
70
60
50
40
30
20
10
0
5
20
50
E:T
P815 CD48+
P815 CD48+ + anti- b mAb
P815 CD48+ + anti- z mAb

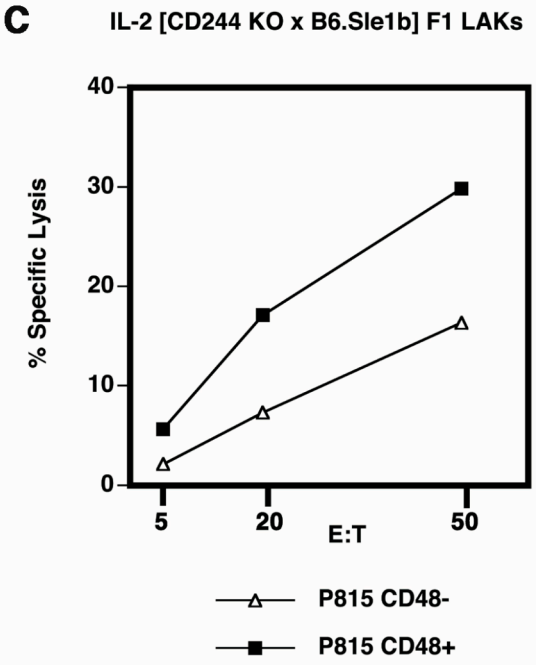

C
IL-2 [CD244 KO x B6.Sle1b] F1 LAKs
% Specific Lysis
40
30
20
10
0
5
20
50
E:T
P815 CD48-
P815 CD48+

Figure 4.5: The *z* and *b* alleles of CD244 function independently in NK cells from F1 mice

IL-2 stimulated [B6 x B6.*Sle1b*] F1 LAK cells were used as effectors in standard 4-hour chromium release assays with P815 CD48- (**Panel A**) and P815 CD48+ (FcR+) target cells (**Panel B**) at the indicated ratios. Anti-*b* and *z* allele specific mAb was added at 10μg/ml where indicated. ($P<0.01$ for CD48- vs CD48- with anti-*z* mAb; $P<0.05$ for CD48+ vs CD48+ with anti-*b* mAb) **Panel C**: IL-2 stimulated [CD244KO x B6.*Sle1b*] F1 LAK cells were used as effectors with P815 CD48- and P815 CD48+ targets. Results are representative of three independent experiments from mice housed in a conventional colony for longer then 5 weeks.

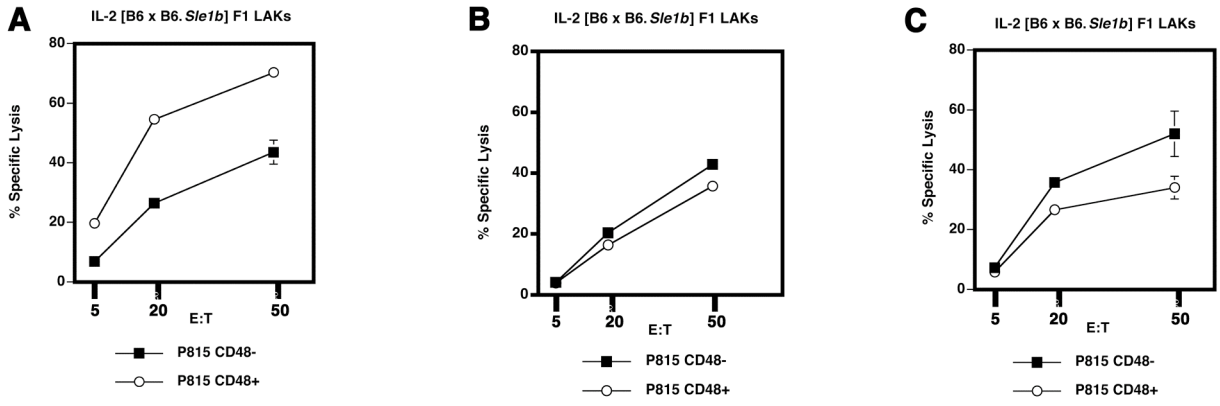


Figure 4.6: Prolonged exposure to conventional housing conditions enhances the inhibitory function of the b allele of CD244

IL-2 stimulated [B6 x B6.Sle1b]F1 LAK cells were used as effectors in chromium release assays with P815 CD48- and P815 CD48+ target cells after being housed in a conventional colony for **Panel A:** 5 weeks, **Panel B:** 6 weeks, and **Panel C:** 7 weeks. ($P<0.001$ for CD48- vs CD48+, 5 weeks; $P<0.05$ for CD48- vs CD48+, 7 weeks)

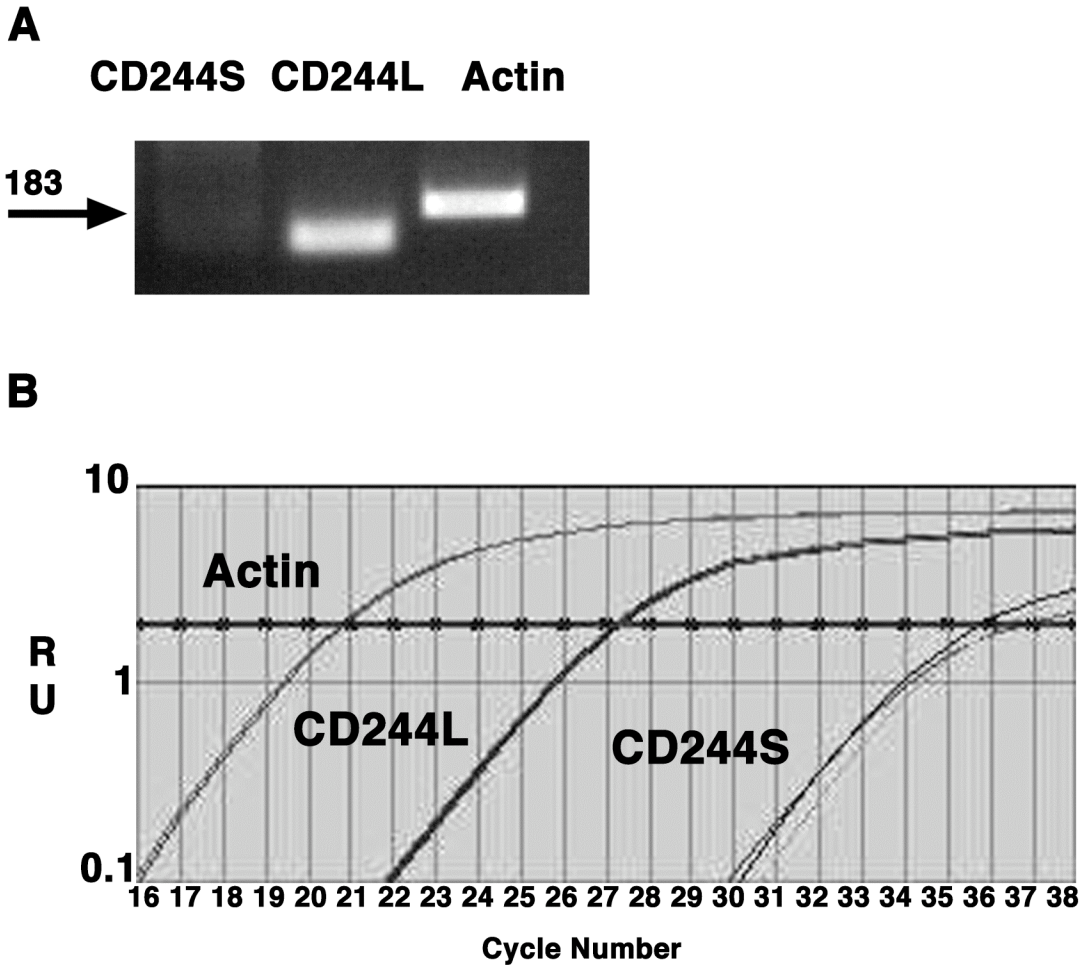


Figure 4.7: CD244L is the predominately expressed version of CD244 in B6.Castc1 NK cells

RNA was isolated from sorted NK1.1+ CD3- IL-2 B6.*Castc1* LAKs and used to synthesize cDNA. **Panel A**: Standard PCR was run at 30 cycles with isoform specific and actin primers where indicated. CD244S migrates at 183 bps; CD244L migrates at 186 bps. **Panel B**: An amplification plot representative of three real-time PCR experiments with isoform specific primes or actin primers is shown. All samples were run in triplicate.

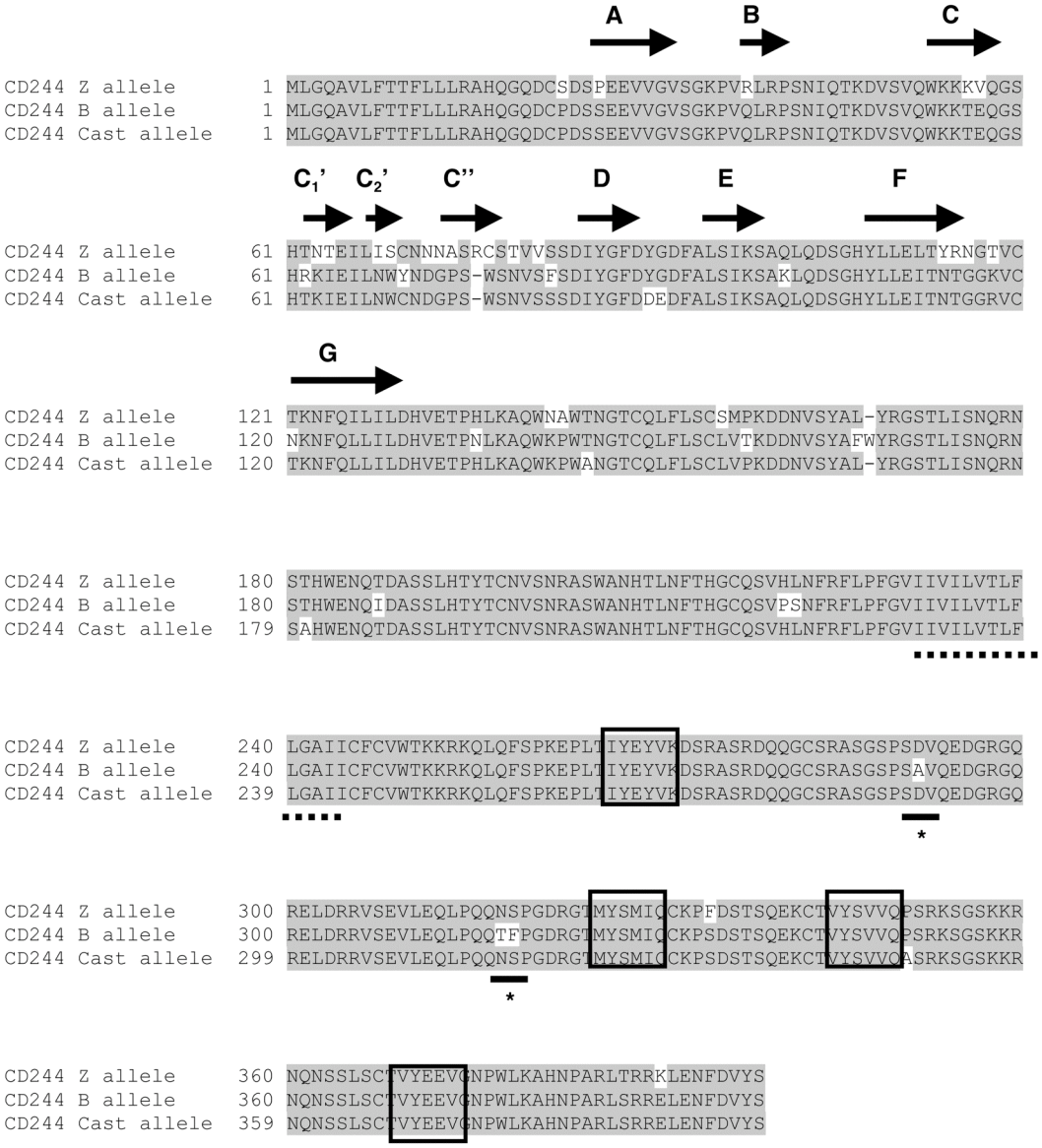

A
B
C
CD244 Z allele 1 MLGQAVLFTTFLLLRAHQGQDCSDSPEEVVGVSGKPVRLRPSNIQTKDVSVQWKKKVQGS
CD244 B allele 1 MLGQAVLFTTFLLLRAHQGQDCPDSSEEVVGVSGKPVQLRPSNIQTKDVSVQWKKTEQGS
CD244 Cast allele 1 MLGQAVLFTTFLLLRAHQGQDCPDSSEEVVGVSGKPVQLRPSNIQTKDVSVQWKKTEQGS
C1' C2' C'' D E F
CD244 Z allele 61 HTNTEILISCNNNASRCSTVVSSDIYGFDYGDFALSIKSAQLQDSGHYLLELTYRNGTVC
CD244 B allele 61 HRKIEILNWYNDGPS-WSNVSFSDIYGFDYGDFALSIKSAKLQDSGHYLLEITNTGGKVC
CD244 Cast allele 61 HTKIEILNWCNDGPS-WSNVSSSDIYGFDDEDFALSIKSAQLQDSGHYLLEITNTGGRVC
G
CD244 Z allele 121 TKNFQILILDHVETPHLKAQWNAWTNGTCQLFLSCSMPKDDNVSYAL-YRGSTLISNQRN
CD244 B allele 120 NKNFQLLILDHVETPNLKAQWKPWTNGTCQLFLSCLVTKDDNVSYAFWYRGSTLISNQRN
CD244 Cast allele 120 TKNFQLLILDHVETPHLKAQWKPWANGTCQLFLSCLVPKDDNVSYAL-YRGSTLISNQRN
CD244 Z allele 180 STHWENQTDASSLHTYTCNVSNRASWANHTLNFTHGCQSVHLNFRFLPFGVIIVILVTLF
CD244 B allele 180 STHWENQIDASSLHTYTCNVSNRASWANHTLNFTHGCQSVPSNFRFLPFGVIIVILVTLF
CD244 Cast allele 179 SAHWENQTDASSLHTYTCNVSNRASWANHTLNFTHGCQSVHLNFRFLPFGVIIVILVTLF
CD244 Z allele 240 LGAIICFCVWTKKRKQLQFSPKEPLTIYEYVKDSRASRDQQGCSRASGSPSDVQEDGRGQ
CD244 B allele 240 LGAIICFCVWTKKRKQLQFSPKEPLTIYEYVKDSRASRDQQGCSRASGSPSAVQEDGRGQ
CD244 Cast allele 239 LGAIICFCVWTKKRKQLQFSPKEPLTIYEYVKDSRASRDQQGCSRASGSPSDVQEDGRGQ
*
CD244 Z allele 300 RELDRRVSEVLEQLPQQNSPGDRGTMYSMIQCKPFDSTSQEKCTVYSVVQPSRKSGSKKR
CD244 B allele 300 RELDRRVSEVLEQLPQQTFPGDRGTMYSMIQCKPSDSTSQEKCTVYSVVQPSRKSGSKKR
CD244 Cast allele 299 RELDRRVSEVLEQLPQQNSPGDRGTMYSMIQCKPSDSTSQEKCTVYSVVQASRKSGSKKR
*
CD244 Z allele 360 NQNSSLSCTVYEEVQNPWLKAHNPARLTRRKLENFDVYS
CD244 B allele 360 NQNSSLSCTVYEEVQNPWLKAHNPARLSRRELENFDVYS
CD244 Cast allele 359 NQNSSLSCTVYEEVQNPWLKAHNPARLSRRELENFDVYS

Figure 4.8: Sequence alignment of the activating (*z* and Castc1) and the inhibitory (*b*) CD244 receptors

A 5' anchored primer common to all versions of CD244 in exon 1 was used in conjunction with 3' RACE to isolate the predominant cDNAs for CD244 expressed in LAK cultures from B6, B6.*Sle1b*, and B6.Castc1 mice. Sequences were aligned and areas of similarity are shaded grey. The β-sheets of the extracellular V-Ig domain are labeled with arrows. The transmembrane domain is underlined by a dotted line. Cytoplasmic regions differing between the activating and inhibitory CD244 forms are denoted by underline and asterisk. Boxes denote ITSM motifs.

CHAPTER V

CHARACTERIZATION OF ALTERED CD4+ T CELL PHENOTYPES DUE TO POLYMORPHISMS AT THE *SLE1B* LOCUS

OBJECTIVE

Recent studies have determined that the SLAM family (SF) exists as two stable haplotypes, *b* and *z*, in common laboratory strains of mice. The *b* haplotype is found in all C57-derived mouse strains. Whereas, the *z* haplotype is found in all other common laboratory mouse strains. C57-derived mouse strains do not spontaneously develop systemic lupus erythematosus (SLE). However, if the *z* haplotype of the SLAM family is crossed onto the C57BL/6 genetic background (B6.*Sle1b* mice), these mice now produce high titers of anti-nuclear autoantibodies (ANA), a phenotype of SLE. Adoptive transfer experiments have shown that the presence of the *z* haplotype (*Sle1b*) in B and T lymphocytes is required for ANA development. Therefore, we wanted to characterize phenotypically T cell function prior to disease onset in B6.*Sle1b* mice compared to non-disease prone B6 mice to identify possible differences in the T cell compartment that could contribute to a loss in tolerance resulting in ANA production. To address this question we wanted to look at specific T cell functions that SLAM family receptors were known to regulate using our congenic mouse

strains. SAP is a signaling molecule critical for SF receptor function, and studies using SAP KO mice described that SF receptor signaling through SAP is required for CD4+ T cell help to B cells. CD4+ T cells from SAP KO mice also have decreased Th2 cytokine production and altered expression of B cell co-stimulatory ligands. These CD4+ T cell defects alter B lymphocyte functions; SAP KO mice have decreased immunoglobulin class switch and lack long-term memory B cells. Based on the altered T and B functions observed in SAP KO mice and the fact that a difference in SF haplotype in the context of the C57BL/6 genome results in ANA production, we hypothesized that polymorphisms at the SLAM family locus alter T cell functions. The studies presented in this chapter examine the role of each haplotype of the SLAM family in CD4+T cell function.

INTRODUCTION

Congenic dissection of the lupus-prone NZM2410 mouse strain identified causative loci involved in SLE disease development (163, 175, 176). These studies determined that the *Sle1* locus (located on chromosome 1 flanked by *B4galt3* and *Copa*) is responsible for a loss of tolerance to chromatin in both T and B cells. In addition to a loss in chromatin tolerance, these mice also had expanded populations of activated CD4+ T cells. Fine mapping of the *Sle1* locus identified four loci, termed *Sle1a-d* (163). Although each locus plays a role in disease, the contribution of the *Sle1b* locus is the strongest, mediating gender-

biased and highly penetrant ANA production in the B6.*Sle1b* congenic mouse strain. Fine mapping of the *Sle1b* locus revealed that the SLAM family of receptors is located in the center of the *Sle1b* interval (166). Sequence analysis of 34 laboratory strains of mice determined that the SLAM family of receptors exists as two stable haplotypes, termed the *b* haplotype, found in C57-derived mouse strains, and the *z* haplotype, found in non-C57 derived mouse strains. Interestingly, the presence of the *z* haplotype in the context of the C57BL/6 genome (B6.*Sle1b* and B6.129 congenic mice) causes ANA production in an otherwise non-autoimmune genetic background. However, the *z* haplotype in the context of non-C57 derived genomes, such as the 129 background, does not result in ANA production. These observations suggest that each SF haplotype in the context of a specific genetic background may cause divergent function in T and B cells.

The SLAM family of receptors includes SLAM (108), CD48 (114), CD84 (110), CS1 (113), Ly108 (112), CD244 (109), and CD229 (111). This family of receptors is expressed on cells of hematopoietic origin. All SLAM family members have a common structure. They contain an extracellular signal sequence followed by a V-Ig domain, a C2-Ig domain, a transmembrane domain, and most SF members contain an intracellular signaling domain. CD48, which is GPI-linked, is the exception (114). The V-Ig and C2-Ig domains are duplicated in

CD229 (111). The SF family of receptors exhibit either homotypic binding or SLAM intrafamilial receptor-ligand binding. SLAM (117), CD299 (118), Ly108 (119, 120), CD84 (121), and CS1 (122) bind homotypically; while CD244-CD48 exhibit intrafamilial binding (123, 124). The signaling domains in the cytoplasmic tails of SF members contain one or more immunoreceptor tyrosine-based switch motifs (ITSM) that are composed of the consensus sequence TxYxxV/I. These ITSM motifs bind SH2 domain containing proteins, such as SLAM-associated protein (SAP), Eat-2a, Eat-2b, SHP-1, SHP-2, and SHIP (reviewed in (115, 127, 129, 130)). ITSM recruitment of these SH2 domain-containing proteins can trigger activating or inhibitory signals depending on which adaptor molecule is recruited. Mice deficient for these signaling molecules have aberrant lymphocyte function (136, 181-183, 256).

B6.*Sle1b* mice have altered T and B cell function leading to ANA production. SAP KO mice also have altered T and B cell function, however the defects observed in SAP KO mice result in decreased immunoglobulin secretion. CD4+ T cells from SAP KO mice have marked defects in CD4+ T cell functions including altered cytokine production. Under polarizing culture conditions, CD4+ SAP KO T cells inherently have the ability to differentiate into Th1 and Th2 cells. However, following TCR stimulation or upon LCMV or Leishmania *major* challenge SAP KO T cells produce increased amounts of IFN-γ and TNF-α, and

decreased amounts of IL-4, 10 and 13 (181, 182, 185). This altered cytokine profile skews B cell class switch in SAP KO mice, resulting in decreased levels of IgG subclasses associated with IL-4 mediated switch, and decreased IgE levels (181-183). In addition to altered cytokine profiles, SAP KO mice have defective B cell help due to a lack of ICOS expression (Schwartzberg unpublished observation). SAP KO mice also have impaired long term B cell immunity and defective plasma cell generation due to the inability of CD4+ T cells to interact with B cells in a productive manner. Although SAP KO and B6.*Sle1b* mice have aberrant T-dependent immune responses, where SAP KO mice have decreased Ig production and B6.*Sle1b* mice have increased ANA Ig production, both mouse strains have normal T-independent immune responses (personal communication C. Mohan, and (190)). Furthermore, mice deficient in Ly108, SLAM, and CD48 expression also have altered CD4+ T cell phenotypes (195, 197, 198). These observations suggest that SF signaling plays an important role in B and T cell interactions critical for the development of a normal humoral immune response.

Based on the aberrant T and B cell immune phenotypes observed in B6.*Sle1b*, SAP KO, and specific SF receptor KO mice, we hypothesized that polymorphisms at the SF locus may alter CD4+ T cell function. Therefore, we characterized CD4+ T cell phenotypes in mice congenic for the *b* and *z* alleles of the SF. B6.*Sle1b* CD4+ T cells containing the *z* haplotype have increased

expression of Ly108-1, CD84, CD48, SLAM and CD229 compared to B6 CD4+ T cells of the *b* haplotype following activation through the TCR. In addition B6.*Sle1b* CD4+ T cells exhibit decreased IFN-γ, IL-4, -5, -6, and IL-10 cytokine production and increased SAP and CD40L expression following stimulation through the TCR. These observations show that polymorphisms at the SF locus do alter CD4+ T cell function in the context of the C57BL/6 genetic background.

RESULTS

Haplotype divergence at the SF locus results in altered SF expression profiles in CD4+ T cells

Previously, Wandstrat *et al* have shown that the SF exists as two stable haplotypes, *b* or *z*. Wandstrat *et al* compared SF expression in freshly isolated splenic CD4+ T cells from B6 mice containing the *b* haplotype of the SF and B6.*Sle1b* mice, a congenic mouse strain containing a 900 kb congenic interval where the *z* haplotype of the SF is located at the center of the interval (166). These studies showed decreased CS1, CD48, SLAM, CD229, and Ly108-2 expression in B6.*Sle1b* mice compared to B6 mice (166). In contrast, CD84 was up regulated in B6.*Sle1b* mice compared to B6 mice. Several SF members have also been shown to have multiple splice-generated isoforms. Wandstrat *et al* also did a comparison of SF isoform levels in B6 and B6.*Sle1b* mice revealing a significant difference in isoform usage for both CD229 and Ly108 (166). A

CD229 splice variation was only found in B6.*Sle1b* mice. Ly108 isoforms differ by expressing alternative exons that encode divergent cytoplasmic domains where Ly108-1 contains 2 ITSMs and Ly108-2 contains 3 ITSMs (166). Preferential Ly108 isoform expression was observed between B6 and B6.*Sle1b* mouse stains, where B6 mice preferentially express isoform Ly108-2 and B6.*Sle1b* mice preferentially express isoform Ly108-1 (166).

To determine if differences in SF expression were maintained following CD4+ T cell activation through the TCR, CD4+ T cells were purified from B6 and B6.*Sle1b* splenocytes and stimulated by plate bound anti-CD3 and anti-CD28 mAb for 24 hours. As shown in Figure 5.1, quantitative real-time PCR analysis determined that CD48, CD84, CD229, SLAM, and Ly108-1 have a one to two-fold increase in expression in B6.*Sle1b* CD4+ T cells compared to B6 CD4+ T cells. In contrast, CS1 and Ly108-2 have a three-fold and five-fold decrease in expression in B6.*Sle1b* CD4+ T cells compared to B6 CD4+ T cells respectively. Therefore, the *b* and *z* haplotypes of the SF have different receptor expression profiles prior to and following CD4+ T cell activation. It is also noteworthy that the magnitude of increased SF expression between freshly isolated and activated CD4+ T cells of the *z* haplotype is greater than that observed in CD4+ T cells of the *b* haplotype.

CD4+ T cells of the z haplotype have altered cytokine production profiles

A critical function of CD4+ T cells is cytokine secretion. The cytokines produced by CD4+ T cells help direct the adaptive immune response toward a cell-mediated immune response via secreting inflammatory cytokines such as IFN-γ and TNF-α, or toward a humoral immune response via providing B cell help and secreting IL-4, 5, and 13 (reviewed in (102)). SF receptors and SAP-dependent signaling have been shown to be critical for CD4+ T cell function. SAP KO CD4+ T cells produce increased amounts of IFN-γ and TNF-α, and decreased IL-4, 5, 10, and 13 following TCR engagement (181-183, 185). Further implicating SF receptors in CD4+ T cell cytokine secretion, SLAM and Ly108 KO mice also have altered CD4+ T cell cytokine profiles (197, 198). CD4+ T cells of SLAM KO mice have decreased IL-4 and increased IFN-γ production when stimulated through the TCR (197). Ly108 KO T cells stimulated via anti-CD3 and anti-CD28 mAb or PMA and Ionomycin also have decreased IL-4 production (198).

To determine if haplotype divergence at the SF locus can also alter CD4+ T cell cytokine profiles, CD4+ T cells from B6 and B6.*Sle1b* mice were stimulated by plate bound anti-CD3 and anti-CD28 mAb for 72 hours, to allow for sufficient cytokine accumulation, and supernatant was analyzed by cytokine bead assays. IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-10, and IL-12p70 cytokine

production was quantitated. As shown in figure 5.2a and b, CD4+ T cells of the z haplotype have deceased IFN-γ, IL-4, IL-5, IL-6, and IL-10 secretion compared to CD4+ T cells of the *b* haplotype. TNF-α secretion was similar between CD4+ T cells of the two SF haplotypes. Differences in CD4+ T cell proliferation between B6 and B6.*Sle1b* mouse strains do not account for the decrease in cytokine production observed in B6.*Sle1b* CD4+ T cells because they both proliferate to an equal extent (data not shown). Under polarizing culture conditions, T cells of the *b* and *z* haplotype inherently differentiate into Th1 and Th2 cells (data not shown). These observations suggest that polymorphisms at the SF locus differentially influence cytokine secretion profiles in CD4+ T cells when stimulated through the TCR. Decreased IL-4 and IFN-γ secretion is also observed following stimulation with anti-CD3 mAb alone (C. Nguyen unpublished observation). However, these defects can be overcome by culturing cells in strong polarizing Th1 and Th2 conditions.

Expression of CD40L is increased and prolonged in CD4+ T cells of the z haplotype

In addition to cytokine production, another major function of CD4+ T cells is the ability to give B cell help via T and B cell interactions through co-stimulatory receptors. These interactions are critical for germinal center formation leading to the development of long-term humoral immune responses and the

production of high affinity Igs. CD40L is a costimulatory receptor expressed on recently activated CD4+ T cells, and CD40L is a main costimulatory receptor involved in B cell help (257, 258). B6.*Sle1b* mice have increased ANA Ig production, suggesting that these mice have more efficient CD4+ T cell help. In contrast, SAP KO mice have decreased Ig production as the result of defective CD4+ T cell help, namely decreased ICOS expression (189, 190, 192). Previous studies have shown that CD40L expression on activated T cells can be separated into an early TCR-dependent phase, which occurs between 0 and 24 hours after activation, and a later extended phase that is reciprocally regulated by the cytokines IL-4 and IL-12, where IL-4 represses CD40L expression (259). Consistent with these results, Th1, but not Th2, cells express CD40L for extended periods following stimulation (259). To examine the kinetics of CD40L expression in CD4+ T cells of the *b* and *z* haplotype of the SF, splenic CD4+ T cells were purified and stimulated via plate bound anti-CD3 and anti-CD28 mAb for 72 hours. CD40L expression on CD4+ T cells was assessed by flow cytometry over a time course, as seen in figure 5.3. CD40L expression was significantly ($p>0.05$) increased during the early TCR-dependent phase (T= 6 hrs) of CD40L expression in CD4+ T cells of the *z* haplotype compared to CD4+ T cells of the *b* haplotype. In addition, CD40L expression was prolonged in CD4+ T cells from B6.*Sle1b* compared to B6 mice. Stimulation with anti-CD3 mAb alone did not induce CD40L expression in B6 or B6.*Sle1b* CD4+ T cells. ICOS is

expressed at similar levels between CD4+ T cells of the *b* and *z* haplotype following CD3 and CD28 stimulation (data not shown). These data suggest that in addition to altered SF expression and cytokine production, polymorphisms at the SF locus alter CD40L expression following TCR stimulation.

Activated CD4+ T cells of the z haplotype have increased SAP expression compared to CD4+ T cells of the b haplotype

SF receptors are known to associate with the signaling adaptor molecule SAP, which is critical for proper SF signaling. In the absence of SAP, CD4+ T cells have increased IFN-γ production and decreased IL-4, 5, and 10 production (181, 182, 185). It has also been shown that over expression of SAP leads to decreased IFN-γ production and increased IL-4 production (185, 187). Thus, SAP expression can alter cytokine production of stimulated CD4+ T cells. Surprisingly, many of the CD4+ T cell phenotypes we have observed for B6.*Sle1b* mice are similar to what has been described for SAP KO mice. Therefore, to determine if differences in SAP expression correlate with the differences in cytokine profiles observed between B6.*Sle1b* and B6 CD4+ T cells SAP expression was determined by PCR following TCR stimulation with plate bound CD3 and CD28 mAbs. RNA isolated from CD4+ T cells stimulated over a time course was converted to cDNA and analyzed for SAP expression. As shown in figure 5.4, after 24 hours of stimulation CD4+ T cells of the *z* haplotype have

increased SAP expression compared to T cells of the *b* haplotype. SAP transcript is undetectable after 24 hours of stimulation in CD4+ T cells from both B6 and B6.*Sle1b* mouse strains (data not shown). Thus altered SAP expression may influence signaling through SF receptors of the *z* haplotype leading to altered cytokine production. Alternatively, increased SAP expression may be the result of changes in the cytokine microenvironment of B6.*Sle1b* mice.

DISCUSSION

Activated CD4+T cells of the *z* haplotype of the SF have increased CD84, CD229, SLAM, and Ly108-1 expression compared to CD4+ T cell of the *b* haplotype. These SF receptors are also expressed on B cells, and exhibit homotypic binding. Therefore, increased expression of SF receptors on B6.*Sle1b* CD4+ T cells could increase the strength of costimulatory signals between T and B cells augmenting both CD4+ T and B cell functions. It has been shown that co-engagement of CD40 and SLAM on B cells results in increased B cell proliferation, and soluble SLAM enhances production of IgM, IgG, and IgA by B cells activated with anti-CD40 mAb (260). Therefore, increased SLAM and CD40L expression on T cells could augment T-B interactions resulting in increased ANA Ig production. The early stage of CD40L expression in mediated by TCR signaling. Because SF receptors can act as costimulatory receptors, increased SF expression could alter TCR signaling resulting in increased CD40L

expression, such as that observed in B6.*Sle1b* CD4+ T cells (Figure 5.3). These observations suggest that altered B-T cell interactions through SF receptors and/or CD40L could influence peripheral B and T cell tolerance by lowering the signaling threshold required for B cell survival. Therefore, autoreactive ANA-specific B cells that would normally be eliminated in the periphery by a lack of T cell help are now able to survive. Thus, altered T-B interactions could ultimately lead to a break in tolerance and ANA production.

CD4+ T cells from B6.*Sle1b* mice have altered cytokine production following TCR engagement compared to CD4+ T cells from B6 mice. As shown in figure 5.2a and b, B6.*Sle1b* CD4+ T cells have decreased IFN-γ, IL-4, IL-5, IL-6, and IL-10 cytokine production compared to B6 CD4+ T cells. This altered cytokine production may influence immune responses elicited in B6.*Sle1b* mice. It has been shown that the ANAs produced by B6.*Sle1b* mice are predominately of Th1 IgG subclasses, which may be due to the decreased IL-4, IL-5, and IL-10 production observed in these mice. Even though IFN-γ is also decreased in B6.*Sle1b* CD4+ T cells, this decrease in IFN-γ production is not as severe as the global decrease in Th2 cytokine production. Furthermore, upon re-stimulation B6.*Sle1b* CD4+ T cells are not defective in IFN-γ production, but remain defective in IL-4 and IL-5 production (data not shown and C. Nguyen unpublished observation).

In addition, we observed an altered expression profile of SAP in B6.*Sle1b* mice. This increased SAP expression following stimulation could result in altered cytokine production or increased SAP expression could be the result of altered cytokine production. B6.*Sle1b* CD4+ T cells have increased SAP expression following TCR engagement that correlates with decreased IFN-γ production observed in these T cells. B6.*Sle1b* CD4+ T cells stimulated by anti-CD3 and CD28 mAb for three days, and then rested for seven days and re-stimulated produce similar amounts of IFN-γ compared to their B6 cohorts (data not shown). Therefore, one might predict that reactivated CD4+ T cells do not express high levels of SAP, explaining comparable IFN-γ production between B6 and B6.*Sle1b* CD4+ T cells. These observations suggest that SAP expression can be modulated by the haplotype divergence of the SF receptors during primary CD4+ T cell stimulation, a previously undefined mechanism of SAP regulation.

Collectively, our results indicate that polymorphisms at the SLAM family locus in the context of the C57BL/6 background account for altered CD4+ T cell phenotypes. Activated CD4+ T cells of the *z* haplotype of the SF have increased SF expression, increased SAP and CD40L expression, and altered cytokine profiles compared to CD4+ T cells of the *b* haplotype. The increased SF expression observed in CD4+ T cells of the *z* haplotype could result in

qualitatively different signaling between T and B cells potentially allowing weakly reactive B cells to survive. Over time, accumulation of these auto-reactive B cells in the context of a Th1 biased environment could lead to ANA production and the inflammatory phenotypes associated with SLE. Recently, it has been shown that the presence of the epistatic suppressive modifier *Sles1* can ablate ANA production in B6.*Sle1b* mice (180). Therefore, comparing CD4+ T cell phenotypes in B6.*Sle1bSles1* mice with those observed in B6.*Sle1b* mice will allow the phenotypes required for ANA production and a break in tolerance to be identified as these should return to a B6 profile in B6.*Sle1b*/*Sles1* mice.

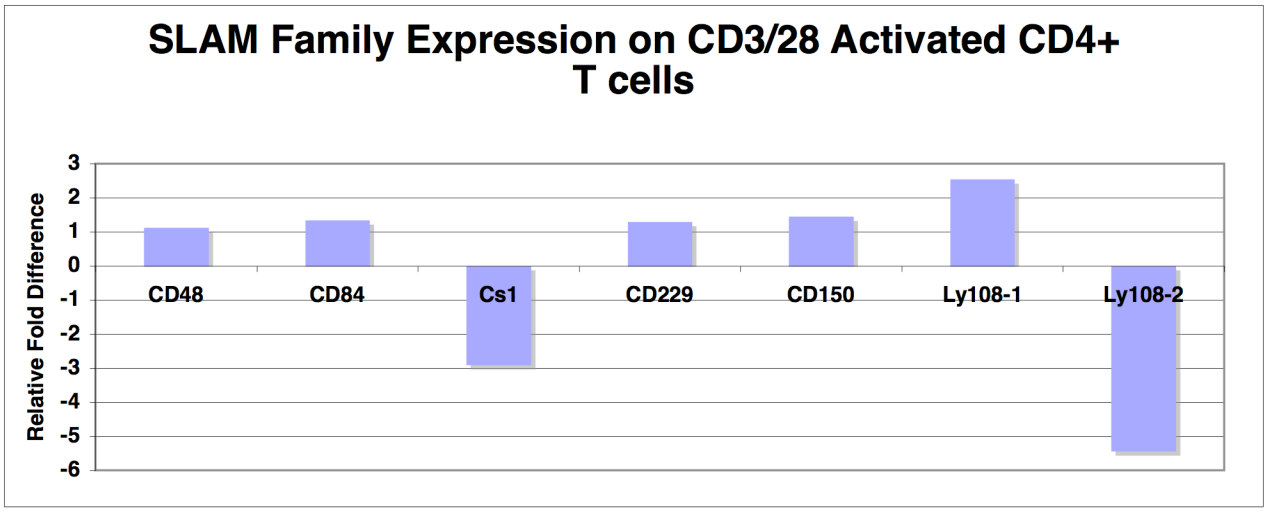
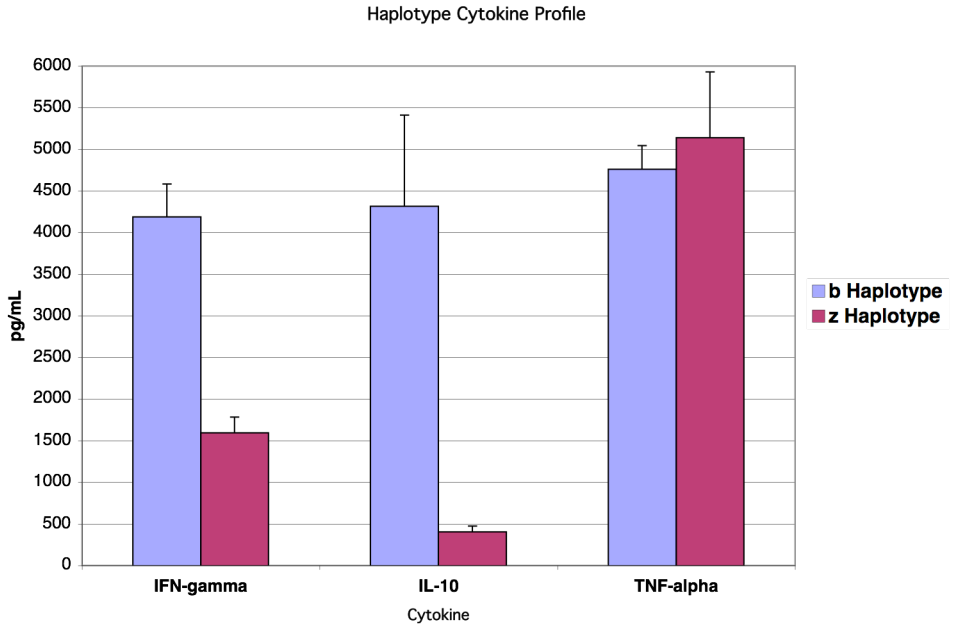


Figure 5.1: SLAM family Expression on CD4+ T cells

Splenic CD4+ T cells were isolated from 6-8 week old B6 and B6.*Sle1b* female mice. Real-time PCR was used to assess SLAM family expression normalized to β2M in activated CD4+ T cells. CD4+ T cells were activated for 24 hrs with plated bound anti-CD3 at 1μg/ml and CD28 at 10μg/ml mAb. Date is representative of 3 independent experiments. (Real Time Data provided by C. Nguyen)


Haplotype Cytokine Profile
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
pg/mL
IFN-gamma
IL-10
TNF-alpha
Cytokine
b Haplotype
z Haplotype

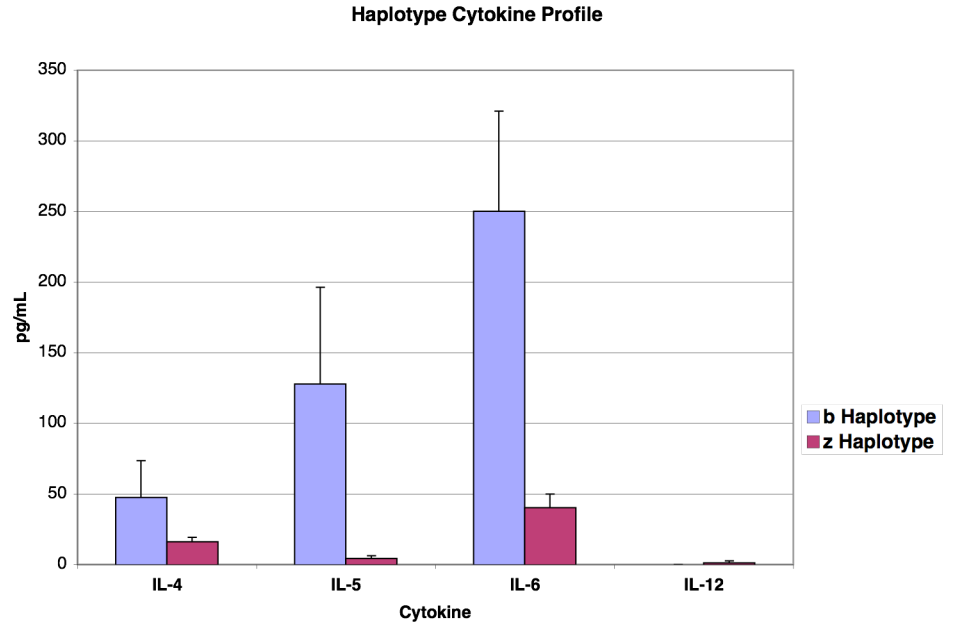

Haplotype Cytokine Profile
350
300
250
200
150
100
50
0
pg/mL
IL-4
IL-5
IL-6
IL-12
Cytokine
b Haplotype
z Haplotype

Figure 5.2: CD4+ T cell Cytokine profiles from B6 and B6.*Sle1b* mice

CD4+ T cells were negatively selected from B6 and B6.*Sle1b* splenocytes. CD4+ T cells were stimulated for 72 hrs with plate bound anti-CD3 at 1μg/ml and CD28 at 10μg/ml mAb. Supernatants were analyzed by CBA. **Panel A:** IFN-γ, IL-10, and TNF-α secretion was analyzed. **Panel B**: IL-4, IL-5, IL-6, and IL-12p70 secretion was analyzed. Data representative of at least 4 individual mice per strain.

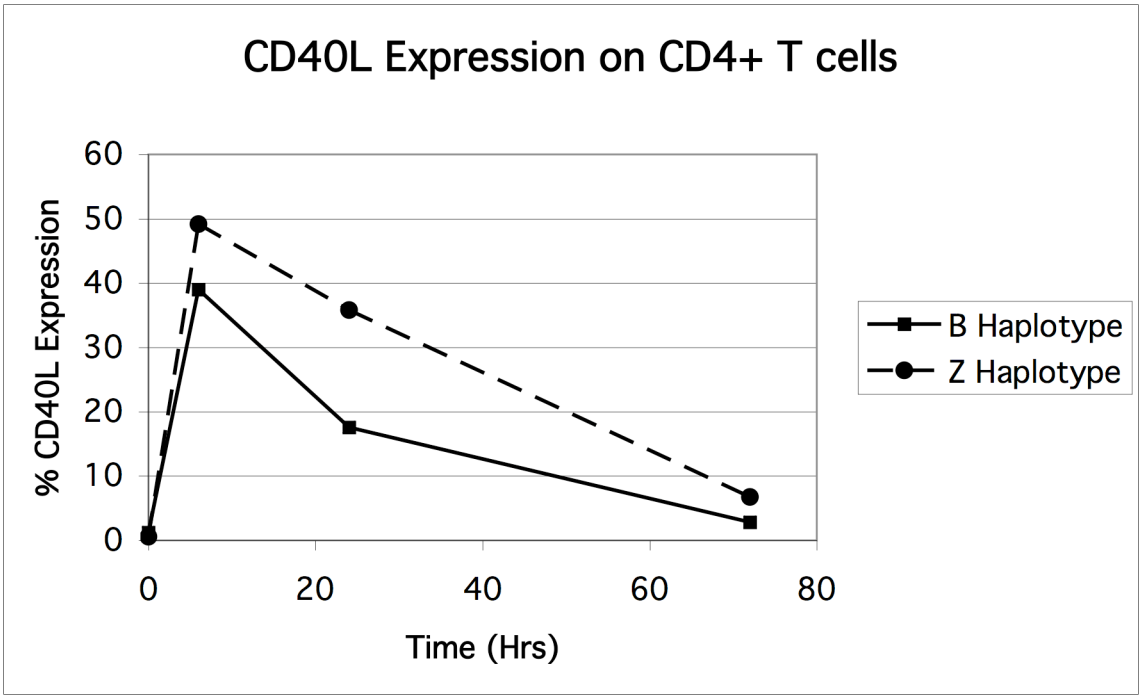


Figure 5.3: B6.*Sle1b* CD4+ T cells have increased and prolonged CD40L expression

CD4+ T cells were negatively selected from B6 and B6.*Sle1b* splenocytes and stimulated over a time course by plate bound anti-CD3 at 1μg/ml and CD28 at 10μg/ml mAb. CD40L expression was analyzed over time by FACS analysis. Data is representative of 5 individual mice per strain. P>0.05 significant at T=6 hrs.

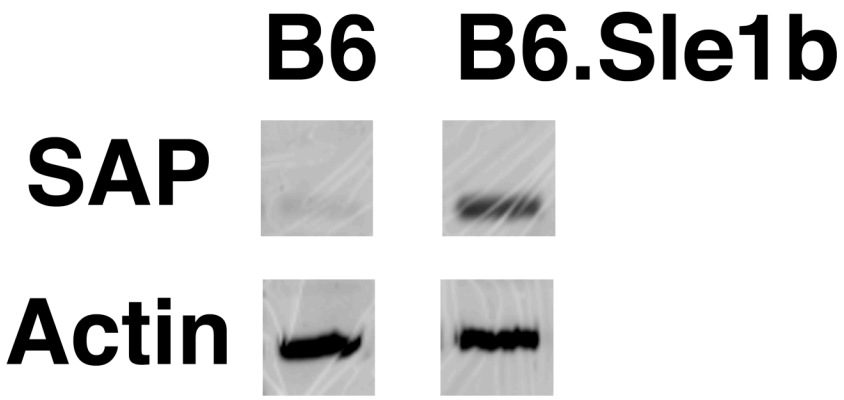


Figure 5.4: B6.*Sle1b* CD4+ T cells have altered SAP expression compared to B6 CD4+ T cells at 24 hrs

CD4+ T cells were isolated and stimulated as described for figure 5.3 for 24 hours. RNA was isolated from T cells and used to make cDNA. SAP and Actin expression was assessed by standard PCR.

DISCUSSION

I. CD244 AND NK CELLS

A. CD244 Function

Prior to the studies on CD244 presented in chapters three and four, murine CD244 was thought to be an activating receptor. Immunization of C57BL/6 and Balb/c mice resulted in the production of two different anti-CD244 antibodies that both identified non-MHC restricted cytotoxic NK and T effector cells (109, 212). In addition to non-MHC restricted cytotoxic NK and T cells, the Balb/c antibody also stained a minor subset of Ig+ splenic B cells (212). Consequential CD244 cloning studies identified multiple CD244 transcripts from Balb/c, CBA, and NZB derived LAK cells compared to B6 derived LAK cells (200). These observations suggested that CD244 differed between mouse strains. Subsequent studies on the B6 version of CD244 identified two alternative splice forms of CD244, in contrast to one transcript found for human CD244 (213). These studies showed that exogenous over expression of these isoforms in the rat NK-16 cell line resulted in opposing functions, suggesting that CD244 function in the murine system may differ from the human. As described in chapter three and by Lee *et al*, CD244 in B6 mice acts as an inhibitory receptor independent of SAP expression (214, 215). In addition, McNereny *et al* also described an inhibitory

role for CD244, where CD244 serves as a non-MHC class I inhibitory receptor preventing the lysis of autologous cells (199). These observations were at odds with the current research detailing an activating function for CD244. A potential explanation for these discrepancies is that the original experiments where conducted before the identification of the ligand for CD244, CD48 (123, 124). Therefore, the addition of anti-CD244 mAb resulting in increased cytotoxicity may actually be due to blocking an inhibitory CD244:CD48 interaction and not the result of engaging an activating CD244. However, a recent study using SAP KO mice have showed that SAP sufficient mice had increased cytotoxicity and IFN-γ production following CD244 engagement by CD48+ target cells or anti-CD244 mAb; whereas, SAP KO mice have decreased cytotoxicity and IFN-γ production following CD244 engagement, complicating the interpretation of CD244 function (218). These conflicting results may be explained by differences in NK cell culture conditions, where prolonged culture of NK cells in high doses of IL-2 leads to altered NK function. Another possible explanation is that adaptor molecule association may regulate CD244 function, as suggested by experiments with SAP, Eat-2a, and Eat-2b KO mice where SAP acts as a positive regulator of CD244 signaling and Eat-2a and Eat-2b function as negative regulators of CD244 signaling (136, 218). Therefore, the question remained: is the predominate role of murine CD244 inhibitory or activating?

The data presented in chapter four may reconcile conflicting results detailing CD244 function in NK cells. As described by Wandstrat *et al*, the SF is found as two stable haplotypes in common laboratory strains of mice (166). Sequence analysis of CD244, along with the entire SF locus in 34 laboratory strains of mice determined that a *b* allele of CD244 is found in all C57-derived mouse strains, which contains a single CD244 gene that can be alternatively spliced into a long and short form (166, 213). The *z* allele of CD244 is found in all non-C57 derived laboratory mouse strains, where the CD244 locus is expanded to contain four CD244 genes (166). Only the extracellular domain of these genes were sequenced and three of these genes appear to encode functional products, while one is a pseudo gene due to a premature stop codon in the V-Ig domain (166). Although the identification of two CD244 alleles may explain previously published conflicting data, reconciling these past functional studies is further complicated by the fact that multiple mouse strains were used which differ at the NK gene complex. The data presented in chapter four details the function of both alleles of CD244 using congenic mouse strains that differ by only a 900 kb interval surrounding the SF locus, where the remainder of the genome is composed of the B6 background. Therefore, these mouse strains share the same NK gene complex allowing for a direct comparison of the function of each CD244 allele in NK cells.

As shown in figure 4.1, the *z* allele of CD244 has activating function following CD244 engagement by mAb or its natural ligand, CD48. This activating function of the *z* allele of CD244 is dependent on SAP expression (figure 4.2), similar to what has been observed for human CD244. Also similar to human CD244, the *z* allele of CD244 is found as a single transcript in NK cells. The *z* allele of CD244 is more common in laboratory strains of mice than the *b* allele (166). These data suggest that the common murine allele of CD244 (*z*) functions as an activating receptor similar to human CD244. Thus, the original data using the Balb/c derived anti-CD244 mAb describing an activating function for CD244 was due to engagement of an activating receptor. In contrast, the *b* allele of CD244 can mediate activating and inhibitory function (figure 4.3), where the function of the *b* allele of CD244 is influenced by environmental factors.

A possible explanation for the divergent function observed between *z* and *b* alleles of CD244 is that the primary structure of the CD244 receptor dictates function. Sequence alignment of the *b* and *z* alleles of CD244 illustrates that the major sequence divergence between receptors occurs at the CD48 binding domain in the extracellular region and at two regions in the cytoplasmic domain. Cytotoxicity assays using [B6 x B6.*Sle1b*] F1 NK cells that express both alleles of CD244 demonstrate that each allele functions independently of other SF receptors or signaling molecule milieu, shown in figure 4.4. Further confirming that the *z*

allele functions as an activating receptor independent of other SF receptors [CD244KO x B6.*Sle1b*] F1 NK cells showed increased lysis of P815 CD48+ targets compared to P815 CD48- targets. These observations further suggest that primary receptor structure dictates function.

Although, this dissertation may have described divergent function for the *b* and *z* alleles of CD244 in the murine system, what selective pressures lead to this divergent CD244 function in the murine system and why an activating or inhibitory CD244 receptor would be advantageous for the murine immune response remains to be determined. One might speculate that pathogen selective pressures may have driven this divergence in CD244 function. Sequence analysis of CD48, CD84, CD229, CD150, and Ly108 in wild mouse populations have shown that polymorphisms of the SLAM family are maintained, although not as discrete haplotypes but rather as conserved single nucleotide polymorphisms (SNPs) (unpublished observation N.Limaye). Phylogenetic analysis done by N. Limaye shows that SLAM family members contain codons under selection in their ligand binding domains most evident in the case of CD48, the ligand for CD244, CD84 and CD229 (unpublished observation N.Limaye). These studies did not examine CD244 in wild mouse populations, but one could presume that the same pressures exist for CD244.

In humans, CD244 functions as an activating receptor. Recent studies have speculated that the activating function of human CD244 has evolved from a once inhibitory CD244 receptor due to the selective pressure of a commonly occurring virus, Epstein-Barr Virus (EBV) (Vaidya *et al*, paper submitted). Epidemiological studies of human populations have shown that EBV has a very high prevalence throughout the human population. A hallmark of EBV infection is the up-regulation of CD48 due to a viral protein interacting with an enhancer element of the *CD48* gene promoter (261). Vaidya *et al* propose that the prevalence of EBV infection induced a selective pressure for an activating receptor that could mediate the clearance of EBV. Thus, CD244 evolved as an activating receptor in humans. In XLP patients, CD244 functions as an inhibitory receptor. The most common cause of mortality in these patients is an inability to clear EBV infection, which leads to a fatal fulminant infectious mononucleosis. It is tempting to speculate that this inhibitory function of CD244 in these patients somehow contributes to disease pathology. Although EBV does not infect mice, examining pathogens found in wild mouse populations could identify a pathogen or pathogens that are currently enforcing a selective pressure on murine CD244, and therefore, murine CD244 may be evolving similarly to what may have occurred for human CD244.

A role for CD244 not only in NK cytotoxicity, but also in lymphocyte interactions has been described in some detail for the *b* allele of CD244. Studies

have shown that CD244 can serve as a ligand for CD48 in NK-NK, NK-T, T-T, and NK-B interactions (reviewed in (220)). What role the *z* allele plays in these interactions remains to be characterized. One might predict that engagement of an activating receptor or activating signal may lower the signaling threshold required for functional outcomes between lymphocyte interactions. For example, it has been shown that CD244-CD48 NK-B cell interactions of the *z* allele result in increased IL-13 production compared to the *b* allele (D. Yuan unpublished observation). Phenotypic studies of NK cell receptor expression have identified a population of NK cells that do not express MHC class I inhibitory receptors but express the *b* allele of CD244 which functions as a non-MHC restricted inhibitory receptor. CD244 may act as a non-MHC inhibitory receptor in this NK cell population maintaining self-tolerance and preventing lysis of normal autologous cells. It is not known if this same population of cells can be found in NK cells expressing the *z* allele of CD244. Similar studies characterizing the function of the *z* allele of CD244 in lymphocyte-lymphocyte interactions are needed and will give insight into what role the activating function of CD244 plays in the immune response.

B. Future Directions

Although the primary structure of CD244 appears to dictate receptor function, the mechanism by which this occurs remains to be determined. Does ligand binding strength regulate activating versus inhibitory function, or does the ability of the cytoplasmic signaling domain to bind adaptor molecules such as SAP or Eat-2a/b regulate function? Reconstitution of CD244 function using CD244 KO mice will determine the answers to these questions. Expression of the *b*, *z*, and Castc1 versions of CD244 on the CD244 KO background will determine if primary structure does determine function. Furthermore, similar experiments using chimeric CD244 receptors where the ligand binding V-Ig domain of the *b* allele has been fused to the C2-Ig, transmembrane, and cytoplasmic domains of the *z* allele and vise versa will determine if ligand binding strength dictates CD244 function. Swapping cytoplasmic domains between alleles or converting the amino acid residues found in the *b* allele to those found in the cytoplasmic domain of the *z* allele will determine if the ability of the cytoplasmic domain to associate with signaling molecules dictates function. Understanding how each allele of CD244 functions will allow for future experiments examining the role of CD244 in XLP disease pathology.

The CD244 locus of the *z* haplotype contains four genes and transcripts for three of these genes are found in the spleen. As described in chapter four, only one transcript is found in NK cells. It remains to be determined which cell types

contain the remaining two transcripts of CD244, and how these CD244 receptors function. Based on FACS analysis, it could be predicted that these additional transcripts may be found in basophils and monocytes and play a role in how these cell types function (212).

The knowledge gained from the experiments described above will give a detailed understanding of how CD244 functions in the immune response. In addition, these experiments will provide a detailed characterization of how polymorphisms within a single receptor can alter immune function, and may give insight into how polymorphisms affect the function of other receptors in the SF and the immune system.

II. CD2/SLAM Family Function

A. Role of the CD2/SLAM Family receptors in viral and bacterial pathogenesis

The CD2/SLAM family of receptors may have originally evolved as pathogen receptors. Several SLAM family receptors are know to directly bind viral or bacterial antigens. SLAM serves as a receptor for measles virus (125), and CD48 recognizes FimH, a bacterial lectin (126). Pathogens have also been shown to encode CD2/SF-like receptor molecules, for example the African swine fever virus (ASFV) encodes a CD2 like molecule shown to be important for

immunosuppression required for ASFV infection (262, 263). Further suggesting that the CD2/SF receptors evolved as pathogen receptors, sequence analysis of SLAM family receptors in wild mouse populations has shown a balancing selection for polymorphisms in the ligand binding domains of these receptors, which is possibly pathogen driven (N. Limaye unpublished observation). The fact that the CD2/SLAM family of receptors are broadly expressed throughout the immune system and that CD2/SLAM family members are known to directly bind pathogens suggests that the primary function of the CD2/SLAM family was to function as pathogen surveillance receptors, similar to what is currently described for Toll-like receptors. In the absence of pathogens, these receptors serve as weakly binding self-ligands. Alternatively, pathogens may have evolved to bind SF receptors whose main functions are to govern lymphocyte-lymphocyte interactions.

B. Role of the SLAM Family receptors in autoimmunity

The development of human autoimmune diseases, such as SLE and MS, has been shown to have a genetic component that predisposes a person to developing disease. However, genetic predisposition alone is not sufficient to cause disease and an environmental component is needed to trigger full autoimmune disease (264). Given the fact that diseases such as SLE and MS do

not follow strict Mendelian inheritance, this would argue that there are epistatic modifiers either genetic or environmental that influence disease progression (reviewed in (264)). It has been hypothesized that a common pathogen may serve as the environmental trigger responsible for disease onset. Therefore, prevalent pathogens in the human population such as EBV or CMV are attractive candidates. EBV has been implicated as a possible environmental trigger for both SLE and MS (265-269). The mechanism/s by which a common pathogen such as EBV may trigger autoimmunity have yet to be identified. Although, it has been suggested that molecular mimicry, where the induction of antibodies and T cells that react against a pathogen can also cross-react with self-antigens is a possible mechanism for triggering autoimmunity (2). Studies have shown that EBV reactive antibody epitopes closely resemble autoantibody Ro epitopes found in SLE (270). An alternative mechanism is that during the development of the immune response to a specific pathogen, autoreactive cells in the presence of self antigen become activated by cytokines and/or APCs participating in the ensuing immune response (reviewed in (271)). Potentially a combination of both of these mechanisms may facilitate a break in tolerance and may explain how multiple pathogens have been implicated in mediating autoimmune disorders (271).

The use of mouse models of autoimmunity, such as the NZM2410 mouse model of lupus, enables the genetic component(s) of autoimmunity to be studied

in detail. In the NZM2410 mouse model of lupus, the ANA causative locus (Sle1b) has been shown to contain the entire SLAM family (166). Further studies have shown that the SF of receptors exists as two stable haplotypes in common laboratory strains of mice (166). A *b* haplotype found in all C57-derived mouse strains and a more common *z* haplotype found in all non-C57 derived mouse strains. Interestingly, introduction of the *z* haplotype of the SF onto the C57BL/6 genetic background leads to ANA production suggesting that polymorphisms at the SF locus are responsible for a break in tolerance resulting in ANA production. However, these polymorphism themselves are not sufficient for a break in tolerance because not all mouse strains containing the *z* haplotype make ANAs, for example Balb/c and 129/Sv mouse strains contain the *z* haplotype of the SF and do not make ANA. Therefore, it is only in a specific genetic context that these polymorphisms at the SF locus are responsible for mediating ANA production. Polymorphisms at the SF locus are maintained in wild mouse populations, suggesting that these polymorphisms may provide a selective advantage and only in the rare genetic backgrounds are these polymorphisms detrimental to survival by causing autoimmunity (N.Limaye unpublished observation). In addition, ANA production alone is not fatal but requires other genetic components to mediate a complete SLE pathology. Therefore, the advantage of maintaining these polymorphisms at the SF locus may out weight the disadvantage of producing increased amounts of ANAs because only in

situations were the SF locus interacts with other SLE disease loci do these polymorphisms become detrimental to survival.

How might polymorphisms at the SF locus alter SF function and lead to a break in tolerance resulting in ANA production? B6.*Sle1b* B cells have inherent defects including a loss in tolerance to ssDNA and chromatin, increased CD69 expression, and increased total IgM serum levels (163, 179). These defects are suggestive of a break in peripheral B cell tolerance. Peripheral B cell tolerance in maintain in several ways: B cells that recognize self antigen arrest their migration in the T cell zone of peripheral lymphoid tissues and are unable to interact with T helper cells resulting in B cell apoptosis, self reactive B cells can undergo B cell anergy, and somatic hypermutation in GCs (2). SF receptors have not been implicated in mechanisms of somatic hypermutation. However, SF receptors are expressed on both T and B cells. Altered, SF expression and/or signaling function could affect B-T interactions and prevent low affinity, self-reactive B cells from being eliminated in the periphery. Adoptive transfer experiments using B6.*Sle1* mice have shown that T cells are required to get full disease (179). As described in chapter V, polymorphisms at the SF locus results in increased SF expression and CD40L expression on CD4+ T cells. CD40L and SF receptors are involved in T cell help and thus increased expression or altered patterns of isoform usage could facilitate a break in B cell peripheral tolerance. Recent studies have

identified a new class of helper T cells called follicular B helper T cells (T_{FH}) (reviewed in (272)). The main function of T_{FH} cells is to provide help to germinal-center B cells (272). T_{FH} have increased expression of CD84 and CD229 and thus it has been postulated that signal-transduction pathways involving these SF receptors and SAP may contribute to T_{FH} effector function in the germinal-center microenvironment (272). Thus, polymorphism at the SF locus could alter T_{FH} effector function and allow self-reactive B cells to escape deletion. Future experiments characterizing the B cell compartment in B6.*Sle1b* and B6 mice will give insight into how self reactive B cells are maintained in the periphery.

Recently, Toll-like receptors have been shown to play a role in B cell function, specifically the generation of T-dependent antigen-specific antibody responses (273). The role of TLR9, which binds CpG DNAs, is of particular interest because evidence has been presented that TLR9 activation can co-stimulate autoreactive B cells, thereby breaking tolerance (reviewed in (274)). These observations suggest that self-DNA containing CpGs from apoptotic cells or necrotic debris could bind TLR9 on autoreactive B cells and provide costimulation resulting in a break in B cell tolerance. TLR9 signaling through MyD88 leads to MAP kinase activation and ultimately to NF-κB and AP1 translocation to the nucleus (274). MAP kinases and NK-κB and AP1 are also involved in SF receptor signaling. Therefore, signaling through TLR9 and/or SF

receptors may synergize to provide a strong costimulatory signal for a weakly autoreactive BCR. Mechanisms preventing anergic B cells from being rescued in this manner have been identified. Rui *et al* show that anergic B cells have uncoupled the BCR pathway from signaling events that synergize with TLR9 ligation (275). In these B cells, continuous ERK signaling inhibits CpG-induced plasma cell differentiation (275). Similarly uncoupled ERK signaling may prevent ANA production in B6.*Sle1Sles1* mice where ERK signaling is up regulated in B6.*Sle1Sles1* mice compared to B6.*Sle1* (180). It has also been observed that unmethylated CpG binding of TLR9 in murine B cells redirects class-switching toward a Th1 Ig isotype response (276). Interestingly, ANAs are also predominantly of the Th1 isotypes. Further studies are needed to determine if TLRs in B cells from B6 and B6.*Sle1b* play a role in the break in tolerance leading to ANA production and if so, how polymorphism at the SF locus might impact or serve as a surrogate for TLR signaling in B cells.

C. Future Directions

Phenotypic studies examining the CD4+ T cell compartment have given some insight into which T cell functions may be altered by polymorphisms at the SF locus in the context of the C57BL/6 background. Further studies characterizing if specific subset of CD4 T helper populations are responsible for these phenotypes is warranted. In addition, studies examining the mechanisms by

which these polymorphisms mediate these phenotypic changes are also needed. It will also be important to determine if the polymorphisms in the entire SF are responsible for these altered phenotypes or if single SF receptors are responsible for altered B and T cell function. Similar phenotypic studies comparing B cell function between B6, B6.*Sle1b*, and B6.*Sle1bSles1* mice will give insight into how anti-ssDNA and anti-chromatin reactive B cells break tolerance. Furthermore, studies comparing phenotypes in B6.*Sle1bSles1*, B6.*Sle1b*, and B6 mice will allow the mechanisms associated with the *z* haplotype responsible for a break in tolerance to be identified. The use of a BCR transgenic mouse model crossed onto B6.*Sle1bSles1*, B6.*Sle1b*, and B6 mice strains to examine B cell tolerance could also give valuable insight into how peripheral autoreactive B cells survive and differentiate into ANA-secreting plasma cells. The knowledge gained from these studies will provide a mechanism for how SF genes may cause a break in tolerance leading to ANA production and how polymorphisms in the appropriate genetic context can alter immune function.

A potential model for how tolerance may be broken in B6.*Sle1b* cells is diagramed in figure 6.1. In this model, naïve mature B cells circulating in the periphery that have weakly self-reactive BCRs migrate to the spleen. Under normal selection, these B cells would be eliminated by apoptosis, rendered anergic, or undergo somatic hypermutation in efforts to avoid autoreactivity.

However, because these self-reactive B cells express the *z* haplotype of the SF receptors in a genetic background lacking repressive loci, these B cells may have altered signaling thresholds required for survival and activation. When these self-reactive B cells interact with B6.*Sle1b* CD4+ T cells that have increased SF expression and CD40L expression they are differentiated into ANA-producing plasma cells.

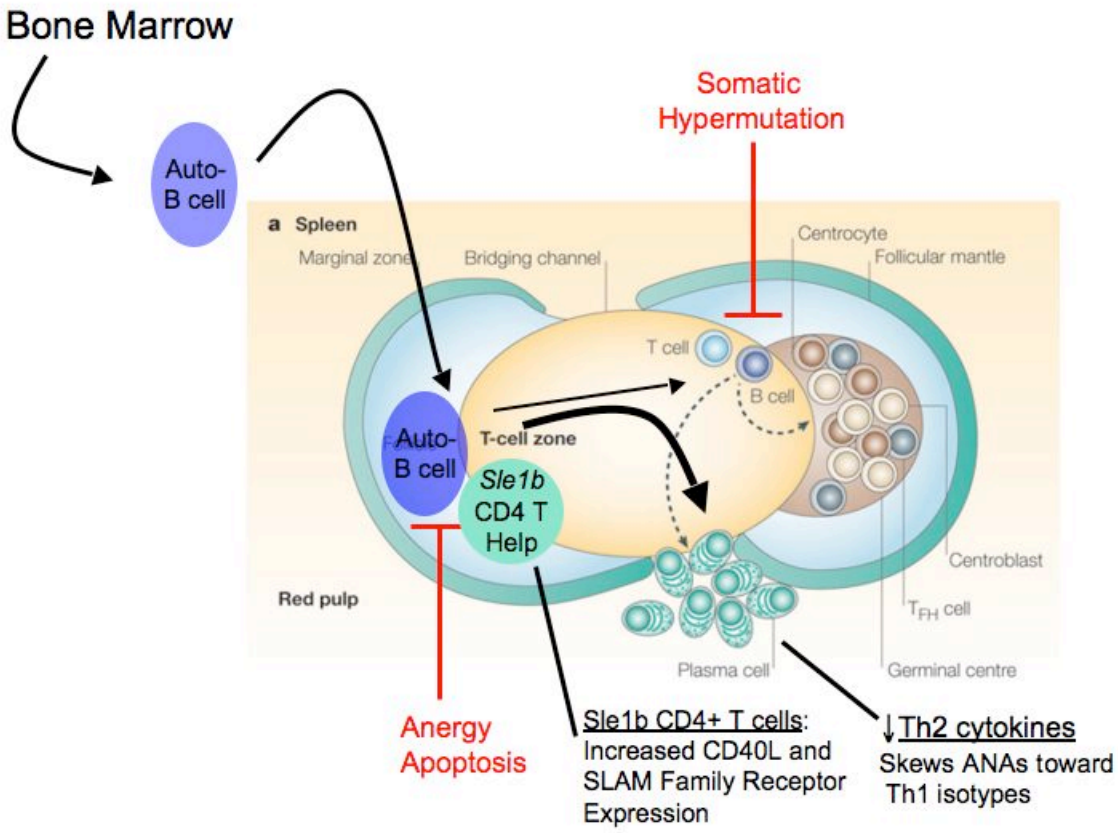


Figure 6.1: Model for How SF Receptors Alter B-T Interactions Leading to a Break in Tolerance

The normal check points for peripheral B cell tolerance are labeled in red. Auto-reactive B cells interact with T cell upon migration through the follicle to the T-cell zone. Once an auto-reactive B cells receive T cell help they can then differentiate into short term low affinity plasma cells or migrate to germinal centers where they differentiate into long-lived plasma cells. Figure adapted from Vinuesa *et al* Nature Reviews (2005).

REFERENCES

1. Blach-Olszewska, Z. 2005. Innate immunity: cells, receptors, and signaling pathways. *Arch Immunol Ther Exp (Warsz) 53:245*.
2. Janeway, C. A., and P. Travers. 1996. *Immunobiology*. Current Biology Limited, New York.
3. Sivakumar, P. V., I. Puzanov, N. S. Williams, M. Bennett, and V. Kumar. 1998. Ontogeny and differentiation of murine natural killer cells and their receptors. *Curr Top Microbiol Immunol 230:161*.
4. Raulet, D. H. 1999. Development and tolerance of natural killer cells. *Curr Opin Immunol 11:129*.
5. Lanier, L. L., S. Cwirla, and J. H. Phillips. 1986. Genomic organization of T cell gamma genes in human peripheral blood natural killer cells. *J Immunol 137:3375*.
6. Trinchieri, G. 1989. Biology of natural killer cells. *Adv Immunol 47:187*.
7. Trinchieri, G., M. Murphy, and B. Perussia. 1987. Regulation of hematopoiesis by T lymphocytes and natural killer cells. *Crit Rev Oncol Hematol 7:219*.
8. Lanier, L. L. 1998. NK cell receptors. *Annu Rev Immunol 16:359*.

9. Cuturi, M. C., I. Anegon, F. Sherman, R. Loudon, S. C. Clark, B. Perussia, and G. Trinchieri. 1989. Production of hematopoietic colony-stimulating factors by human natural killer cells. *J Exp Med 169:569.*

10. Gray, J. D., M. Hirokawa, and D. A. Horwitz. 1994. The role of transforming growth factor beta in the generation of suppression: an interaction between CD8+ T and NK cells. *J Exp Med 180:1937.*

11. Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai. 1983. Natural killer (NK) cells as a responder to interleukin 2 (IL 2). II. IL 2-induced interferon gamma production. *J Immunol 130:988.*

12. Hoshino, T., R. T. Winkler-Pickett, A. T. Mason, J. R. Ortaldo, and H. A. Young. 1999. IL-13 production by NK cells: IL-13-producing NK and T cells are present in vivo in the absence of IFN-gamma. *J Immunol 162:51.*

13. Mehrotra, P. T., R. P. Donnelly, S. Wong, H. Kanegane, A. Geremew, H. S. Mostowski, K. Furuke, J. P. Siegel, and E. T. Bloom. 1998. Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. *J Immunol 160:2637.*

14. Andrews, D. M., C. E. Andoniou, A. A. Scalzo, S. L. van Dommelen, M. E. Wallace, M. J. Smyth, and M. A. Degli-Esposti. 2005. Cross-talk between dendritic cells and natural killer cells in viral infection. *Mol Immunol 42:547.*

15. Raulet, D. H. 2004. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat Immunol 5:996.*

16. Piccioli, D., S. Sbrana, E. Melandri, and N. M. Valiante. 2002. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med 195:335.*

17. Cudkowicz, G., and G. E. Cosgrove. 1960. Immunologically competent cells in adult mouse liver: Studies with parent:to:hybrid radiation chimeras. *Proc. Soc. Exp. Biol. Med. 105:366.*

18. Cudkowicz, G., and G. B. Rossi. 1972. Hybrid resistance to parental DBA-2 grafts: independence from the H-2 locus. I. Studies with normal hematopoietic cells. *J Natl Cancer Inst 48:131.*

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

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

21. Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature 319:675.*

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

23. Yu, Y. Y., T. George, J. R. Dorfman, J. Roland, V. Kumar, and M. Bennett. 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*.

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

25. Anderson, S. K., J. R. Ortaldo, and D. W. McVicar. 2001. The ever-expanding Ly49 gene family: repertoire and signaling. *Immunol Rev 181:79*.

26. Yokoyama, W. M., and W. E. Seaman. 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annu Rev Immunol 11:613*.

27. Correa, I., and D. H. Raulet. 1995. Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. *Immunity 2:61*.

28. Hanke, T., H. Takizawa, C. W. McMahon, D. H. Busch, E. G. Pamer, J. D. Miller, J. D. Altman, Y. Liu, D. Cado, F. A. Lemonnier, P. J.

Bjorkman, and D. H. Raulet. 1999. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity 11:67*.

29. Orihuela, M., D. H. Margulies, and W. M. Yokoyama. 1996. The natural killer cell receptor Ly-49A recognizes a peptide-induced conformational determinant on its major histocompatibility complex class I ligand. *Proc Natl Acad Sci U S A 93:11792*.

30. Nakamura, M. C., E. C. Niemi, M. J. Fisher, L. D. Shultz, W. E. Seaman, and J. C. Ryan. 1997. Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase. *J Exp Med 185:673*.

31. Lanier, L. L., and A. B. Bakker. 2000. The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function. *Immunol Today 21:611*.

32. McVicar, D. W., L. S. Taylor, P. Gosselin, J. Willette-Brown, A. I. Mikhael, R. L. Geahlen, M. C. Nakamura, P. Linnemeyer, W. E. Seaman, S. K. Anderson, J. R. Ortaldo, and L. H. Mason. 1998. DAP12-mediated signal transduction in natural killer cells. A dominant role for the Syk protein-tyrosine kinase. *J Biol Chem 273:32934*.

33. Sivakumar, P. V., M. Bennett, and V. Kumar. 1997. Fetal and neonatal NK1.1+ Ly-49- cells can distinguish between major histocompatibility

complex class I(hi) and class I(lo) target cells: evidence for a Ly-49-independent negative signaling receptor. *Eur J Immunol 27:3100.*

34. Dorfman, J. R., and D. H. Raulet. 1998. Acquisition of Ly49 receptor expression by developing natural killer cells. *J Exp Med 187:609.*

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

36. Saleh, A., G. E. Davies, V. Pascal, P. W. Wright, D. L. Hodge, E. H. Cho, S. J. Lockett, M. Abshari, and S. K. Anderson. 2004. Identification of probabilistic transcriptional switches in the Ly49 gene cluster: a eukaryotic mechanism for selective gene activation. *Immunity 21:55.*

37. Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature 436:709.*

38. Vance, R. E., J. R. Kraft, A. J. D., J. P. E., and D. H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1b. *J Exp Med 188:1841.*

39. Braud, V. M., D. S. Allan, and A. J. McMichael. 1999. Functions of nonclassical MHC and non-MHC-encoded class I molecules. *Curr Opin Immunol 11:100.*

40. Vance, R. E., A. M. Jamieson, D. Cado, and D. H. Raulet. 2002. Implications of CD94 deficiency and monoallelic NKG2A expression for natural killer cell development and repertoire formation. *Proc Natl Acad Sci U S A 99:868.*

41. Houchins, J. P., T. Yabe, C. McSherry, and F. H. Bach. 1991. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J Exp Med 173:1017.*

42. Lanier, L. L., B. Corliss, J. Wu, and J. H. Phillips. 1998. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity 8:693.*

43. Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol 3:781.*

44. Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science 285:730.*

45. Chang, C., J. Dietrich, A. G. Harpur, J. A. Lindquist, A. Haude, Y. W. Loke, A. King, M. Colonna, J. Trowsdale, and M. J. Wilson. 1999. Cutting edge: KAP10, a novel transmembrane adapter protein genetically

linked to DAP12 but with unique signaling properties. *J Immunol 163:4651.*

46. Diefenbach, A., E. Tomasello, M. Lucas, A. M. Jamieson, J. K. Hsia, E. Vivier, and D. H. Raulet. 2002. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol 3:1142.*

47. Gilfillan, S., E. L. Ho, M. Cella, W. M. Yokoyama, and M. Colonna. 2002. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol 3:1150.*

48. Gasser, S., S. Orsulic, E. J. Brown, and D. H. Raulet. 2005. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature 436:1186.*

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

50. Boyington, J. C., A. G. Brooks, and P. D. Sun. 2001. Structure of killer cell immunoglobulin-like receptors and their recognition of the class I MHC molecules. *Immunol Rev 181:66.*

51. Westgaard, I. H., S. F. Berg, J. T. Vaage, L. L. Wang, W. M. Yokoyama, E. Dissen, and S. Fossum. 2004. Rat NKp46 activates natural killer cell cytotoxicity and is associated with FcepsilonRIgamma and CD3zeta. *J Leukoc Biol 76:1200.*

52. Biassoni, R., C. Cantoni, D. Pende, S. Sivori, S. Parolini, M. Vitale, C. Bottino, and A. Moretta. 2001. Human natural killer cell receptors and co-receptors. *Immunol Rev 181:203*.

53. Gerosa, F., B. Baldani-Guerra, C. Nisii, V. Marchesini, G. Carra, and G. Trinchieri. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med 195:327*.

54. Spaggiari, G. M., R. Carosio, D. Pende, S. Marcenaro, P. Rivera, M. R. Zocchi, L. Moretta, and A. Poggi. 2001. NK cell-mediated lysis of autologous antigen-presenting cells is triggered by the engagement of the phosphatidylinositol 3-kinase upon ligation of the natural cytotoxicity receptors NKp30 and NKp46. *Eur J Immunol 31:1656*.

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

56. Smyth, M. J., E. Cretney, J. M. Kelly, J. A. Westwood, S. E. Street, H. Yagita, K. Takeda, S. L. van Dommelen, M. A. Degli-Esposti, and Y. Hayakawa. 2005. Activation of NK cell cytotoxicity. *Mol Immunol 42:501*.

57. Page, L. J., A. J. Darmon, R. Uellner, and G. M. Griffiths. 1998. L is for lytic granules: lysosomes that kill. *Biochim Biophys Acta 1401:146*.

58. Trapani, J. A., K. A. Browne, M. J. Smyth, and D. A. Jans. 1996. Localization of granzyme B in the nucleus. A putative role in the

mechanism of cytotoxic lymphocyte-mediated apoptosis. *J Biol Chem 271:4127*.

59. Motyka, B., G. Korbutt, M. J. Pinkoski, J. A. Heibein, A. Caputo, M. Hobman, M. Barry, I. Shostak, T. Sawchuk, C. F. Holmes, J. Gauldie, and R. C. Bleackley. 2000. Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell 103:491*.

60. Pinkoski, M. J., M. Hobman, J. A. Heibein, K. Tomaselli, F. Li, P. Seth, C. J. Froelich, and R. C. Bleackley. 1998. Entry and trafficking of granzyme B in target cells during granzyme B-perforin-mediated apoptosis. *Blood 92:1044*.

61. Kawasaki, Y., T. Saito, Y. Shirota-Someya, Y. Ikegami, H. Komano, M. H. Lee, C. J. Froelich, N. Shinohara, and H. Takayama. 2000. Cell death-associated translocation of plasma membrane components induced by CTL. *J Immunol 164:4641*.

62. Metkar, S. S., B. Wang, M. Aguilar-Santelises, S. M. Raja, L. Uhlin-Hansen, E. Podack, J. A. Trapani, and C. J. Froelich. 2002. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity 16:417*.

63. Keefe, D., L. Shi, S. Feske, R. Massol, F. Navarro, T. Kirchhausen, and J. Lieberman. 2005. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity 23:249*.

64. Wulfing, C., B. Purtic, J. Klem, and J. D. Schatzle. 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*.

65. Wulfing, C., I. Tskvitaria-Fuller, N. Burroughs, M. D. Sjaastad, J. Klem, and J. D. Schatzle. 2002. Interface accumulation of receptor/ligand couples in lymphocyte activation: methods, mechanisms, and significance. *Immunol Rev 189:64*.

66. Smyth, M. J., and R. W. Johnstone. 2000. Role of TNF in lymphocyte-mediated cytotoxicity. *Microsc Res Tech 50:196*.

67. Bodmer, J. L., K. Burns, P. Schneider, K. Hofmann, V. Steiner, M. Thome, T. Bornand, M. Hahne, M. Schroter, K. Becker, A. Wilson, L. E. French, J. L. Browning, H. R. MacDonald, and J. Tschopp. 1997. TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and Fas(Apo-1/CD95). *Immunity 6:79*.

68. Degli-Esposti, M. 1999. To die or not to die--the quest of the TRAIL receptors. *J Leukoc Biol 65:535*.

69. Liu, C. C., C. M. Walsh, N. Eto, W. R. Clark, and J. D. Young. 1995. Morphologic and functional characterization of perforin-deficient lymphokine-activated killer cells. *J Immunol 155:602*.

70. Montel, A. H., M. R. Bochan, J. A. Hobbs, D. H. Lynch, and Z. Brahmi. 1995. Fas involvement in cytotoxicity mediated by human NK cells. *Cell Immunol 166:236*.

71. Kashii, Y., R. Giorda, R. B. Herberman, T. L. Whiteside, and N. L. Vujanovic. 1999. Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. *J Immunol 163:5358*.

72. Arase, H., N. Arase, and T. Saito. 1995. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med 181:1235*.

73. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell 66:233*.

74. van Engeland, M., L. J. Nieland, F. C. Ramaekers, B. Schutte, and C. P. Reutelingsperger. 1998. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry 31:1*.

75. Screpanti, V., R. P. Wallin, A. Grandien, and H. G. Ljunggren. 2005. Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells. *Mol Immunol 42:495*.

76. Wallin, R. P., V. Screpanti, J. Michaelsson, A. Grandien, and H. G. Ljunggren. 2003. Regulation of perforin-independent NK cell-mediated cytotoxicity. *Eur J Immunol 33:2727*.

77. Alderson, M. R., T. W. Tough, T. Davis-Smith, S. Braddy, B. Falk, K. A. Schooley, R. G. Goodwin, C. A. Smith, F. Ramsdell, and D. H. Lynch. 1995. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med 181:71*.

78. Ju, S. T., D. J. Panka, H. Cui, R. Ettinger, M. el-Khatib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T- cell activation. *Nature 373:444*.

79. Cohen, P. L., and R. A. Eisenberg. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu Rev Immunol 9:243*.

80. Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature 356:314*.

81. Takahashi, T., M. Tanaka, C. I. Brannan, N. A. Jenkins, N. G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell 76:969.*

82. Bossi, G., and G. M. Griffiths. 1999. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat Med 5:90.*

83. Kojima, Y., A. Kawasaki-Koyanagi, N. Sueyoshi, A. Kanai, H. Yagita, and K. Okumura. 2002. Localization of Fas ligand in cytoplasmic granules of CD8+ cytotoxic T lymphocytes and natural killer cells: participation of Fas ligand in granule exocytosis model of cytotoxicity. *Biochem Biophys Res Commun 296:328.*

84. Bluman, E. M., K. J. Bartynski, B. R. Avalos, and M. A. Caligiuri. 1996. Human natural killer cells produce abundant macrophage inflammatory protein-1 alpha in response to monocyte-derived cytokines. *J Clin Invest 97:2722.*

85. Oliva, A., A. L. Kinter, M. Vaccarezza, A. Rubbert, A. Catanzaro, S. Moir, J. Monaco, L. Ehler, S. Mizell, R. Jackson, Y. Li, J. W. Romano, and A. S. Fauci. 1998. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. *J Clin Invest 102:223.*

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

87. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol 162:4511*.

88. Orange, J. S., and C. A. Biron. 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J Immunol 156:1138*.

89. Berg, R. E., C. J. Cordes, and J. Forman. 2002. Contribution of CD8+ T cells to innate immunity: IFN-gamma secretion induced by IL-12 and IL-18. *Eur J Immunol 32:2807*.

90. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu Rev Immunol 15:749*.

91. Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to

lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol 5:1260*.

92. Yuan, D. 2004. Interactions between NK cells and B lymphocytes. *Adv Immunol 84:1*.

93. Brigl, M., and M. B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annu Rev Immunol 22:817*.

94. Godfrey, D. I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest 114:1379*.

95. Mattner, J., K. L. Debord, N. Ismail, R. D. Goff, C. Cantu, 3rd, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, B. Beutler, L. Teyton, P. B. Savage, and A. Bendelac. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature 434:525*.

96. von Freeden-Jeffry, U., P. Vieira, L. A. Lucian, T. McNeil, S. E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med 181:1519*.

97. Kappes, D. J., and X. He. 2005. CD4-CD8 lineage commitment: an inside view. *Nat Immunol 6:761*.

98. Anderson, G., N. C. Moore, J. J. Owen, and E. J. Jenkinson. 1996. Cellular interactions in thymocyte development. *Annu Rev Immunol 14:73*.

99. Schwartzberg, P. L., L. D. Finkelstein, and J. A. Readinger. 2005. TEC-family kinases: regulators of T-helper-cell differentiation. *Nat Rev Immunol 5:284.*

100. Morris, A. G., Y. L. Lin, and B. A. Askonas. 1982. Immune interferon release when a cloned cytotoxic T-cell line meets its correct influenza-infected target cell. *Nature 295:150.*

101. Ramsay, A. J., J. Ruby, and I. A. Ramshaw. 1993. A case for cytokines as effector molecules in the resolution of virus infection. *Immunol Today 14:155.*

102. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat Rev Immunol 2:933.*

103. Kashiwakura, J., N. Suzuki, H. Nagafuchi, M. Takeno, Y. Takeba, Y. Shimoyama, and T. Sakane. 1999. Txk, a nonreceptor tyrosine kinase of the Tec family, is expressed in T helper type 1 cells and regulates interferon gamma production in human T lymphocytes. *J Exp Med 190:1147.*

104. Miller, A. T., H. M. Wilcox, Z. Lai, and L. J. Berg. 2004. Signaling through Itk promotes T helper 2 differentiation via negative regulation of T-bet. *Immunity 21:67.*

105. Sidorenko, S. P., and E. A. Clark. 2003. The dual-function CD150 receptor subfamily: the viral attraction. *Nat Immunol 4:19.*

106. Kingsbury, G. A., L. A. Feeney, Y. Nong, S. A. Calandra, C. J. Murphy, J. M. Corcoran, Y. Wang, M. R. Prabhu Das, S. J. Busfield, C. C. Fraser, and J. L. Villeval. 2001. Cloning, expression, and function of BLAME, a novel member of the CD2 family. *J Immunol 166:5675.*

107. Sewell, W. A., R. W. Palmer, N. K. Spurr, D. Sheer, M. H. Brown, Y. Bell, and M. J. Crumpton. 1988. The human LFA-3 gene is located at the same chromosome band as the gene for its receptor CD2. *Immunogenetics 28:278.*

108. Cocks, B. G., C. C. Chang, J. M. Carballido, H. Yssel, J. E. de Vries, and G. Aversa. 1995. A novel receptor involved in T-cell activation. *Nature 376:260.*

109. Garni-Wagner, B. A., A. Purohit, P. A. Mathew, M. Bennett, and V. Kumar. 1993. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol 151:60.*

110. de la Fuente, M. A., P. Pizcueta, M. Nadal, J. Bosch, and P. Engel. 1997. CD84 leukocyte antigen is a new member of the Ig superfamily. *Blood 90:2398.*

111. Sandrin, M. S., T. P. Gumley, M. M. Henning, H. A. Vaughan, L. J. Gonez, J. A. Trapani, and I. F. McKenzie. 1992. Isolation and characterization of cDNA clones for mouse Ly-9. *J Immunol 149:1636.*

112. Peck, S. R., and H. E. Ruley. 2000. Ly108: a new member of the mouse CD2 family of cell surface proteins. *Immunogenetics 52:63*.

113. Boles, K. S., and P. A. Mathew. 2001. Molecular cloning of CS1, a novel human natural killer cell receptor belonging to the CD2 subset of the immunoglobulin superfamily. *Immunogenetics 52:302*.

114. Staunton, D. E., and D. A. Thorley-Lawson. 1987. Molecular cloning of the lymphocyte activation marker Blast-1. *Embo J 6:3695*.

115. Nichols, K. E., C. S. Ma, J. L. Cannons, P. L. Schwartzberg, and S. G. Tangye. 2005. Molecular and cellular pathogenesis of X-linked lymphoproliferative disease. *Immunol Rev 203:180*.

116. Tangye, S. G., J. H. Phillips, and L. L. Lanier. 2000. The CD2-subset of the Ig superfamily of cell surface molecules: receptor-ligand pairs expressed by NK cells and other immune cells. *Semin Immunol 12:149*.

117. Mavaddat, N., D. W. Mason, P. D. Atkinson, E. J. Evans, R. J. Gilbert, D. I. Stuart, J. A. Fennelly, A. N. Barclay, S. J. Davis, and M. H. Brown. 2000. Signaling lymphocytic activation molecule (CDw150) is homophilic but self-associates with very low affinity. *J Biol Chem 275:28100*.

118. Romero, X., N. Zapater, M. Calvo, S. G. Kalko, M. A. de la Fuente, V. Tovar, C. Ockeloen, P. Pizcueta, and P. Engel. 2005. CD229 (Ly9) lymphocyte cell surface receptor interacts homophilically through its N-

terminal domain and relocalizes to the immunological synapse. *J Immunol 174:7033*.

119. Falco, M., E. Marcenaro, E. Romeo, F. Bellora, D. Marras, F. Vely, G. Ferracci, L. Moretta, A. Moretta, and C. Bottino. 2004. Homophilic interaction of NTBA, a member of the CD2 molecular family: induction of cytotoxicity and cytokine release in human NK cells. *Eur J Immunol 34:1663*.

120. Flaig, R. M., S. Stark, and C. Watzl. 2004. Cutting edge: NTB-A activates NK cells via homophilic interaction. *J Immunol 172:6524*.

121. Martin, M., X. Romero, M. A. de la Fuente, V. Tovar, N. Zapater, E. Esplugues, P. Pizcueta, J. Bosch, and P. Engel. 2001. CD84 functions as a homophilic adhesion molecule and enhances IFN-gamma secretion: adhesion is mediated by Ig-like domain 1. *J Immunol 167:3668.*

122. Kumaresan, P. R., W. C. Lai, S. S. Chuang, M. Bennett, and P. A. Mathew. 2002. CS1, a novel member of the CD2 family, is homophilic and regulates NK cell function. *Mol Immunol 39:1*.

123. Latchman, Y., P. F. McKay, and H. Reiser. 1998. Identification of the 2B4 molecule as a counter-receptor for CD48. *J Immunol 161:5809*.

124. Brown, M. H., K. Boles, P. A. van der Merwe, V. Kumar, P. A. Mathew, and A. N. Barclay. 1998. 2B4, the natural killer and T cell

immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med 188:2083.*

125. Tatsuo, H., N. Ono, K. Tanaka, and Y. Yanagi. 2000. SLAM (CDw150) is a cellular receptor for measles virus. *Nature 406:893*.

126. Baorto, D. M., Z. Gao, R. Malaviya, M. L. Dustin, A. van der Merwe, D. M. Lublin, and S. N. Abraham. 1997. Survival of FimH-expressing enterobacteria in macrophages relies on glycolipid traffic. *Nature 389:636*.

127. Engel, P., M. J. Eck, and C. Terhorst. 2003. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat Rev Immunol 3:813.*

128. Dustin, M. L., P. Selvaraj, R. J. Mattaliano, and T. A. Springer. 1987. Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. *Nature 329:846.*

129. Veillette, A., and S. Latour. 2003. The SLAM family of immune-cell receptors. *Curr Opin Immunol 15:277*.

130. Veillette, A. 2004. Specialised adaptors in immune cells. *Curr Opin Cell Biol 16:146*.

131. Klem, J., P. C. Verrett, V. Kumar, and J. D. Schatzle. 2002. 2B4 is constitutively associated with linker for the activation of T cells in glycolipid-enriched microdomains: properties required for 2B4 lytic function. *J Immunol 169:55*.

132. Morra, M., J. Lu, F. Poy, M. Martin, J. Sayos, S. Calpe, C. Gullo, D. Howie, S. Rietdijk, A. Thompson, A. J. Coyle, C. Denny, M. B. Yaffe, P. Engel, M. J. Eck, and C. Terhorst. 2001. Structural basis for the interaction of the free SH2 domain EAT-2 with SLAM receptors in hematopoietic cells. *Embo J 20:5840.*

133. Sayos, J., C. Wu, M. Morra, N. Wang, X. Zhang, D. Allen, S. van Schaik, L. Notarangelo, R. Geha, M. G. Roncarolo, H. Oettgen, J. E. De Vries, G. Aversa, and C. Terhorst. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM [see comments]. *Nature 395:462.*

134. Latour, S., R. Roncagalli, R. Chen, M. Bakinowski, X. Shi, P. L. Schwartzberg, D. Davidson, and A. Veillette. 2003. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat Cell Biol 5:149.*

135. Chan, B., A. Lanyi, H. K. Song, J. Griesbach, M. Simarro-Grande, F. Poy, D. Howie, J. Sumegi, C. Terhorst, and M. J. Eck. 2003. SAP couples Fyn to SLAM immune receptors. *Nat Cell Biol 5:155.*

136. Roncagalli, R., J. E. Taylor, S. Zhang, X. Shi, R. Chen, M. E. Cruz-Munoz, L. Yin, S. Latour, and A. Veillette. 2005. Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor. *Nat Immunol 6:1002.*

137. Purtilo, D. T., C. K. Cassel, J. P. Yang, and R. Harper. 1975. X-linked recessive progressive combined variable immunodeficiency (Duncan's disease). *Lancet 1:935*.

138. Skare, J. C., H. L. Grierson, J. L. Sullivan, R. L. Nussbaum, D. T. Purtilo, B. S. Sylla, G. M. Lenoir, D. S. Reilly, B. N. White, and A. Milunsky. 1989. Linkage analysis of seven kindreds with the X-linked lymphoproliferative syndrome (XLP) confirms that the XLP locus is near DXS42 and DXS37. *Hum Genet 82:354*.

139. Skare, J. C., J. L. Sullivan, and A. Milunsky. 1989. Mapping the mutation causing the X-linked lymphoproliferative syndrome in relation to restriction fragment length polymorphisms on Xq. *Hum Genet 82:349*.

140. Purtilo, D. T., and H. L. Grierson. 1991. Methods of detection of new families with X-linked lymphoproliferative disease. *Cancer Genet Cytogenet 51:143*.

141. Sumegi, J., D. Huang, A. Lanyi, J. D. Davis, T. A. Seemayer, A. Maeda, G. Klein, M. Seri, H. Wakiguchi, D. T. Purtilo, and T. G. Gross. 2000. Correlation of mutations of the SH2D1A gene and epstein-barr virus infection with clinical phenotype and outcome in X-linked lymphoproliferative disease. *Blood 96:3118*.

142. Seemayer, T. A., T. G. Gross, R. M. Egeler, S. J. Pirruccello, J. R. Davis, C. M. Kelly, M. Okano, A. Lanyi, and J. Sumegi. 1995. X-linked

lymphoproliferative disease: twenty-five years after the discovery. *Pediatr Res 38:471*.

143. Grierson, H., and D. T. Purtilo. 1987. Epstein-Barr virus infections in males with the X-linked lymphoproliferative syndrome. *Ann Intern Med 106:538*.

144. Harrington, D. S., D. D. Weisenburger, and D. T. Purtilo. 1987. Malignant lymphoma in the X-linked lymphoproliferative syndrome. *Cancer 59:1419*.

145. Grierson, H. L., J. Skare, J. Hawk, M. Pauza, and D. T. Purtilo. 1991. Immunoglobulin class and subclass deficiencies prior to Epstein-Barr virus infection in males with X-linked lymphoproliferative disease. *Am J Med Genet 40:294*.

146. Coffey, A. J., R. A. Brooksbank, O. Brandau, T. Oohashi, G. R. Howell, J. M. Bye, A. P. Cahn, J. Durham, P. Heath, P. Wray, R. Pavitt, J. Wilkinson, M. Leversha, E. Huckle, C. J. Shaw-Smith, A. Dunham, S. Rhodes, V. Schuster, G. Porta, L. Yin, P. Serafini, B. Sylla, M. Zollo, B. Franco, D. R. Bentley, and et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene [see comments]. *Nat Genet 20:129*.

147. Nichols, K. E., D. P. Harkin, S. Levitz, M. Krainer, K. A. Kolquist, C. Genovese, A. Bernard, M. Ferguson, L. Zuo, E. Snyder, A. J. Buckler, C.

Wise, J. Ashley, M. Lovett, M. B. Valentine, A. T. Look, W. Gerald, D. E. Housman, and D. A. Haber. 1998. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc Natl Acad Sci U S A 95:13765.*

148. Morra, M., O. Silander, S. Calpe, M. Choi, H. Oettgen, L. Myers, A. Etzioni, R. Buckley, and C. Terhorst. 2001. Alterations of the X-linked lymphoproliferative disease gene SH2D1A in common variable immunodeficiency syndrome. *Blood 98:1321.*

149. Sayos, J., K. B. Nguyen, C. Wu, S. E. Stepp, D. Howie, J. D. Schatzle, V. Kumar, C. A. Biron, and C. Terhorst. 2000. Potential pathways for regulation of NK and T cell responses: differential X-linked lymphoproliferative syndrome gene product SAP interactions with SLAM and 2B4. *Int Immunol 12:1749.*

150. Wu, C., J. Sayos, N. Wang, D. Howie, A. Coyle, and C. Terhorst. 2000. Genomic organization and characterization of mouse SAP, the gene that is altered in X-linked lymphoproliferative disease. *Immunogenetics 51:805.*

151. Nagy, N., C. Cerboni, K. Mattsson, A. Maeda, P. Gogolak, J. Sumegi, A. Lanyi, L. Szekely, E. Carbone, G. Klein, and E. Klein. 2000. SH2D1A and SLAM protein expression in human lymphocytes and derived cell lines. *Int J Cancer 88:439.*

152. Shlapatska, L. M., S. V. Mikhalap, A. G. Berdova, O. M. Zelensky, T. J. Yun, K. E. Nichols, E. A. Clark, and S. P. Sidorenko. 2001. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. *J Immunol 166:5480.*

153. Feldhahn, N., I. Schwering, S. Lee, M. Wartenberg, F. Klein, H. Wang, G. Zhou, S. M. Wang, J. D. Rowley, J. Hescheler, M. Kronke, K. Rajewsky, R. Kuppers, and M. Muschen. 2002. Silencing of B cell receptor signals in human naive B cells. *J Exp Med 196:1291.*

154. Halasa, N. B., J. A. Whitlock, T. L. McCurley, J. A. Smith, Q. Zhu, H. Ochs, T. S. Dermody, and J. E. Crowe, Jr. 2003. Fatal hemophagocytic lymphohistiocytosis associated with Epstein-Barr virus infection in a patient with a novel mutation in the signaling lymphocytic activation molecule-associated protein. *Clin Infect Dis 37:e136.*

155. Bottino, C., M. Falco, S. Parolini, E. Marcenaro, R. Augugliaro, S. Sivori, E. Landi, R. Biassoni, L. D. Notarangelo, L. Moretta, and A. Moretta. 2001. NTB-A [correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *J Exp Med 194:235.*

156. Nakajima, H., M. Cella, A. Bouchon, H. L. Grierson, J. Lewis, C. S. Duckett, J. I. Cohen, and M. Colonna. 2000. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur J Immunol 30:3309.*

157. Parolini, S., C. Bottino, M. Falco, R. Augugliaro, S. Giliani, R. Franceschini, H. D. Ochs, H. Wolf, J. Y. Bonnefoy, R. Biassoni, L. Moretta, L. D. Notarangelo, and A. Moretta. 2000. X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill epstein-barr virus-infected cells [In Process Citation]. *J Exp Med 192:337.*

158. Tangye, S. G., H. Cherwinski, L. L. Lanier, and J. H. Phillips. 2000. 2B4-mediated activation of human natural killer cells. *Mol Immunol 37:493.*

159. Benoit, L., X. Wang, H. F. Pabst, J. Dutz, and R. Tan. 2000. Defective NK cell activation in X-linked lymphoproliferative disease. *J Immunol 165:3549.*

160. Wakeland, E. K., K. Liu, R. R. Graham, and T. W. Behrens. 2001. Delineating the genetic basis of systemic lupus erythematosus. *Immunity 15:397.*

161. Ruiz-Irastorza, G., M. A. Khamashta, G. Castellino, and G. R. Hughes. 2001. Systemic lupus erythematosus. *Lancet 357:1027.*

162. Morel, L., K. R. Blenman, B. P. Croker, and E. K. Wakeland. 2001. The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proc Natl Acad Sci U S A 98:1787.*

163. Morel, L., B. P. Croker, K. R. Blenman, C. Mohan, G. Huang, G. Gilkeson, and E. K. Wakeland. 2000. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. *Proc Natl Acad Sci U S A 97:6670.*

164. Tsao, B. P., R. M. Cantor, K. C. Kalunian, C. J. Chen, H. Badsha, R. Singh, D. J. Wallace, R. C. Kitridou, S. L. Chen, N. Shen, Y. W. Song, D. A. Isenberg, C. L. Yu, B. H. Hahn, and J. I. Rotter. 1997. Evidence for linkage of a candidate chromosome 1 region to human systemic lupus erythematosus. *J Clin Invest 99:725.*

165. Moser, K. L., B. R. Neas, J. E. Salmon, H. Yu, C. Gray-McGuire, N. Asundi, G. R. Bruner, J. Fox, J. Kelly, S. Henshall, D. Bacino, M. Dietz, R. Hogue, G. Koelsch, L. Nightingale, T. Shaver, N. I. Abdou, D. A. Albert, C. Carson, M. Petri, E. L. Treadwell, J. A. James, and J. B. Harley. 1998. Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American pedigrees. *Proc Natl Acad Sci U S A 95:14869.*

166. Wandstrat, A. E., C. Nguyen, N. Limaye, A. Y. Chan, S. Subramanian, X. H. Tian, Y. S. Yim, A. Pertsemlidis, H. R. Garner, Jr., L. Morel, and E. K.

Wakeland. 2004. Association of extensive polymorphisms in the SLAM/CD2 gene cluster with murine lupus. *Immunity 21:769*.

167. Martin, R., H. F. McFarland, and D. E. McFarlin. 1992. Immunological aspects of demyelinating diseases. *Annu Rev Immunol 10:153*.

168. Ferrante, P., M. L. Fusi, M. Saresella, D. Caputo, M. Biasin, D. Trabattoni, A. Salvaggio, E. Clerici, J. E. de Vries, G. Aversa, C. L. Cazzullo, and M. Clerici. 1998. Cytokine production and surface marker expression in acute and stable multiple sclerosis: altered IL-12 production and augmented signaling lymphocytic activation molecule (SLAM)-expressing lymphocytes in acute multiple sclerosis. *J Immunol 160:1514*.

169. Valdez, P. A., H. Wang, D. Seshasayee, M. van Lookeren Campagne, A. Gurney, W. P. Lee, and I. S. Grewal. 2004. NTB-A, a new activating receptor in T cells that regulates autoimmune disease. *J Biol Chem 279:18662*.

170. Scofield, R. H., G. R. Bruner, J. A. Kelly, J. Kilpatrick, D. Bacino, S. K. Nath, and J. B. Harley. 2003. Thrombocytopenia identifies a severe familial phenotype of systemic lupus erythematosus and reveals genetic linkages at 1q22 and 11p13. *Blood 101:992*.

171. Morel, L., and E. K. Wakeland. 1998. Susceptibility to lupus nephritis in the NZB/W model system. *Curr Opin Immunol 10:718*.

172. Rozzo, S. J., T. J. Vyse, C. G. Drake, and B. L. Kotzin. 1996. Effect of genetic background on the contribution of New Zealand black loci to autoimmune lupus nephritis. *Proc Natl Acad Sci U S A 93:15164.*

173. Drake, C. G., S. J. Rozzo, T. J. Vyse, E. Palmer, and B. L. Kotzin. 1995. Genetic contributions to lupus-like disease in (NZB x NZW)F1 mice. *Immunol Rev 144:51.*

174. Vyse, T. J., S. J. Rozzo, C. G. Drake, S. Izui, and B. L. Kotzin. 1997. Control of multiple autoantibodies linked with a lupus nephritis susceptibility locus in New Zealand black mice. *J Immunol 158:5566.*

175. Morel, L., U. H. Rudofsky, J. A. Longmate, J. Schiffenbauer, and E. K. Wakeland. 1994. Polygenic control of susceptibility to murine systemic lupus erythematosus. *Immunity 1:219.*

176. Mohan, C., E. Alas, L. Morel, P. Yang, and E. K. Wakeland. 1998. Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes. *J Clin Invest 101:1362.*

177. Morel, L., C. Mohan, Y. Yu, B. P. Croker, N. Tian, A. Deng, and E. K. Wakeland. 1997. Functional dissection of systemic lupus erythematosus using congenic mouse strains. *J Immunol 158:6019.*

178. Sobel, E. S., C. Mohan, L. Morel, J. Schiffenbauer, and E. K. Wakeland. 1999. Genetic dissection of SLE pathogenesis: adoptive transfer of Sle1

mediates the loss of tolerance by bone marrow-derived B cells. *J Immunol 162:2415*.

179. Sobel, E. S., L. Morel, R. Baert, C. Mohan, J. Schiffenbauer, and E. K. Wakeland. 2002. Genetic dissection of systemic lupus erythematosus pathogenesis: evidence for functional expression of Sle3/5 by non-T cells. *J Immunol 169:4025.*

180. Subramanian, S., Y. S. Yim, K. Liu, K. Tus, X. J. Zhou, and E. K. Wakeland. 2005. Epistatic suppression of systemic lupus erythematosus: fine mapping of Sles1 to less than 1 mb. *J Immunol 175:1062*.

181. Wu, C., K. B. Nguyen, G. C. Pien, N. Wang, C. Gullo, D. Howie, M. R. Sosa, M. J. Edwards, P. Borrow, A. R. Satoskar, A. H. Sharpe, C. A. Biron, and C. Terhorst. 2001. SAP controls T cell responses to virus and terminal differentiation of TH2 cells. *Nat Immunol 2:410*.

182. Czar, M. J., E. N. Kersh, L. A. Mijares, G. Lanier, J. Lewis, G. Yap, A. Chen, A. Sher, C. S. Duckett, R. Ahmed, and P. L. Schwartzberg. 2001. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc Natl Acad Sci U S A 98:7449.*

183. Yin, L., U. Al-Alem, J. Liang, W. M. Tong, C. Li, M. Badiali, J. J. Medard, J. Sumegi, Z. Q. Wang, and G. Romeo. 2003. Mice deficient in the X-linked lymphoproliferative disease gene sap exhibit increased

susceptibility to murine gammaherpesvirus-68 and hypo-gammaglobulinemia. *J Med Virol 71:446.*

184. Chen, G., A. K. Tai, M. Lin, F. Chang, C. Terhorst, and B. T. Huber. 2005. Signaling lymphocyte activation molecule-associated protein is a negative regulator of the CD8 T cell response in mice. *J Immunol 175:2212.*

185. Cannons, J. L., L. J. Yu, B. Hill, L. A. Mijares, D. Dombroski, K. E. Nichols, A. Antonellis, G. A. Koretzky, K. Gardner, and P. L. Schwartzberg. 2004. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity 21:693.*

186. Ma, C. S., N. J. Hare, K. E. Nichols, L. Dupre, G. Andolfi, M. G. Roncarolo, S. Adelstein, P. D. Hodgkin, and S. G. Tangye. 2005. Impaired humoral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by CD4+ T cells. *J Clin Invest 115:1049.*

187. Davidson, D., X. Shi, S. Zhang, H. Wang, M. Nemer, N. Ono, S. Ohno, Y. Yanagi, and A. Veillette. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. *Immunity 21:707.*

188. Marsland, B. J., T. J. Soos, G. Spath, D. R. Littman, and M. Kopf. 2004. Protein kinase C theta is critical for the development of in vivo T helper (Th)2 cell but not Th1 cell responses. *J Exp Med 200:181.*

189. Crotty, S., E. N. Kersh, J. Cannons, P. L. Schwartzberg, and R. Ahmed. 2003. SAP is required for generating long-term humoral immunity. *Nature 421:282*.

190. Hron, J. D., L. Caplan, A. J. Gerth, P. L. Schwartzberg, and S. L. Peng. 2004. SH2D1A regulates T-dependent humoral autoimmunity. *J Exp Med 200:261*.

191. Morra, M., R. A. Barrington, A. C. Abadia-Molina, S. Okamoto, A. Julien, C. Gullo, A. Kalsy, M. J. Edwards, G. Chen, R. Spolski, W. J. Leonard, B. T. Huber, P. Borrow, C. A. Biron, A. R. Satoskar, M. C. Carroll, and C. Terhorst. 2005. Defective B cell responses in the absence of SH2D1A. *Proc Natl Acad Sci U S A 102:4819*.

192. Al-Alem, U., C. Li, N. Forey, F. Relouzat, M. C. Fondaneche, S. V. Tavtigian, Z. Q. Wang, S. Latour, and L. Yin. 2005. Impaired Ig class switch in mice deficient for the X-linked lymphoproliferative disease gene Sap. *Blood 106:2069*.

193. Nichols, K. E., J. Hom, S. Y. Gong, A. Ganguly, C. S. Ma, J. L. Cannons, S. G. Tangye, P. L. Schwartzberg, G. A. Koretzky, and P. L. Stein. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med 11:340*.

194. Chung, B., A. Aoukaty, J. Dutz, C. Terhorst, and R. Tan. 2005. Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J Immunol 174:3153.*

195. Gonzalez-Cabrero, J., C. J. Wise, Y. Latchman, G. J. Freeman, A. H. Sharpe, and H. Reiser. 1999. CD48-deficient mice have a pronounced defect in CD4(+) T cell activation. *Proc Natl Acad Sci U S A 96:1019.*

196. Vaidya, S. V., S. E. Stepp, M. E. McNerney, J. K. Lee, M. Bennett, K. M. Lee, C. L. Stewart, V. Kumar, and P. A. Mathew. 2005. Targeted disruption of the 2B4 gene in mice reveals an in vivo role of 2B4 (CD244) in the rejection of B16 melanoma cells. *J Immunol 174:800.*

197. Wang, N., A. Satoskar, W. Faubion, D. Howie, S. Okamoto, S. Feske, C. Gullo, K. Clarke, M. R. Sosa, A. H. Sharpe, and C. Terhorst. 2004. The cell surface receptor SLAM controls T cell and macrophage functions. *J Exp Med 199:1255.*

198. Howie, D., F. S. Laroux, M. Morra, A. R. Satoskar, L. E. Rosas, W. A. Faubion, A. Julien, S. Rietdijk, A. J. Coyle, C. Fraser, and C. Terhorst. 2005. Cutting edge: the SLAM family receptor Ly108 controls T cell and neutrophil functions. *J Immunol 174:5931.*

199. McNerney, M. E., D. Guzior, and V. Kumar. 2005. 2B4 (CD244)-CD48 interactions provide a novel MHC class I-independent system for NK-cell self-tolerance in mice. *Blood 106:1337.*

200. Mathew, P. A., B. A. Garni-Wagner, K. Land, A. Takashima, E. Stoneman, M. Bennett, and V. Kumar. 1993. Cloning and characterization of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated natural killer cells and T cells. *J Immunol 151:5328.*

201. Valiante, N. M., and G. Trinchieri. 1993. Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *J Exp Med 178:1397.*

202. Nakajima, H., M. Cella, H. Langen, A. Friedlein, and M. Colonna. 1999. Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur J Immunol 29:1676.*

203. Boles, K. S., H. Nakajima, M. Colonna, S. S. Chuang, S. E. Stepp, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens 54:27.*

204. Romero, X., D. Benitez, S. March, R. Vilella, M. Miralpeix, and P. Engel. 2004. Differential expression of SAP and EAT-2-binding leukocyte cell-surface molecules CD84, CD150 (SLAM), CD229 (Ly9) and CD244 (2B4). *Tissue Antigens 64:132.*

205. Munitz, A., I. Bachelet, S. Fraenkel, G. Katz, O. Mandelboim, H. U. Simon, L. Moretta, M. Colonna, and F. Levi-Schaffer. 2005. 2B4 (CD244) is expressed and functional on human eosinophils. *J Immunol 174:110.*

206. Schuhmachers, G., K. Ariizumi, P. A. Mathew, M. Bennett, V. Kumar, and A. Takashima. 1995. 2B4, a new member of the immunoglobulin gene superfamily, is expressed on murine dendritic epidermal T cells and plays a functional role in their killing of skin tumors. *J Invest Dermatol 105:592.*

207. Kubota, K. 2002. A structurally variant form of the 2B4 antigen is expressed on the cell surface of mouse mast cells. *Microbiol Immunol 46:589.*

208. Kambayashi, T., E. Assarsson, J. C. Chambers, and H. Ljunggren. 2001. Cutting edge: Regulation of CD8+ T cell proliferation by 2B4/CD48 interactions. *J Immunol 167:6706.*

209. Peritt, D., D. A. Sesok-Pizzini, R. Schretzenmair, R. R. Macgregor, N. M. Valiante, X. Tu, G. Trinchieri, and M. Kamoun. 1999. C1.7 antigen expression on CD8+ T cells is activation dependent: increased proportion of C1.7+CD8+ T cells in HIV-1-infected patients with progressing disease. *J Immunol 162:7563.*

210. Stepp, S. E., J. D. Schatzle, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Gene structure of the murine NK cell receptor 2B4: presence of two

alternatively spliced isoforms with distinct cytoplasmic domains. *Eur J Immunol 29:2392*.

211. Kubin, M. Z., D. L. Parshley, W. Din, J. Y. Waugh, T. Davis-Smith, C. A. Smith, B. M. Macduff, R. J. Armitage, W. Chin, L. Cassiano, L. Borges, M. Petersen, G. Trinchieri, and R. G. Goodwin. 1999. Molecular cloning and biological characterization of NK cell activation-inducing ligand, a counterstructure for CD48. *Eur J Immunol 29:3466*.

212. Kubota, K., H. Katoh, K. Muguruma, and K. Koyama. 1999. Characterization of a surface membrane molecule expressed by natural killer cells in most inbred mouse strains: monoclonal antibody C9.1 identifies an allelic form of the 2B4 antigen. *Immunology 96:491*.

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

214. Mooney, J. M., J. Klem, C. Wulfing, L. A. Mijares, P. L. Schwartzberg, M. Bennett, and J. D. Schatzle. 2004. The murine NK receptor 2B4 (CD244) exhibits inhibitory function independent of signaling lymphocytic activation molecule-associated protein expression. *J Immunol 173:3953*.

215. Lee, K. M., M. E. McNerney, S. E. Stepp, P. A. Mathew, J. D. Schatzle, M. Bennett, and V. Kumar. 2004. 2B4 acts as a non-major

histocompatibility complex binding inhibitory receptor on mouse natural killer cells. *J Exp Med 199:1245*.

216. Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood 105:4416*.

217. Chen, R., F. Relouzat, R. Roncagalli, A. Aoukaty, R. Tan, S. Latour, and A. Veillette. 2004. Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. *Mol Cell Biol 24:5144*.

218. Bloch-Queyrat, C., M. C. Fondaneche, R. Chen, L. Yin, F. Relouzat, A. Veillette, A. Fischer, and S. Latour. 2005. Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn. *J Exp Med 202:181*.

219. Lee, K. M., J. P. Forman, M. E. McNerney, S. Stepp, S. Kuppireddi, D. Guzior, Y. E. Latchman, M. H. Sayegh, H. Yagita, C. K. Park, S. B. Oh, C. Wulfing, J. Schatzle, P. A. Mathew, A. H. Sharpe, and V. Kumar. 2005. Requirement of homotypic NK cell interactions through 2B4(CD244)/CD48 in the generation of NK effector functions. *Blood*.

220. Assarsson, E., T. Kambayashi, C. M. Persson, B. J. Chambers, and H. G. Ljunggren. 2005. 2B4/CD48-mediated regulation of lymphocyte activation and function. *J Immunol 175:2045*.

221. Assarsson, E., T. Kambayashi, J. D. Schatzle, S. O. Cramer, A. von Bonin, P. E. Jensen, H. G. Ljunggren, and B. J. Chambers. 2004. NK cells stimulate proliferation of T and NK cells through 2B4/CD48 interactions. *J Immunol 173:174.*

222. Lee, K. M., S. Bhawan, T. Majima, H. Wei, M. I. Nishimura, H. Yagita, and V. Kumar. 2003. Cutting edge: the NK cell receptor 2B4 augments antigen-specific T cell cytotoxicity through CD48 ligation on neighboring T cells. *J Immunol 170:4881.*

223. Gao, N., T. Dang, W. A. Dunnick, J. T. Collins, B. R. Blazar, and D. Yuan. 2005. Receptors and counterreceptors involved in NK-B cell interactions. *J Immunol 174:4113.*

224. Costello, R. T., S. Sivori, F. Mallet, D. Sainty, C. Arnoulet, D. Reviron, J. A. Gastaut, A. Moretta, and D. Olive. 2002. A novel mechanism of antitumor response involving the expansion of CD3+/CD56+ large granular lymphocytes triggered by a tumor-expressed activating ligand. *Leukemia 16:855.*

225. Tangye, S. G., J. H. Phillips, L. L. Lanier, and K. E. Nichols. 2000. Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. *J Immunol 165:2932.*

226. Sivori, S., S. Parolini, M. Falco, E. Marcenaro, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2000. 2B4 functions as a co-receptor in human NK cell activation. *European Journal of Immunology 30:787.*

227. Bryceson, Y. T., M. E. March, D. F. Barber, H. G. Ljunggren, and E. O. Long. 2005. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med 202:1001.*

228. Watzl, C., and E. O. Long. 2003. Natural killer cell inhibitory receptors block actin cytoskeleton-dependent recruitment of 2B4 (CD244) to lipid rafts. *J Exp Med 197:77.*

229. Bryceson, Y. T., M. E. March, H. G. Ljunggren, and E. O. Long. 2005. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood.*

230. Nagy, N., K. Mattsson, A. Maeda, A. Liu, L. Szekely, and E. Klein. 2002. The X-linked lymphoproliferative disease gene product SAP is expressed in activated T and NK cells. *Immunol Lett 82:141.*

231. Sivori, S., M. Falco, E. Marcenaro, S. Parolini, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2002. Early expression of triggering receptors and regulatory role of 2B4 in human natural killer cell precursors undergoing in vitro differentiation. *Proc Natl Acad Sci U S A 99:4526.*

232. Morandi, B., R. Costa, M. Falco, S. Parolini, A. De Maria, G. Ratto, M. C. Mingari, G. Melioli, A. Moretta, and G. Ferlazzo. 2005. Distinctive lack

of CD48 expression in subsets of human dendritic cells tunes NK cell activation. *J Immunol 175:3690.*

233. Jevremovic, D., D. D. Billadeau, R. A. Schoon, C. J. Dick, B. J. Irvin, W. Zhang, L. E. Samelson, R. T. Abraham, and P. J. Leibson. 1999. Cutting edge: a role for the adaptor protein LAT in human NK cell-mediated cytotoxicity. *J Immunol 162:2453.*

234. Bottino, C., R. Augugliaro, R. Castriconi, M. Nanni, R. Biassoni, L. Moretta, and A. Moretta. 2000. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. *Eur J Immunol 30:3718.*

235. Eissmann, P., L. Beauchamp, J. Wooters, J. C. Tilton, E. O. Long, and C. Watzl. 2005. Molecular basis for positive and negative signaling by the natural killer cell receptor 2B4 (CD244). *Blood 105:4722.*

236. Jevremovic, D., D. D. Billadeau, R. A. Schoon, C. J. Dick, and P. J. Leibson. 2001. Regulation of NK cell-mediated cytotoxicity by the adaptor protein 3BP2. *J Immunol 166:7219.*

237. Saborit-Villarroya, I., J. M. Del Valle, X. Romero, E. Esplugues, P. Lauzurica, P. Engel, and M. Martin. 2005. The Adaptor Protein 3BP2 Binds Human CD244 and Links this Receptor to Vav Signaling, ERK Activation, and NK Cell Killing. *J Immunol 175:4226.*

238. Chuang, S. S., R. K. Pappanaicken, and P. A. Mathew. 2001. 2B4 (CD244)-mediated activation of cytotoxicity and IFN-gamma release in human NK cells involves distinct pathways. *Journal of Immunology 167:6210.*

239. Colucci, F., E. Rosmaraki, S. Bregenholt, S. I. Samson, V. Di Bartolo, M. Turner, L. Vanes, V. Tybulewicz, and J. P. Di Santo. 2001. Functional dichotomy in natural killer cell signaling: Vav1-dependent and -independent mechanisms. *J Exp Med 193:1413.*

240. Aoukaty, A., and R. Tan. 2005. Role for glycogen synthase kinase-3 in NK cell cytotoxicity and X-linked lymphoproliferative disease. *J Immunol 174:4551.*

241. George, T. C., J. R. Ortaldo, S. Lemieux, V. Kumar, and M. Bennett. 1999. Tolerance and alloreactivity of the Ly49D subset of murine NK cells. *J Immunol 163:1859.*

242. Calpe, S., E. Erdos, G. Liao, N. Wang, S. Rietdijk, M. Simarro, B. Scholtz, J. Mooney, C. H. Lee, M. S. Shin, E. Rajnavolgyi, J. Schatzle, H. C. Morse, 3rd, C. Terhorst, and A. Lanyi. 2006. Identification and characterization of two related murine genes, Eat2a and Eat2b, encoding single SH2-domain adapters. *Immunogenetics:1.*

243. Tskvitaria-Fuller, I., A. L. Rozelle, H. L. Yin, and C. Wulfing. 2003. Regulation of sustained actin dynamics by the TCR and costimulation as a mechanism of receptor localization. *J Immunol 171:2287*.

244. Lanier, L. L. 1997. Natural killer cell receptors and MHC class I interactions. *Curr Opin Immunol 9:126*.

245. Seed, B., and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc Natl Acad Sci U S A 84:3365*.

246. Fennelly, J. A., B. Tiwari, S. J. Davis, and E. J. Evans. 2001. CD2F-10: a new member of the CD2 subset of the immunoglobulin superfamily. *Immunogenetics 53:599*.

247. Wallner, B. P., A. Z. Frey, R. Tizard, R. J. Mattaliano, C. Hession, M. E. Sanders, M. L. Dustin, and T. A. Springer. 1987. Primary structure of lymphocyte function-associated antigen 3 (LFA-3). The ligand of the T lymphocyte CD2 glycoprotein. *J Exp Med 166:923*.

248. Davis, D. M., I. Chiu, M. Fassett, G. B. Cohen, O. Mandelboim, and J. L. Strominger. 1999. The human natural killer cell immune synapse. *Proc Natl Acad Sci U S A 96:15062*.

249. Schuhmachers, G., K. Ariizumi, P. A. Mathew, M. Bennett, V. Kumar, and A. Takashima. 1995. Activation of murine epidermal gamma delta T cells through surface 2B4. *Eur J Immunol 25:1117*.

250. Veillette, A. 2003. SAP: a molecular switch regulating the immune response through a unique signaling mechanism. *Eur J Immunol 33:1141*.

251. Kumaresan, P. R., V. T. Huynh, and P. A. Mathew. 2000. Polymorphism in the 2B4 gene of inbred mouse strains. *Immunogenetics 51:758*.

252. Ames, J. B., V. Vyas, J. D. Lusin, and R. Mariuzza. 2005. NMR structure of the natural killer cell receptor 2B4 (CD244): implications for ligand recognition. *Biochemistry 44:6416*.

253. Taylor, M. A., B. Ward, J. D. Schatzle, and M. Bennett. 2002. Perforin- and Fas-dependent mechanisms of natural killer cell-mediated rejection of incompatible bone marrow cell grafts. *Eur J Immunol 32:793*.

254. Mathew, S. O., P. R. Kumaresan, J. K. Lee, V. T. Huynh, and P. A. Mathew. 2005. Mutational Analysis of the Human 2B4 (CD244)/CD48 Interaction: Lys68 and Glu70 in the V Domain of 2B4 Are Critical for CD48 Binding and Functional Activation of NK Cells. *J Immunol 175:1005*.

255. Hamerman, J. A., N. K. Tchao, C. A. Lowell, and L. L. Lanier. 2005. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat Immunol 6:579*.

256. Park, I. K., L. D. Shultz, J. J. Letterio, and J. D. Gorham. 2005. TGF-beta1 inhibits T-bet induction by IFN-gamma in murine CD4+ T cells through

the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1. *J Immunol 175:5666.*

257. Grewal, I. S., J. Xu, and R. A. Flavell. 1995. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature 378:617.*

258. van Essen, D., H. Kikutani, and D. Gray. 1995. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. *Nature 378:620.*

259. Lee, B. O., L. Haynes, S. M. Eaton, S. L. Swain, and T. D. Randall. 2002. The biological outcome of CD40 signaling is dependent on the duration of CD40 ligand expression: reciprocal regulation by interleukin (IL)-4 and IL-12. *J Exp Med 196:693.*

260. Punnonen, J., B. G. Cocks, J. M. Carballido, B. Bennett, D. Peterson, G. Aversa, and J. E. de Vries. 1997. Soluble and membrane-bound forms of signaling lymphocytic activation molecule (SLAM) induce proliferation and Ig synthesis by activated human B lymphocytes. *J Exp Med 185:993.*

261. Klaman, L. D., and D. A. Thorley-Lawson. 1995. Characterization of the CD48 gene demonstrates a positive element that is specific to Epstein-Barr virus-immortalized B-cell lines and contains an essential NF-kappa B site. *J Virol 69:871.*

262. Borca, M. V., G. F. Kutish, C. L. Afonso, P. Irusta, C. Carrillo, A. Brun, M. Sussman, and D. L. Rock. 1994. An African swine fever virus gene

with similarity to the T-lymphocyte surface antigen CD2 mediates hemadsorption. *Virology 199:463*.

263. Borca, M. V., C. Carrillo, L. Zsak, W. W. Laegreid, G. F. Kutish, J. G. Neilan, T. G. Burrage, and D. L. Rock. 1998. Deletion of a CD2-like gene, 8-DR, from African swine fever virus affects viral infection in domestic swine. *J Virol 72:2881*.

264. Wandstrat, A., and E. Wakeland. 2001. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. *Nat Immunol 2:802*.

265. James, J. A., B. R. Neas, K. L. Moser, T. Hall, G. R. Bruner, A. L. Sestak, and J. B. Harley. 2001. Systemic lupus erythematosus in adults is associated with previous Epstein-Barr virus exposure. *Arthritis Rheum 44:1122*.

266. Moon, U. Y., S. J. Park, S. T. Oh, W. U. Kim, S. H. Park, S. H. Lee, C. S. Cho, H. Y. Kim, W. K. Lee, and S. K. Lee. 2004. Patients with systemic lupus erythematosus have abnormally elevated Epstein-Barr virus load in blood. *Arthritis Res Ther 6:R295*.

267. Kang, I., T. Quan, H. Nolasco, S. H. Park, M. S. Hong, J. Crouch, E. G. Pamer, J. G. Howe, and J. Craft. 2004. Defective control of latent Epstein-Barr virus infection in systemic lupus erythematosus. *J Immunol 172:1287*.

268. Gross, A. J., D. Hochberg, W. M. Rand, and D. A. Thorley-Lawson. 2005. EBV and systemic lupus erythematosus: a new perspective. *J Immunol 174:6599.*

269. Cepok, S., D. Zhou, R. Srivastava, S. Nessler, S. Stei, K. Bussow, N. Sommer, and B. Hemmer. 2005. Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. *J Clin Invest 115:1352.*

270. McClain, M. T., L. D. Heinlen, G. J. Dennis, J. Roebuck, J. B. Harley, and J. A. James. 2005. Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry. *Nat Med 11:85.*

271. von Herrath, M. G., R. S. Fujinami, and J. L. Whitton. 2003. Microorganisms and autoimmunity: making the barren field fertile? *Nat Rev Microbiol 1:151.*

272. Vinuesa, C. G., S. G. Tangye, B. Moser, and C. R. Mackay. 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol 5:853.*

273. Pasare, C., and R. Medzhitov. 2005. Control of B-cell responses by Toll-like receptors. *Nature 438:364.*

274. Peng, S. L. 2005. Signaling in B cells via Toll-like receptors. *Curr Opin Immunol 17:230.*

275. Rui, L., C. G. Vinuesa, J. Blasioli, and C. C. Goodnow. 2003. Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. *Nat Immunol 4:594.*

276. Lin, L., A. J. Gerth, and S. L. Peng. 2004. CpG DNA redirects class-switching towards "Th1-like" Ig isotype production via TLR9 and MyD88. *Eur J Immunol 34:1483.*

VITAE

Jill Marie Mooney was born in Eugene, Oregon, on January 5, 1978, the daughter of Ellen Marie and Christopher Joseph Mooney Jr. She has one younger brother, Shane Thomas. She graduated from Marist High School in Eugene, Oregon in June of 1996. Jill then obtained an Honors Bachelor of Science degree in Biochemistry and Biophysics from Oregon State University in Corvallis, Oregon in June of 2000. The following fall she was admitted to the doctorate program at the University of Texas Southwestern Medical Center in Dallas, Texas. She received her Doctorate of Philosophy degree in immunology on the 13th of January, 2006.

Permanent Address: 93614 Swamp Creek Road
Blachly, Oregon 97412