The Role of Vav-1, Vav-2 and Lsc in NK T cell development and NK cell cytotoxicity

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Summary

The hematopoietic-specific Rho-family GTP exchange factor (GEF) Vav-1 is a regulator of lymphocyte antigen receptor signaling and mediates normal maturation and activation of B and T cells. Recent findings suggest that Vav-1 also forms part of signaling pathways required for natural and antibody dependent cellular cytotoxicity (ADCC) of human NK cells. In this study, I show that Vav-1 is also expressed in murine NK cells. Vav-$1^{-/-}$ mice had normal numbers of splenic NK cells, and these displayed a similar expression profile of NK cell receptors as cells from wild type mice. Unexpectedly, IL-2-activated Vav-$1^{-/-}$ NK cells retained normal ADCC. Fc-receptor mediated activation of ERK, JNK, and p38 was also normal. In contrast, Vav-$1^{-/-}$ NK cells exhibited reduced natural cytotoxicity against EL4, C4.4.25, RMA and RMA/S. Together, these results demonstrate that Vav-1 is dispensable for mainstream NK cell development, but is required for NK cell natural cytotoxicity. Vav-2, a protein homologous to Vav-1 has also been implicated in NK cell functions. However, NK cells from Vav-$2^{-/-}$ mice have normal cytotoxic activities and NK cells that lack both Vav-1 and Vav-2 exhibit similar defect as Vav-$1^{-/-}$ cells. Thus Vav-2 has no apparent function in the development and the activation of NK cells. Although NK cell development is normal in Vav-$1^{-/-}$ mice, their numbers of NKT cells were dramatically diminished. Furthermore, NKT cells from Vav-1 mutant mice failed to produce IL-4 and IFNγ following *in vivo* CD3 stimulation. A similar loss of NKT cells was observed in Vav-$1^{-/-}$Vav-$2^{-/-}$ mice, but not in Vav-$2^{-/-}$ mice,

suggesting that only Vav-1, and not Vav-2, is an essential regulator of NKT cell development and NK cell cytotoxicity.

Similar to Vav-1, Lsc is a Rho GEF that is expressed specifically in the hematopoietic system. It contains a regulator of G-protein signaling (RGS) domain which negatively regulates the Gα12 and Gα13 subunits of G-protein coupled receptors (GPCRs). This study shows that NK and NKT cell development are normal in $Lsc^{-/-}$ mice. However, NK cells from mutant mice display enhanced cytotoxic responses towards a panel of tumor cells. These data implicate for the first time a RGS-containing Rho GEF in cytotoxic responses and suggest that Lsc down-modulate NK cell activation.

Zusammenfassung

Vav-1 ist ein spezifisch in hämatopoetischen Zellen exprimierter Guanin-Nukleotid-Exchange-Faktor (GEF) für Rho-GTPasen, der die Antigenrezeptor-vermittelte Signaltransduktion in Lymphocyten reguliert und essentiell für der Reifung und Aktivierung von B- und T-Zellen ist. Untersuchungen an menschlichen Zellen lassen vermuten, dass Vav1 auch für Antikörper-unabhängige, natürliche Zytotoxizität und die Antikörper-abhängige zellvermittelte Zytoxizität (ADCC) von „Natürlichen-Killerzellen“ (NK-Zellen) wichtig ist. In der vorliegenden Arbeit zeige ich, dass Vav-1 auch in murinen NK-Zellen exprimiert ist. Analysen von Vav-1$^{-/-}$-Mäuse zeigen eine normale Anzahl von NK-Zellen, die wiederum ein ähnliches Expressionsprofil von typischen NK-Zell-Rezeptoren im Vergleich zu wildtypischen Mäusen aufweisen. Die ADCC von Vav-1$^{-/-}$ NK-Zellen ist unverändert, wie auch die Fc-Rezeptor vermittelte Aktivierung von ERK, JNK und p38. Im Gegensatz dazu zeigen Vav-1$^{-/-}$ NK-Zellen eine reduzierte natürliche Cytotoxizität gegenüber EL4-, C4.4.25-, RMA- und RMA/S-Zielzellen. Vav-1 ist daher nicht für die Entwicklung, sondern für den Aufbau der natürlichen Cytotoxizität von NK-Zellen von Bedeutung. Für Vav-2 wurde ebenfalls eine Rolle in NK-Zellfunktionen wahrscheinlich gemacht. Dennoch zeigen Vav-2$^{-/-}$ Mäuse ein normales zytotoxisches Verhalten. NK-Zellen von Vav-1/Vav-2-doppeldefizienten Tieren weisen ähnliche Defekte wie NK-Zellen von Vav-1-defizienten Tieren. Somit besitzt Vav-2 keine entscheidende Funktion für die Entwicklung und Aktivierung von NK-Zellen. Im Gegensatz zu NK-Zellen ist die Anzahl der NKT-Zellen in Vav-1$^{-/-}$ Mäusen drastisch reduziert. Außerdem

sind NKT-Zellen Vav-1-defizienter Mäuse nicht in der Lage IL-4 und INFγ nach CD3-Stimulierung *in vivo* zu produzieren. Ein ähnlicher Verlust der NKT-Zell-Population wurde in Vav-$1^{-/-}$- Vav-$2^{-/-}$-Mäusen beobachtet, nicht aber in Vav-$2^{-/-}$ Mäusen. Daher scheint nur Vav-1, nicht aber Vav-2, ein essentieller Regulator sowohl der NKT-Zell-Entwicklung als auch der NK-Zell-Cytotoxizität zu sein. Ein weiterer Rho-GEF, Lsc, ist ebenfalls spezifisch im hämatopoetischen System exprimiert. Lsc besitzt auch eine negativ-regulatorische RGS-Domäne (regulator of G-protein signaling) für die Gα12- und Gα13-Untereinheiten von G-Protein-gekoppelten Rezeptoren. NK- und NKT-Zellen von Lsc-defizienten Mäusen entwickeln sich normal, weisen aber eine erhöhte Cytotoxizität gegenüber einer Reihe von Tumor-Zellen auf. Diese Daten zeigen zum ersten mal die Beteiligung eines RGS-Rho-GEF an zytotoxischen Reaktionen und deuten auf eine negative Modulation der NK-Zell-Aktivierung durch Lsc hin.

Abbreviations

AD	acidic domain
ADCC	Antibody-dependent cellular cytotoxicity
BCR	B cell receptor
BM	bone marrow
BMMC	bone marrow derived mast cell
BSA	bovine serum albumin
CD	cluster of differentiation
CH	calponin homology
Cr	chromium
CTL	cytotoxic T lymphocyte
DH	Dbl homology
DN	double negative
DP	double positive
DTH	delayed type hyersensitivity
ECL	enhance chemilumescence
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescein-activated cell sorter
FcR	Fc receptor
FCS	fetal calf serum
FITC	fluorescein isothiocynanate
GAP	GTPase activating protein
GEF	guanosine nucleotide exchange factor
GDP	guanosine diphosphate
GPI	glycosyl-phoshatidyl-inositol
GTP	guanosine triphosphate
Grb2	growth factor receptor-bound protein-2
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
JNK	c-Jun N-terminal kinase
KIR	killer immunologlobin-related receptor
LAK	lymphokine activated killer
LAT	linker for activation of T cells
LT	lymphotoxin

MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
NF-AT	nuclear factor of activated T cells
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PAK	p21 activated kinase
PBS	phosphate buffered saline
PE	phycoerythrin
PH	pleckstrin homology
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3 kinase
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinase
RAG	recombinase activating gene
Rho	ras homolog
SAP	SLAM-associated protein
SDS	sodium dodecyl sulfate
SH	Src homology
SH2D1A	SH2 domain-containing protein
SHP	SH2-containing phosphatase
Sos	son of sevenless
SP	single positive
TCF	T-cell factor
TCR	T-cell receptor
TD	T-cell-dependent
TI	T-cell-independent
XLP	X-linked lymphoproliferative disease
ZAP	ζ-chain associated protein

1. Introduction

1.1 NKT cells

Natural killer (NK) T cells are important for tumor rejection, prevention of autoimmunity, protection against bacterial infections, and modulating T cells cytokine profiles. Mouse NKT cells express typical NK markers such as NK1.1, members of the Ly49 family, and IL-2Rβ, demonstrating developmental and/or activation characteristics reminiscent of NK cells (Godfrey et al., 2000). Many NKT cells are positively selected by CD1, a β_2-microglobulin (β_2m) associated MHC class I like molecule (Porcelli and Modlin, 1999). The CD1-restricted NKT cells are localized mostly in the thymus and the liver, but also present in the bone marrow and the spleen in lower numbers. These NKT cells express a TCR repertoire with a biased Vα chain usage: in humans mainly Vα24JαQ pairing with Vβ11, and in mice mainly Vα14Jα281 paired with Vβ8.2,Vβ7 and Vβ2 (Godfrey et al., 2000). CD1d restricted NKT cells are predominately $CD4^+$ or $CD4^-CD8^-$(DN). CD1-independent NKT cells, on the contrary, are either $CD8^+$ or DN. They have a diverse TCR repertoire, and are mostly found in the spleen and the bone marrow. Whether there exist any functional difference between $CD4^+$ and $CD8^+$ NKT cells is not known (Eberl et al., 1999b; Emoto et al., 2000; Godfrey et al., 2000; Hammond et al., 1999; Hammond et al., 2001).

Gene-ablation studies revealed many molecules are necessary for NKT cells development. These mutants exhibit profound NKT cell deficiencies (Table 1.1). Interestingly one naturally occurring mice strain, SJL, also exhibit a selective reduction of NKT cells (Yoshimoto et al., 1999). The cause of this defect is still unknown. Although the ontology of NKT cells is not well characterized, mice deficient in Lck, pre-TCRα, CD45, CD3ζ and ZAP-70 exhibit developmental defect in both T cell and NKT cell compartments, highlighting possible common signaling pathways in maturation of these two lineages. However, $Fyn^{-/-}$, $LT\alpha^{-/-}$ and $LT\beta^{-/-}$ mice showed a normal maturation of conventional T cells but a mark reduction in the number of NKT cells. These observations indicate that the ontology of conventional T cells and NKT cells have similar but not identical signaling requirements.

Although NKT cells have been implicated in various immune responses, their precise roles and their mechanisms of activation remain largely unknown. *In vitro*, CD3 ligation of thymic and splenic NK T cells leads to the release of IL-4 (Hammond et al., 1999). Thymic NKT cells but not splenic NKT cells can also secrete moderate amount of IFNγ in response to CD3 stimulation. *In vivo*, injection of CD3 lead to a prompt and robust release of IL-4 by splenic NKT cells (Yoshimoto and Paul, 1994). This response is largely abrogated in mice that are deficient in NKT cells (Chen et al., 1997; Cui et al., 1999; Gadue et al., 1999; Mendiratta et al., 1997; Yoshimoto et al., 1999). Since NKT cells are capable of releasing large amount of IL-4, it was speculated that their activation might polarize a Th2 response. However, activation of NKT cells can also trigger NK cells to release large amount of IFNγ

Table 1.1. Gene-ablation mutations that lead to aberration of NK T cell ontology

Mice mutation	NKT cells	Reference
β_2M	Severely reduced	(Bendelac et al., 1992; Coles and Raulet, 1994)
CD1d	Severely reduced	(Chen et al., 1997; Mendiratta et al., 1997; Smiley et al., 1997)
pre-TCRα	Severely reduced	(Eberl et al., 1999a)
CD3ζ	Severely reduced	(Arase et al., 1995)
ZAP-70	Severely reduced	(Iwabuchi et al., 2001)
CD45	Reduced	(Martin et al., 2001)
Lck	Reduced	(Eberl et al., 1999c; Gadue et al., 1999)
Fyn	Reduced	(Eberl et al., 1999c; Gadue et al., 1999)
Vav-1	Reduced	(Chan et al., 2001; Colucci et al., 2001)
Jα281	Severely reduced	(Cui et al., 1997)
TCF-1	Reduced	(Ohteki et al., 1996)
IRF-1	Reduced	(Ohteki et al., 1998)
LTα	Severely reduced	(Elewaut et al., 2000)
LTβ	Severely reduced	(Elewaut et al., 2000)
IL-7Rα	Reduced	(Boesteanu et al., 1997)
IL-2Rβ/IL-15Rβ	Reduced	(Ohteki et al., 1997)
γc	Severely reduced	(Boesteanu et al., 1997)
IL-15Rα	Reduced	(Lodolce et al., 1998)
IL-15	Reduced	(Kennedy et al., 2000)
SJL (naturally occuring stain)	Reduced	(Yoshimoto et al., 1999)

(Carnaud et al., 1999). Whether NKT cell activation polarize a Th2 response *in vivo* remain unresolved.

Natural occurring antigens that could trigger NKT cell activation remains obscure. CD1d independent NKT cells have a diverse TCR expression and it was suggested that they bind to conventional MHC-peptide complexes (Eberl et al., 1999b; Hammond et al., 1999). While CD1d dependent NKT cells probably recognize CD1d in conjunction with hydrophobic ligands (Benlagha et al., 2000; Burdin and Kronenberg, 1999). Until now, no natural ligand for CD1d restricted NKT cells has been identified. α-galactosylceramide (αGalCer), a glycosphingolipid derived from marine sponge *Agelas mauritanius* can bind to CD1d and strongly stimulate NKT cells (Burdin et al., 1998; Kawano et al., 1997; Yamaguchi et al., 1996). Exposure to αGalCer to NKT cells leads to massive production of both IFNγ and IL-4, NK cell proliferation and cytotoxic responses (Carnaud et al., 1999; Eberl and MacDonald, 2000). *In vivo*, αGalCer treatment lead to anti-tumor immunity (Kawano et al., 1998; Nakagawa et al., 1998), protection against murine malaria (Gonzalez-Aseguinolaza et al., 2000) and prevention of type 1 diabetes in mice (Hong et al., 2001; Sharif et al., 2001). Recent evidence suggested NKT cells also participate an immunosuppressive function. Terabe et al. (2000) showed that a subset of $CD4^+$ NKT cells can act as suppressors of CTL mediated anti-tumor immunity. Furthermore, Moodycliffe et al. (2000) showed $CD4^+DX5^+$ cells plays a critical role in suppressing delayed type hypersensitivity (DTH). It is therefore possible that different populations of NKT cells may exhibit unique cytokine profiles, allowing them to execute different functions *in vivo*.

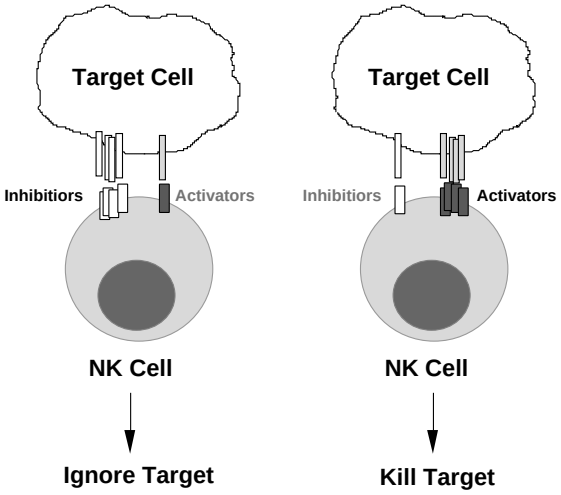
1.2 NK cells

Natural killer (NK) cells are subset of lymphocytes that can contribute protective responses against a variety of infections and tumors (Biron et al., 1999). They are bone marrow derived and share a common progenitor with T cells. This is demonstrated by a subset of $CD34^+$ human thymic precursors capable of differentiating into NK and T cells in fetal thymic organ cultures (Sanchez et al., 1994). In both mice and human, NK cells comprise up to 5-15% of peripheral blood lymphocytes and are found in the spleen, bone marrow, liver, lymph nodes and lungs (Trinchieri, 1989). Unlike T and B cells, NK cells do not have antigen specific receptors, instead they express a plethora of surface molecules that could discriminate abnormal cells from healthy ones. These receptors deliver either stimulatory or inhibitory signals, and it is the net balance of these signals that ultimately lead to promotion or suppression of NK activation (Figure 1.1 and Table 1.2).

1.2.1 Inhibitory receptors of NK cell activation

Ly49 protein family

A



B

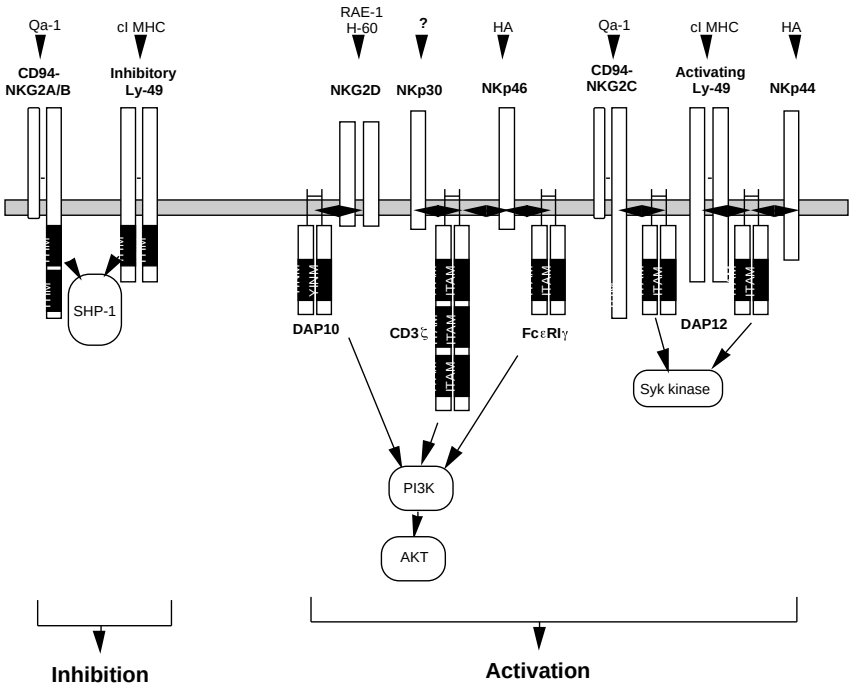


Figure 1.1 A. NK cell acitivation in mediated by signal integration of stimulatory and inhibitory receptors on the cell surface. Normal target cells express more inhibitors leading to tolerance. Tumor and viral infected targets down-regulate inhibitors and up-regulation of activators, thereby rendering them sensitive to NK cell lysis. **B.** Major NK cell stimulatory and inhibitory receptors.
◄► Interaction of charge residues in the transmembrane of receptors.

Table 1.2. Activators and Inhibitors on NK cells

NK cell Receptors	Species	Ligands	Signaling Components
Activators			
NKR-P1C (CD161)	M	unknown	FcεRγI
Ly49D	M	H-2D^{d}	DAP12,Syk, ZAP70
Ly49H	M	unknown	DAP12
CD94/NKG2C	M, H	Qa-1^{b} (M), HLA-E (H)	DAP12
KIR2DS	H	HLA-C	DAP12
NKG2D	M, H	RAE-1 (M), H-60 (M), MICA/B (H)	DAP10, PI3K
2B4 (CD244)	M, H	CD48	SAP, LAT
NKp46	M, H	Viral hemaglutinin	FcεRγI, CD3ζ, PI3K, AKT
NKp30	M	unknown	CD3ζ, PI3K, AKT
NKp44	M	Viral hemaglutinin	DAP12
Lag-3	M, H	MHC class II	?
Inhibitors			
Ly49A	M	H-2D^{b,d,k,p}, H-2^{s}	SHP-1
Ly49C	M	H-2K^{b,d},H-2D^{b,d,k},H-2^{s}	SHP-1
Ly49G2	M	H-2D^{d,r}, H-2^{k}	SHP-1
Ly49I	M	H-2K^{d},H-2^{b,d,k,s}	SHP-1
Ly49F	M	H-2^{d}	SHP-1
CD94/NKG2A/B	M, H	Qa-1^{b} (M), HLA-E (H)	SHP-1

M, mouse. H, human.

NK cells express a variety of different inhibitory receptors. In humans, inhibition is mediated by killer immunologlobin-related receptors (KIR), a type I transmembrane protein belonging to the Ig superfamily that binds specific HLA class I molecules (Long, 1999). In mice, inhibition is mediated by Ly49 family members, which are type II transmembrane proteins of the c-type lectin superfamily (Long, 1999). Despite the difference in membrane topology, both of these inhibitors transduce their signals via a conserved mechanism. They contain immunoreceptor tyrosine-base inhibitory motifs (ITIMs), which are phosphorylated upon receptor engagement. This leads to the recruitment and activation SHP-1, a phosphatase that dephosphorylates proteins in the activation signaling cascade of NK cells, thus inhibiting cell activation (Long, 1999).

Ly49 family comproses of at least 21 members. Among these, 13 are likely to be inhibitors, based on the presence of an ITIM in their cytoplasmic domain. They include Ly49 A, B, C, E, F, G, I, J, O, Q, S, T and V. The rest of the family members are activators (Kane et al., 2001)(see below). All Ly49 proteins bind to class I MHC, but each one is distinct in their allelic specificities. For example, Ly49A recognize H-2D^{b,d,k,p} and H-2^{s}, while Ly49G2 recognize H-2D^{d,r} and H-2^{k} (Hanke et al., 1999; Takei et al., 1997). The expression of Ly49 members is mouse strain specific. For example, Ly49A and Ly49B are express on C57BL/6, BALB/c, NOD, but not 129/J, 129/Svj, NZB, and CBA/J, while Ly49C is expressed in C57BL/6, BALB/c and 129/Svj but not other strains (Kane et al., 2001). It is partly due to the difference in Ly49 expression that NK cells of various mouse strains display unique target specificity and susceptibility.

CD94/NKG2

NKG2 proteins belong to another lectin-like receptor family known to act as inhibitors in NK cells. These include NKG2A/B, NKG2C, and NKG2E (NKG2A and B represent alternative splice variants). In contrast to Ly49 proteins, which bind to conventional MHC class I, these receptors bind to non-classical MHC molecules such as HLA-E in humans and Qa-1 in mice (Braud et al., 1998; Vance et al., 1999; Vance et al., 1998). Both HLA-E and Qa-1 present MHC class I-derived leader peptides, which are peptides cleaved by signal peptidases in the ER membrane during protein synthesis (Braud et al., 1998; Vance et al., 1998). All NKG2 proteins (except NKG2D) heterodimerize with CD94. CD94 contains a very short cytoplasmic domain with no identifiable motifs (Lanier et al., 1998a). In contrast, NKG2A/B posses cytoplasmic ITIMs which can recruit SHP-1 upon receptor engagement (Lanier et al., 1998a). NKG2C and NKG2E are structurally different from NKG2A/B and act as NK stimulatory receptors (see below).

1.2.2 Stimulatory receptors of NK cell activation

Similar to inhibitory receptors, stimulatory NK cells receptors bind to MHC class I molecules. They contain in their transmembrane domains charged residues that could associate with adapter proteins. These adapters include DAP10 and DAP12. The latter contains

immunoreceptor tyrosine-based activation motifs (ITAMs) in its cytoplasmic tail, which allow it to associate with Syk family kinases (Lanier et al., 1998b; Olcese et al., 1997). DAP10, on the other hand, contains in its cytoplasmic tail a YxxM motif which upon tyrosine phosphorylation can recruit the p85 subunit of PI3-kinase (PI3K) (Wu et al., 1999). Pathways downstream of DAP12 and DAP10 are not known.

NKp30, NKp44 and NKp46

A group of NK stimulatory receptors belonging to the Ig- superfamily were identified in human (Moretta et al., 2001). These include NKp46, NKp44, and NKp30. NKp46 and NKp30 are expressed in both resting and activated NK cells. They contain in their transmembrane domains positive charged arginines which interact with aspartic acid residues in the transmembrane domains of CD3ζ and FcεRIγ (Pende et al., 1999; Sivori et al., 1997). Both NKp46 and NKp30 can activate PI3K and AKT (Spaggiari et al., 2001). To date, only the NKp46 mouse homologue has been identified as MAR-1 (Biassoni et al., 1999). Unlike NKp46 and NKp30, NKp44 is not expressed on resting human NK cells but is upregulated after IL-2 stimulation. Therefore, it might participate in LAK cell mediated cytotoxicity (Vitale et al., 1998). NKp44 is also found in a subset of γδ T cells (Vitale et al., 1998) and it associates with KARAP/DAP12 (Cantoni et al., 1999). Antibodies directed against NKp46, NKp44 and NKp30 can trigger intracellular Ca^{2+} mobilization and lysis of tumor targets in an antibody redirected cytotoxic assay. Conversely cytotoxicity against tumor targets can be

blocked by including neutralize antibodies against these molecules in the assay, indicating that NKp30, NKp44 and NKp46 are key receptors for NK cell mediated tumor lysis (Moretta et al., 2001).

Ly49D and Ly49H

In contrast to the negative regulatory receptors of the Ly49 family, ligation of Ly49D and Ly49H stimulate NK cytotoxicity (Mason et al., 1996; Smith et al., 2000). These activating receptors do not contain cytoplasmic ITIMs, but instead they have transmembrane basic residue enabling them to associate with adapter DAP12, an ITAM containing adapter (Smith et al., 1998). DAP12 is indispensable for Ly49D and Ly49H expression as indicated by a complete loss of these receptors on the surface of $DAP12^{-/-}$ NK cells (Bakker et al., 2000; Tomasello et al., 2000). The ligand for Ly49D was identified as $H\text{-}2\text{-}D^{d}$ (Nakamura et al., 1999) whereas the ligand for Ly49H is not known. Ly49D not only activates cytotoxicity against allogenic targets, it is also involved in the killing of xenogenic targets such as CHO cells (Idris et al., 1999). The importance of DAP12 and Ly49D in this process is further supported by the analysis of $DAP12^{-/-}$ NK cells, which showed compromised capacity in killing CHO targets (Bakker et al., 2000; Tomasello et al., 2000). Ly49H is present in C57BL/6 mice but absent in DBA/2 mice. The absence of Ly49H on the surface of NK cells render DBA/2 susceptible to mouse cytomegalovirus (MCMV) infection. Conversely, the depletion of $Ly49H^{+}$ NK cells from C57BL/6 mice, which are MCMV-resistant, leads to a

MCMV-susceptible phenotype. These data demonstrated Ly49H is the essential in controlling MCMV infection in mice (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001).

CD94/NKG2C and NKG2E

NKG2 family members heterodimerize with CD94 and bind to the non-classical MHC class I molecules HLA-E and Qa-1. In contrast to NKG2A/B, NKG2C and NKG2E are activating receptors. They posses charged residues in their transmembrane domains that enable them to associate with adapter protein KARAP/DAP12 (Lanier et al., 1998a). Recruitment of DAP12 to the activated receptors leads to further activation of Syk family kinases (Lanier et al., 1998b; Olcese et al., 1997).

NKG2D

Another activator expressed on NK cells is NKG2D, a type II c-type lectin molecule. Unlike NKG2A, NKG2C, or NKG2E, NKG2D does not heterodimerized with CD94. Instead it is expressed as a homodimer on NK cells, αβ and γδ T cells and LPS stimulated macrophages (Bauer et al., 1999; Diefenbach et al., 2000). NKG2D has two charged residues in its transmembrane domain which associate DAP10/KAP10, which can in turn recruit PI3K (Chang et al., 1999; Wu et al., 1999). NKG2D bind to MHC class I-like molecules H60 and

Rae1 in mouse cells (Cerwenka et al., 2000; Diefenbach et al., 2000) and MICA/B and ULBPs on human cells (Bauer et al., 1999; Cosman et al., 2001). Interestingly, a HCMV (human cytomegalovirus)-encoded glycoprotein, UL16, was recently identified to be a ligand of MICB and ULBPs, thus highlighting a possible mechanism use by viruses to mask NK cell activation signals (Cosman et al., 2001).

NKR-P1

Four highly related genes - *nkr-p1a*, *nkr-p1b*, *nk-p1c* and *nk-p1d* - have been identified in rodents and they encode receptors of the c-type lectin family. NKR-P1A and NKR-P1C are stimulatory isoforms and are widely express on NK cells, whereas NKR-P1B and NKR-P1D are inhibitory isoforms and exhibit limited expression in C57BL/6 mice (Carlyle et al., 1999; Chambers et al., 1989; Giorda et al., 1990; Giorda and Trucco, 1991; Giorda et al., 1992; Ryan et al., 2001; Yokoyama et al., 1991). NKP-P1B mediates inhibition through the recruitment of SHP-1 to its cytoplasmic ITIM (Carlyle et al., 1999). The best characterized member, NKP-P1C (also known as NK1.1) associates with FcεRγI in mediating its downstream signaling (Arase et al., 1997). Rat NKP-P1C also has a CxCP motif in its cytoplasmic domain that constitutively associates with $p56^{lck}$ (Campbell and Giorda, 1997). Cross-linking of NKR-P1C activates NK cells (Arase et al., 1996; Karlhofer and Yokoyama, 1991; Ryan et al., 1991). The physiological ligands for NKR-P1 receptors remain unknown.

2B4

2B4 (CD244) is expressed in NK cells, monocytes and a subset of $CD8^+$ T cells (Garni-Wagner et al., 1993; Valiante and Trinchieri, 1993). It binds to CD48, a broadly expressed GPI-anchored molecule of unknown function (Brown et al., 1998; Kubin et al., 1999; Latchman et al., 1998; Nakajima et al., 1999). Ligation of 2B4 leads to phosphorylation of the tyrosine motif (TxYxxI/V) in the cytoplasmic tail, resulting in the recruitment of the adapter SH2D1A/SAP (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998), SHP-1 (Parolini et al., 2000) and SHP-2 (Tangye et al., 1999). It was recently reported that ligation of 2B4 could also lead to phosphorylation of the membrane adapter LAT and the association PLCγ and Grb2 with the receptor (Bottino et al., 2000). Since 2B4 binds both SH2D1A/SAP and SHP-2, it was suggested that 2B4 triggers NK cells activation by recruitment of SH2D1A/SAP, which displaces SHP-2 from 2B4 (Parolini et al., 2000; Tangye et al., 1999). Consistent with this hypothesis, mutation of SH2D1A/SAP in XLP (X-linked lymphoproliferative disease) patients leads to an impairment of this adapter to associate with 2B4. As a result, 2B4 bound SHP-2 exclusively and functions as an inhibitory rather than a stimulatory receptor (Parolini et al., 2000).

CD16

IgG Ab-coated targets trigger natural killer cells cytotoxicity. This process, known as antibody dependent cell mediated cytotoxicity (ADCC) is mediated by the low affinity receptor FcγRIIIA (or CD16), the only Fc receptor expressed in NK cells (Takai et al., 1994). Ligation of CD16 leads to Lck and Syk activation, increase in intracellular Ca^{2+} and PLCγ-1 and PLC-γ2 activation (Azzoni et al., 1992; Ting et al., 1995; Ting et al., 1992). Triggering of CD16 also leads to the phosphorlyaton of Vav-1 (Billadeau et al., 1998; Galandrini et al., 1999), activation of p21Ras (Galandrini et al., 1996), PI3K (Bonnema et al., 1994; Kanakaraj et al., 1994), NFATp and NFATc (Aramburu et al., 1995) and the activation of ERK, JNK and p38 pathways, leading to subsequent mobilization cytotoxic granules and the secretion of IFNγ and TNFα (Chini et al., 2000; Milella et al., 1997; Trotta et al., 2000). Indeed both CD16-mediated cytotoxic degranulation and IFNγ and TNFα mRNA accumulation are in part dependent on ERK and p38 activation (Chini et al., 2000; Milella et al., 1997; Trotta et al., 2000).

1.2.3 Mechanisms of NK cell mediated tumor lysis

Mechanisms of NK cytotoxicity can be classified into secretory and non-secretory pathways. The secretory pathway involved the mobilization and polarization of cytotoxic granules to the sites of cell-cell contact upon NK cell activation. These granules are secreted, releasing perforin which homopolymerizes to form pores on target cell membranes (Liu et al., 1995). This leads to osmotic leakage of the target cells, thus allowing granzymes, another components of cytotoxic granules, to enter and induce caspases-dependent and caspases-

independent cell death (Smyth and Trapani, 1995). NK cells also secrete TNFα in promoting tumor cytotoxicity, as indicated by TNFα$^{-/-}$ NK cells which show reduced tumor lysis *in vitro* and *in vivo* (Baxevanis et al., 2000). Activation of the non-secretory pathway is mainly due to FasL on NK cells engaging with Fas on target cells (Kagi et al., 1994a; Lowin et al., 1994). In addition, TRAIL was also implicated in the induction of target cell death by NK cells (Kayagaki et al., 1999). Mechanism of NK cytotoxicity might be target specific, but the most conserved mechanisms involve perforin and FasL (Kagi et al., 1994a; Kagi et al., 1994b; Lowin et al., 1994). Incidentally, these are also used by CTL to lyse target cells (Kagi et al., 1994a; Kagi et al., 1994b; Lowin et al., 1994).

1.3 Vav family of Rho GEFs

The Vav family belongs to the Dbl family of Rho guanine nucleoide exchange factors (GEFs). Currently three members have been identified in mammals. These are Vav-1, Vav-2 and Vav-3. Vav-1 is primary expressed in the hematopoietic system, whereas Vav-2 transcripts are expressed in mouse liver, spleen, thymus, lung and testis (Schuebel et al., 1996). Interestingly, Vav-2 protein is expressed in higher amount in B cells compared to T cells (Tedford et al., 2001). Vav-3 show a broad patterns of expression. Two species of Vav-3 mRNA, 3.4 kb and 5.4 kb, are present in all tissues except the ovaries and the skeletal muscles. Placenta, kidney, pancreas, and colon express highest level of the 3.4 kb

transcripts, while spleen and peripheral blood lymphocytes express the highest level of the 5.4 kb species (Movilla and Bustelo, 1999; Zeng et al., 2000).

All Vav proteins have the same order of protein domains in their primary structure (Figure 1.2). These include a calponin homology (CH) domain; PH (pleckstrin homology) domain; one Src homology (SH) 2 domain and two SH3 domains, for mediating protein-protein interactions; and a Dbl homology (DH) domain, which catalyzes guanine nucleotide exchange for Rho-family GTPases (Bustelo, 1996; Cantrell, 1998; Fischer et al., 1998b).

Vav-1 was discovered by its ability to transform fibroblasts (Katzav et al., 1989). Expression of N-terminal truncated mutant of Vav-1 and Vav-2 leads to high levels of transformation in NIH3T3 cells (Abe et al., 2000; Liu and Burridge, 2000; Schuebel et al., 1996; Schuebel et al., 1998). Whereas elimination of the CH domain is sufficient to activate transformation by Vav-1 (Coppola et al., 1991), deletion of both CH and AD domain is required to activate the oncogenic potential of Vav-2 (Schuebel et al., 1996). Contentious data exist in regard to whether Vav-3 can also transform rodent fibroblasts. Movilla and Bustelo (1999) showed that both full length (FL) and truncated Vav-3 (Δ1-444, CH-AD-DH and Δ1-184, CH-AD) fail to transform NIH 3T3 fibroblasts (Movilla and Bustelo, 1999), while Zeng et al (2000) showed

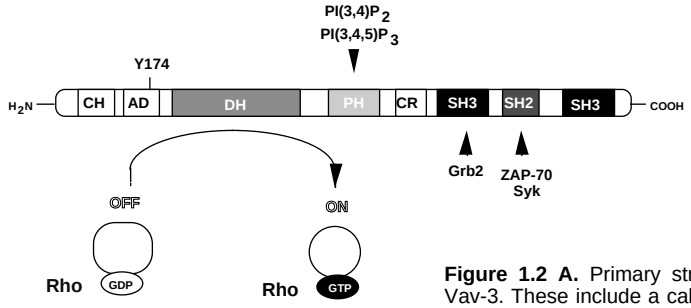


Figure 1.2 A. Primary structures of Vav-1, Vav-2 and Vav-3. These include a calponin homology (CH) domain; PH (pleckstrin homology) domain; one Src homology (SH) 2 domain and two SH3 domains, for mediating protein-protein interactions; and a Dbl homology (DH) domain, which catalyzes guanine nucleotide exchange for Rho-family GTPases. The SH3-SH2-containing adaptor domains of Vav-1 were shown to associate with many signaling intermediates such as SLP-76, Syk, ZAP-70, BLNK, CD19, Grb2. Vav-1 GEF activity could be positively modified by Lck-induced tyrosine phosphorylation of Y174 and by the binding of PI(3,4)P2, and PI(3,4,5)P3 to its PH domain.

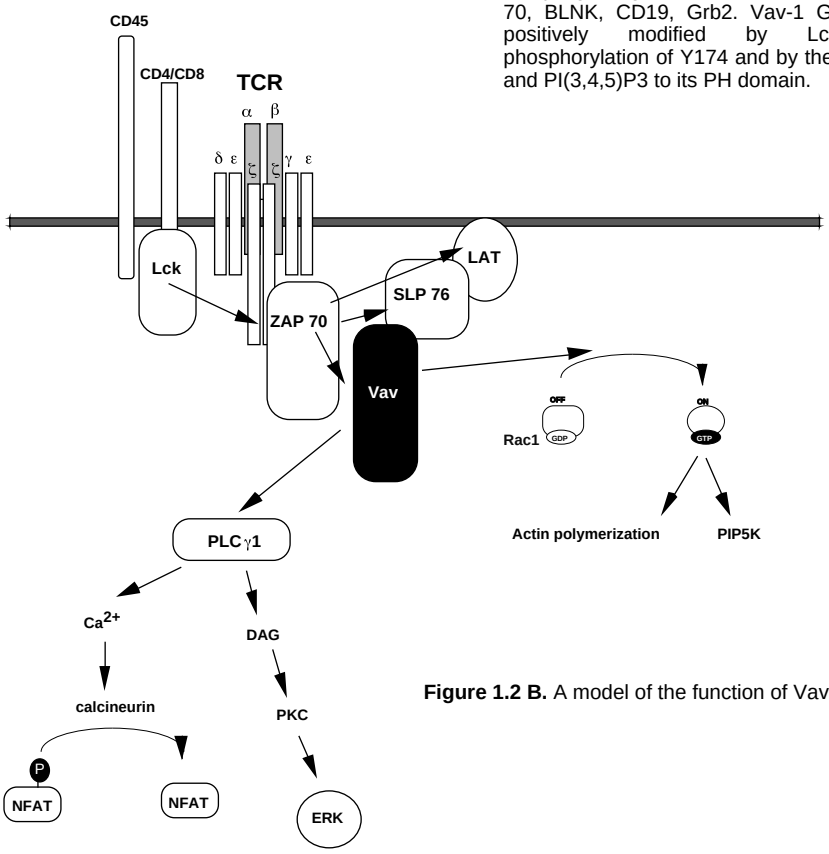


Figure 1.2 B. A model of the function of Vav-1 in T cells.

that over-expression of Vav-3 (Δ1-114, CH) can lead to foci formation in these cells (Zeng et al., 2000). In summary, these data led to the speculation that the CH and the AD domains are involved in negative regulation of Vav activity (see below).

Vav-1 GEF activity can be activated by Lck-mediated tyrosine phosphorylation of Y174 and modulated by the binding of $PI(3,4)P_2$, and $PI(3,4,5)P_3$ to its PH domain (Crespo et al., 1997; Han et al., 1997; Han et al., 1998). Tyrosine phosphorylation of Vav-1 relieves autoinhibition by exposing its DH domain, leading to an enhanced GEF activity towards Rho GTPases (Aghazadeh et al., 2000). A similar mechanism is expected but not shown in the regulation of Vav-2 and Vav-3.

Vav-1 preferentially activates Rac1 and RhoA, although catalytic activities towards Cdc42 has also been noted (Crespo et al., 1997; Han et al., 1997). Upon phosphorylation or N-terminal truncation, Vav-2 expressed in insect cells can catalyze GDP-GTP exchange of RhoA, RhoB and RhoG but not Rac1 and Cdc42 (Schuebel et al., 1998). However, bacterial expressed Vav-2 DH fragment exhibited strong GEF activity for Rac1, Cdc42 and RhoA (Abe et al., 2000). It is not known which GTPase is specially activated by Vav-3. Baculovirus-expressed N-terminal truncated Vav-3 (Δ1-144) can promote GDP-GTP exchange of Rac1, RhoA, RhoG but not Cdc42 (Movilla and Bustelo, 1999). Lck phosphorylated full length (FL)-Vav-3 can also catalyze Rac1 and RhoA but not Cdc42 (Movilla and Bustelo, 1999). In sharp contrast, Zeng et al. (2000) showed that over-expression of FL-Vav-3 in NHI3T3 can lead to the activation of Rac1 and Cdc42 but not RhoA (Zeng et al., 2000). It is possible that both

methodology and cell background might account for activation of different GTPase by Vav family proteins. Interestingly these studies implicate Rac1 is a downstream target of Vav-3. This notion is further supported by the observation that IgM-mediated Rac1 activation is compromised in Vav-3$^{-/-}$ DT40 chicken B cells (Inabe et al., 2002). Taken together, Vav-1, Vav-2 and Vav-3 can catalyse Rac1 activation.

Vav family proteins function not only as Rho GEF, but they also contain adapter SH3-SH2-SH3 domains that could recruit other signaling intermediates. Vav-1 adapter domains were reported to associate with SLP-76 (Onodera et al., 1996; Wu et al., 1996), Syk (Deckert et al., 1996), ZAP-70 (Katzav et al., 1994) and Grb2 (Ramos-Morales et al., 1995; Ye and Baltimore, 1994). Vav-2 and Vav-3, on the other hand, are less well characterized. It was shown that both Vav-2 and Vav-3 undergo tyrosine phoshorylation upon ligation of EGFR and PDGFR and can directly bind to these receptors (Moores et al., 2000; Pandey et al., 2000).

1.3.1 Vav-1

Vav-1 is a crucial regulator of T cell activation. Over expression of Vav-1 in Jurkat cells activates NF-AT transcriptional activity. Interestingly, this process seems to be independent of Vav-1 GEF catalytic activity, since a DH-dead version of Vav-1 displayed a similar effect, suggesting that other domains are involved in Vav-1 mediated NFAT activation (Kuhne et al.,

2000). Over-expressing Vav-1 also leads to the CD69 upregulation, JNK activation, membrane translocation of PKCθ and actin polymerization (Villalba et al., 2000). Furthermore, Vav-1 and PKCθ synergistically activate IKKβ and NFκB transcriptional activities (Dienz et al., 2000). These observations show that Vav-1 is an important regulator of T cell activation. This notion is further supported by genetic evidence from the analysis of knock-out mice. Gene ablation of *vav-1* results in severe impairment of thymic maturation. In these mice, thymocytes are impaired in the transition from the $CD4^-CD8^-$ (double negative, DN) stage to $CD4^+CD8^+$ (double positive, DP) stage, and from the DP to $CD4^+$ or $CD8^+$ (single positive, SP) stage. This results in a reduction of SP cells and mature T cells in the periphery (Fischer et al., 1998a; Fischer et al., 1995; Turner et al., 1997; Zhang et al., 1995). The defect in the transition from DP to SP could be explained by an aberrant thymic positive selection (Kong et al., 1998; Turner et al., 1997) and negative selection in Vav-$1^{-/-}$ mice (Kong et al., 1998). Mutant thymocytes are also more resistant to superantigen mediated deletion (Turner et al., 1997). Vav-$1^{-/-}$ T cells from the periphery show a compromised response to CD3-induced proliferation and IL-2 secretion (Costello et al., 1999; Fischer et al., 1998a; Tarakhovsky et al., 1995; Zhang et al., 1995). They are also impaired in primary responses to antigen *in vivo* (Penninger et al., 1999). On a molecular level, the defects in the differentiation and activation of mutant T cells can be in part explained by a failure of Vav-$1^{-/-}$ T cells to elicit TCR clustering following ligation (Fischer et al., 1998a) (Holsinger et al., 1998). Furthermore, there is marked reduction of CD3-induced production of IP_3, intracellular calcium release, ERK and NFκB activation in Vav-1-deficient thymocytes and peripheral T cells (Costello et al., 1999; Fischer et al., 1998a; Fischer et al., 1995; Holsinger et al., 1998;

Kong et al., 1998; Turner et al., 1997). Recently it was reported that introducing *cbl-c* null mutation in Vav-1$^{-/-}$ background can rescue the activation of CD3-mediated Cdc42 activation, TCR clustering, T cell proliferation and T cell cytotoxicity. It does not however restore CD3-mediated Ca^{2+} mobilization and IL-2 production (Krawczyk et al., 2000). These observations led to the hypothesis that two signaling pathways exist downstream of Vav-1: one mediates intracellular Ca^{2+} mobilization leading to NFAT activation and the other regulates cytoskeletal re-organization via Cdc42/WASP. Both pathways are necessary in T cell activation.

The Vav-1 null mutation does not only affect T cells, but also other cells of the hematopoietic system. Vav-1$^{-/-}$ mice have normal numbers of conventional B cells, but a diminished amount of $CD5^+$ B1 cells in the peritoneum (Fischer et al., 1998a; Turner et al., 1997). Mutant B cells are impaired in IgM-induced proliferation and they show a reduction in CD19-mediated Ca^{2+} flux, $PI(4,5)P_2$ production and Ig- and CD19-mediated JNK activation (O'Rourke et al., 1998). Furthermore, bone marrow derived mast cells (BMMC) from Vav-1$^{-/-}$ mice exhibits reduced antigen mediated degranulation and IL-2 and IFNγ production. FcεR-mediated PLCγ1 and PLCγ2 phosphorylation, IP_3 and PIP_3 production, Ca^{2+} mobilization and AKT activation are also markedly compromised in mutant BMMC (Manetz et al., 2001). Taken together, Vav-1 is essential for development of T and B1 cells and the activation of T cells, B cells and mast cells. On a molecular level, Vav-1 traffics signals derived from antigen receptors. Common to these receptors is the presence of ITAMs that can recruit Src- and Syk family kinases. This suggest that other ITAMs containing receptors may also lie up-stream of Vav-1 in other cell types of the hematopoietic system.

1.3.2 Vav-2

In Jurkat cells, CD3 ligation leads to tyrosine phosphorylation of both Vav-1 and Vav-2 (Doody et al., 2000). However, Vav-2 differs from Vav-1 in that it does not potentiate TCR-mediated NFAT and NFκB-dependent transcriptional activity (Doody et al., 2000; Moores et al., 2000). In fact, over-expression of Vav-2 dramatically suppressed TCR-mediated NFAT activation (Tartare-Deckert et al., 2001). Vav-1 but not Vav-2 enhances TCR-induced Ca^{2+} mobilization (Doody et al., 2000). In B cells, BCR and CD19 engagement lead to tyrosine phosphorylation of Vav-2 (Doody et al., 2000). Furthermore, both Vav-1 and Vav-2 can synergize with IgM in potentiating NFAT transcriptional activity (Doody et al., 2000). Over-expression of either Vav-1 or Vav-2 in Bal-17 B cells also enhances IgM-mediated Ca^{2+} mobilization (Doody et al., 2000).

Vav-2 knockout mice have normal development of T and B cells, however, they display a mild impairment of TD and TI antigen responses and BCR-induced calcium flux (Doody et al., 2001; Tedford et al., 2001). Vav-1/Vav-2 double knock out mice show similar T cell developmental defects as observed in Vav-$1^{-/-}$ mice, however, the numbers of peripheral T cells is more severely reduced in Vav-$1^{-/-}$Vav-$2^{-/-}$ mice compared to Vav-$1^{-/-}$ mice. This suggests that Vav-2 has an unrecognized function in T cells. In contrast to Vav-1 and Vav-2 single knockout mice, Vav-$1^{-/-}$Vav-$2^{-/-}$ mice have a severe impairment of splenic B cell

development in the transition from $IgM^{hi}IgD^{lo}$ to $IgM^{lo}IgD^{hi}$ stages. In addition, mature double knockout mutant B cells are refractory to IgM-induced proliferation and they show a complete abolishment of BCR-induced intracellular calcium mobilization (Doody et al., 2001; Tedford et al., 2001). These data suggest that in T cells, TCR mediated Ca^{2+} flux is Vav-1 dependent, whereas in splenic B cells, both Vav-1 and Vav-2 are necessary components of the signalsome which regulates intracellular calcium release. A functional compensation of Vav-1 and Vav-2 during B cell development in the spleen alludes the possibility that Vav proteins may also complement each other in other hematopoietic cell types such as NK cells.

1.3.3 Vav-3

In Jurkat T cells, ligation of CD3 can leads to tyrosine phosphorylaton of exogenous expressed Vav-3. In constrast to Vav-1, Vav-3 fails to potentiate TCR-mediated NFAT-dependent transcription, but it is able to enhance TCR-mediated NFκB-dependent transcriptional activity (Moores et al., 2000). In chicken DT40 B cells, gene disruption of Vav-3 leads to a reduction of IP_3 and Ca^{2+} flux in respond to BCR stimulation. Rac1 activation, PI3K and AKT activities are also attenuated in the $Vav\text{-}3^{-/-}$ B cells following BCR ligation (Inabe et al., 2002). The roles of Vav-3 in murine T and B- development and activation remain to be identified.

1.4 Vav-1, Vav-2 and NK cells

Vav family members are known to facilitate many NK cell functions. Vav-1 was implicated in potentiating human NK cell cytotoxicity (Billadeau et al., 1998; Billadeau et al., 2000; Galandrini et al., 1999) and β1 integrin-mediated IL-8 secretion via the activation of the Rac-PAK-MKK3-p38 pathway (Mainiero et al., 2000). In human NK cells, ectopic expression of Vav-1 potentiates ADCC (Billadeau et al., 1998), while inhibition of Vav-1 expression with anti-sense oligonucleotides markedly reduced FcγR mediated cytotoxic activity (Galandrini et al., 1999). Furthermore, ligation of CD16 (FcRγIII) leads to rapid tyrosine phosphorylation of Vav-1 (Billadeau et al., 1998; Galandrini et al., 1999; Xu and Chong, 1996), implicating Vav-1 as a downstream target. Enhancement of NK tumorcidal activities mediated by over-expression of Vav-1 requires a functional DH and PH domain, but not the CH domain (Billadeau et al., 2000). Interestingly mutant carrying Y->F substitution of Y142, Y160, and Y174 in the AD domain can lead to greater increase in ADCC compare to wild type Vav-1, implicating these as negative regulatory residues. Taken together, Vav-1 is implicated in CD16 signals in human NK cells.

NK cells exhibit spontaneous cytotoxicity toward selective tumors targets. Upon engagement of target cells with NK cells, Vav-1 is rapidly phosphorylated (Billadeau et al., 1998; Galandrini et al., 1999). Human NK cell cytotoxic activity against K562 cells is also potentiated by the over-expression of Vav-1 (Billadeau et al., 1998), while reduction of Vav-1 expression by anti-sense oligonucleotides markedly reduces NK cytotoxic activity (Galandrini

et al., 1999). The DH domain is essential for mediating these responses, while the PH and the CH domains are not required. Similar to the increase in ADCC, mutating Y142, Y160, and Y174 to phenylalanine also leads to an enhancement of cytoxicity, highlighting again the negative regulatory roles of these three tyrosine residues (Billadeau et al., 2000).

Vav-2 also has been described to participate in both NK natural cytotoxicity and ADCC since FcR ligation leads to Vav-2 phosphorylation and ectopic expression of Vav-2 increases NK natural cytotoxicity and ADCC. This process requires functional DH and SH2 domains of Vav-2. The PH domain is also required for ADCC and natural cytotoxicity against selective tumor targets (Billadeau et al., 2000). Together, these observations suggest both Vav-1 and Vav-2 are involved in NK cell activation.

Several lines of evidence suggested that upstream modulators and downstream targets of Vav-1 in T cells are also crucial signaling components in mediating NK cell activation. These include LAT, PI3K and ERK. Vav-1 can be recruited to the adapter LAT following TCR stimulation via the interactions of phosphorylated LAT with the SLP-76 SH2 domain and phoshorylated SLP-76 binding to Vav-1 SH2 domain (Zhang et al., 1998). Coincidently, over-expression of LAT in NK cells augments spontaneous cytotoxicity (Jevremovic et al., 1999). Similar to LAT, PI3K is also a modulator of Vav-1 as indicated by the ability of its substrates $PI(3,4)P_2$, and $PI(3,4,5)P_3$ to bind directly to Vav-1 PH domain and thereby enhance its GEF activity (Han et al., 1998). PI3K also has a pivotal role in the regulation of NK cell activation as the presence of PI3K inhibitors Ly294002 or wortmannin compromises NK cell-mediated

tumor lysis (Jiang et al., 2000). ERK, another key component of T cells activation, is a downstream target of Vav-1 in T cells derived from some mice strains (C57BL/6 and 129 mice but not CD1 mice) (my unpublished observation) (Costello et al., 1999; Fischer et al., 1998a). Interesting ERK is also required for perforin and serine proteases release in NK cells (Trotta et al., 1998; Wei et al., 1998). Together these observations suggest that Vav-1 dependent signaling pathways are involved in activation of both T cells and NK cells.

1.5 Lsc/p115 Rho GEF

Similar to Vav-1, Lsc/p115 Rho GEF is expressed primarily in cells of the hematopioetic system (Girkontaite et al., 2001). Lsc was identified as a GEF specific for RhoA with minimum catalytic activities towards Cdc42, Rac1 and Ras (Hart et al., 1996). Similar to other GEFs, Lsc contains a tandem DH-PH domain for catalyzing activation of RhoA GTPases (Hart et al., 1998). It also has a RGS domain that stimulates the intrinsic GTPase activity of Gα12 and Gα13 (Kozasa et al., 1998) (Figure 1.3). Co-immunoprecipitation experiments demonstrated a direct interaction between Gα13 and Lsc via its RGS and DH domains (Hart et al., 1998; Wells et al., 2002). The binding of activated Gα13 to Lsc also stimulate its Rho GEF activities (Hart et al., 1998). In NIH3T3 cells, Lsc and Gα13 can synergistically activate serum response factor (SRF) (Mao et al., 1998b), and in astrocytomas, over-expression of Lsc is involved in cell rounding induced by thrombin stimulation and the overexpression of Gα12 (Majumdar et al., 1999). Based on these

observations, it was postulated that Gα12 and Gα13 activates Lsc, which then catalyzes RhoA activation, leading to SRF-dependent transcriptional activation. As Lsc is specifically expressed in the hematopioetic system, it remains uncertain whether conclusions derived from ectopic expression studies in non-lymphoid cells can be extended to lymphocytes.

Of interest is that Lsc assumes both positive and negative regulatory functions mediated by its GEF domain and RGS domain respectively. How these two branches of signal integrate remains an open question. One possibility is that Lsc down-regulates its own activation, thus generating only short live signals. Experiments with Lsc mutants carrying mutations in the GEF or the RGS domains may resolve these issues.

Lsc is shown to act downstream of Gα12 and Gα13 coupled receptors (Kozasa et al., 1998) (Girkontaite et al., 2001). One such receptor is the lymphoid-expressed G-protein coupled receptor (GPCR) G2A, a receptor that mediates actin rearrangement in a Gα13-dependent manner in murine embryonic fibroblasts (Kabarowski et al., 2000). Another set of receptors

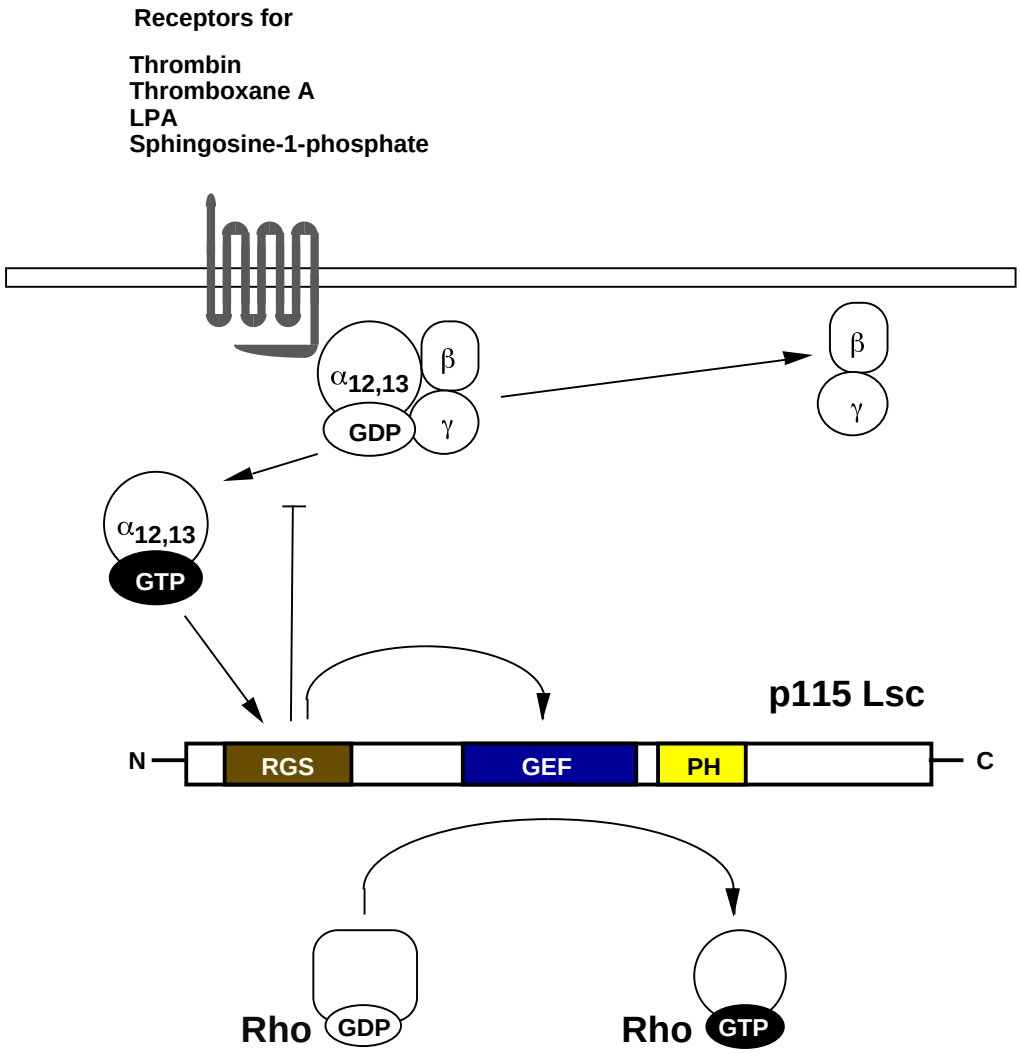


Figure 1.3. Primary structures of p115 Lsc. These include a regulator of G protein signaling (RGS) domain which stimulate the intrinsic GTPase activity of Gα12 and Gα13 and a PH (pleckstrin homology) domain and a Dbl homology (DH) domain, which catalyzes guanine nucleotide exchange for RhoA. Ligands of several Gα12/13 couple receptors are indicated.

that signal through Gα12/13 include lysophospholipid receptors (Fukushima et al., 2001), and receptors for sphingosine-1-phosphate (Pyne and Pyne, 2000), thrombin (Majumdar et al., 1999), and thromboxane A_2 (TXA_2) (Djellas et al., 1999). The function of these molecules in lymphocytes remain largely obscure, with the exception of G2A, which regulates lymphocyte homeostasis (Le et al., 2001).

Although Gα12/13 are known to mediate crucial functions of platelets (Klages et al., 1999), their roles in hematopoietic cells is unclear. Some evidence suggest that Tec family kinases such as Tec, Bmx and BTK act downstream of Gα12/13 (Jiang et al., 1998; Mao et al., 1998a). Several Tec family kinases play vital roles in immune system. BTK is a crucial regulator of B cell development and activation, whereas Itk/Emt and Rlk/Txk are involved in activation and maturation of T cells (Schaeffer and Schwartzberg, 2000; Yang et al., 2000). The fact that Gα12/13 act upstream of Tec family kinases, and that these kinases regulate immune responses suggest that other downstream effectors of Gα12/13, such as Lsc, may also modulate immune functions.

First evidence for a function of Lsc in lymphocytes came from analysis of $Lsc^{-/-}$ mice (Girkontaite et al., 2001). These mutant mice have a reduced number of marginal zone B (MZB) cells in the spleen and reduced numbers of recirculating ($B220^{hi}IgM^{lo}$) B cells in the bone marrow. They also exhibit a defective humoral responses to TI and TD antigens. Furthermore, $Lsc^{-/-}$ T and B cells show a decrease in LPA- and TXA_2-induced actin

polymerization, thus providing direct genetic evidence that Lsc acts downstream of Gα12/13 coupled receptors in remodeling of the cell cytoskeleton. Interestingly, Lsc mutant T and B cells also show a reduced basal migration in a transwell migration assay. Together these data indicate that Lsc is a positive regulator of homing or maturation of MZB cells and recirculating B cells, actin polymerization mediated by Gα12/13 coupled receptors, and basal motility of T and B cells. These positive regulatory roles are likely mediated by the GEF domain of Lsc which catalyses the activaton of Rho GTPases. Lsc also has a negative regulatory role in the immune system. This is highlighted by the observation that $Lsc^{-/-}$ lymph node T cells display an increase basal proliferation (Girkontaite et al., 2001). In addition, $Lsc^{-/-}$ MZB cells exhibit enhanced migration after serum activation. On a molecular level, the negative regulatory role of Lsc is probably executed by the RGS domain which may be involved in GPCR desensitization. Further experiments are required to define the signaling pathways downstream of Lsc in lymphocytes.

1.6 Aimes of the project

Rho GTPase are important regulators of a variety of cellular functions and they are implicated in NK cell mediated cytotoxicity. However, until now, there is no genetic evidence for the role of Rho GTPases and GEFs in the development and the activation of NKT and NK cells. The aims of this project were to characterize the functions of the GTPase activators Vav-1, Vav-2 and Lsc/p115 Rho GEF in NKT and NK cell development and activation. I took

advantage of Vav- and Lsc-deficient mice generated in the laboratory to address whether Vav-1 and Vav-2 are involved in the maturation of NK and NKT cells and in the induction of spontaneous cytotoxicity and ADCC of murine NK cells. In addition, since Vav-1 and Vav-2 can compensate each other in B cell development, I wanted to examine whether this compensation is extended to the ontology of NK and NKT cells and the activation of NK cells. Finally, since Lsc is expressed in LAK cells, this study investigated whether Lsc is also expressed in freshly purified NK cells and whether it has a role in the development of NK and NKT cells and NK cell cytotoxicity.

2. Materials and Methods

2.1 Mice

The generation of Vav-1, Vav-2, and Vav-1/Vav-2 deficient mice is described elsewhere (Fischer et al., 1998a; Tedford et al., 2001). All Vav mutant mice were crossed onto a C57BL/6 mouse background for 8-9 generations. Mice were maintained under pathogen-free conditions in accordance with institutional guidelines and were used at 8-12 weeks of age. $Lsc^{-/-}$ mice (Girkontaite et al., 2001) were on an 129 background and used for experiments at the age of 9-12 weeks.

2.2 Cell lines

See Table 2.1 for a list of cell lines. All cell lines were grown in RPMI 1640 and 10% FCS.

2.3 Antibodies

See Table 2.2 for a list of antibodies. To generate anti-CD4 and anti-CD8 antibodies, hybridomas were grown in RPMI 1640, 10% FCS, Glutamine, penicillin and streptomycin. Cell free supernatants were harvested when the medium turns slightly yellow. Optimal concentrations of antibodies used in experiments were determined by incubating with 100 µl, 300 µl or 500 µl of antibody supernatant for 30 minutes on ice. The cells were washed and

subjected to complement lysis for 50 minutes at 37°C. The efficiency of purification was determined by FACS analysis of $CD4^+$ and $CD8^+$ cells. A normal spleen contains around 30-40% $CD3^+$ cells. After complement lysis, this is reduced to 2-5%.

2.4 Tissue harvesting and cell culture

2.4.1 Splenectomy

The mice were sacrificed by cervical dislocation. Mice were placed on their right side and an incision was made on the left side skin with a pair of scissors. The incision was made approximately 2.5 cm long, from between the last rib and the hip joint. Another incision (1-2 cm) was made in the peritoneal wall and the spleen, a red enlongated bean-shape organ, was pulled onto the exterior wall. The spleen was removed by cutting off the mesentery and connective tissues attaching the spleen to the abdominal cavity.

2.4.2 Splenic NK cells enrichment

Single cell suspensions were generated by incubating 3-5 ml of 0.16 M ammonium chloride for 2-5 minutes at room temperature to lyse erythrocytes. The incubation was terminated with 5 ml of cold RPMI 1640 and cells were spun down. Cell pellets were resuspended in 2 ml of

Table 2.1 Cell lines

Cell lines	MHC Class I	Source	Comments
C4.4.25	H-2b	C57Bl/6 T lymphoma	C4.4.25 is derived from EL4 by selecting MHC class I^- variant of EL4 (Glas et al., 1992).
EL4	H-2b	C57Bl/6 T lymphoma	EL4 was established from a lymphoma induced in a C57Bl/6 mouse by 9,10-dimethyl-1,2-benzanthracene (Gorer, 1950). They are $CD4^-$ $CD8^-$.
RMA	H-2b	C56Bl/6 T lymphoma	RMA cells were derived from RBL-5, a Raucscher virus-induced T lymphoma (Ljunggren and Karre, 1985). RMA cells were used for ADCC because they are resistant to NK- and LAK-mediated spontaneous cytotoxicity.
RMA/S	H-2b	C56Bl/6 T lymphoma	RMA/S is derived from RMA by selecting MHC-class I^- variant of RMA (Ljunggren and Karre, 1985). It is a putative target for NK cytotoxictiy *in vitro* and *in vivo*.
YAC-1	H-2a	A/Sn lymphoma	YAC-1 is a lymphoma which was induced by inoculation of the Moloney leukemia virus (MLV) into a newborn A/Sn mouse (Kiessling et al., 1975). The cells are sensitive to NK- and LAK-mediated spontaneous cytotoxicity.

Table 2.2 Antibodies

Antibodies against	Source	Clone	Dilutions	Assay
anti-mouse HRP	Amersham	cat. nr. NA 9340	1:10000	Western blot
anti-mouse HRP	Amersham	cat. nr. NA 931	1:10000	Werstern blot
CD3	Pharmingen	145-2C11	1:200	FACS
CD3	Purified antibodies	145-2C11	2 µg	Injection
CD4	T. Winkler (Germany)	172	300 µl/spleen	complement lysis
CD8	T. Winkler (Germany)	31M	300 µl/spleen	complement lysis
CD16/CD32 (FcR)	Pharmingen	2.4G2	1:200	FACS
CD16/CD32 (FcR)	Purified antibodies	2.4G2	1:50	Blocking FcR (FACS)
CD44	Pharmingen	IM7	1:200	FACS
CD45	Pharmingen	16A	1:200	FACS
CD69	Pharmingen	H1.2F3	1:200	FACS
DX5	Pharmingen	DX5	1:200	FACS
ERK	Santa Cruz	K-23	1 µg/ml	Western blot
pERK	Santa Cruz	E4	1 µg/ml	Western blot
FasL	Pharmingen	MFL3	1:200	FACS
HSA	Pharmingen	M1/69	1.200	FACS
IFNγ	Pharmingen	R4-6A2	1 µg/ml	ELISA
IFNγ (biotinylated)	Pharmingen	XMG1.2	0.666 mg/ml	ELISA
IL-2Rβ	M.Miyasak (Japan)	rabbit polyclonal	1:200	FACS
IL-4	Pharmingen	11B11	1 µg/ml	ELISA
IL-4 (biotinylated)	Pharmingen	BVD4-1D11	0.5 µg/ml	ELISA
JNK	Pharmingen	G151-666	0.1 µg/ml	Western blot
pJNK	Pharmingen	cat. nr.9251	1:1000	Western blot
Lsc	(Girkontaite et al., 2001)	Lsc24	0.1 µg/ml	Western blot
Ly49D	Pharmingen	4E5	1:200	FACS
Ly49G2	Pharmingen	LGL-1	1:200	FACS
NK1.1	Pharmingen	PK136	1:200	FACS
p38	Santa Cruz	C-20	0.1 µg/ml	Western blot
pp38	NEB	cat nr. 9211	1:1000	Western blot
streptavidin alkaline phosphate	Amersham	-	1:2000	ELISA
streptavidin-APC	Pharmingen	-	1:200	FACS
streptavidin-Cy-chrome	Pharmingen	-	1:200	FACS
streptavidin-PerCP	Pharmingen	-	1:200	FACS
TNFα	Pharmingen	G281-2626	2 µg/ml	ELISA
TNFα (biotinylated)	Pharmingen	MP6-XT3	1 µg/ml	ELISA
Vav-1	UBI	cat. nr. 05219	0.1 µg/ml	Western blot
Vav-2	M. Barbacid (Spain)	BR82α	1:5000	Western blot

pre-warmed RPMI 1640 with 10% FCS. This suspension was pipetted into nylon wool (NEN) columns, which has been pre-saturated with RPMI 1640 and 10% FCS for 1 hour at 37°C. After the cell suspension was incubated in the column for 1 hour at 37°C, pre-warmed medium is added into the column to displace unattached cells. Cells were eluted with 15 ml of medium, spun down and resuspended in rat anti-mouse CD4 (clone 172) and rat anti-mouse CD8 (clone 31M) antibodies (0.3 ml/spleen). The cells were stained for 30 minutes at 4°C before washed and incubated with baby rabbit complement (Cederlane, 0.5 ml/spleen) for an additional 50 minutes at 37°C. The remaining cells were either used directly used in cytotoxic assay or they were cultured to generate LAK cells. LAK cells were generated by incubating enriched splenocytes for 6 days in 6-well plates with 2 ml of complete medium (RPMI 1640, 10% FCS, Glutamine, penicillin, streptomycin, 5 x 10^{-5} M 2-mercapto-ethanol) and 10% supernatant from X63-Ag8-653 plasmacytoma cells expressing recombinant mIL-2 cDNA (Karasuyama and Melchers, 1988). New IL-2 (10% of total volume) is added every two to three days. At day 6, LAK cells were harvested and the purity of $CD3^{-}NK1.1^{+}$ cells was determined by FACS analysis. The optimal concentration of IL-2 supernatant was determined by incubating splenocytes with 5%, 10% or 20% of IL-2 supernatant for 6 days. Titration that yield the highest purity of $CD3^{-}NK1.1^{+}$ cell (96-98%) with minimum doses of IL-2 were used.

2.4.3 Thymectomy and thymocyte preparation

The mice were sacrificed by cervical dislocation and were placed on their back. A small incision was made in the skin just over the second and the third rib with a pair of scissors. The incision was elongated toward the clavicles. Once the chest cavity was exposed, an another incision was made along both sides of the sternum. The sternum was cut away, exposing the heart and the lungs. The white lobed tissue located under the ribs, on top of the hearts and toward the head is the thymus. This was pulled away with a pair of forceps. The thymi were meshed with a 5 ml syringe plunger. The tissues were washed with 10 ml of RPMI 1640.

2.4.4 Harvesting liver lymphocytes

Solutions:

I. **100% Percoll:** Dilute 90% stock Percoll solution (Pharmacia) with 10% 10x PBS.

II. **40% Percoll:** Dilute 100% Percoll to 40% with RPMI 1640 + 5% FCS.

III. **80% Percoll:** Dilute 100% Percoll to 80% with RPMI 1640 + 5% FCS.

The abdominal cavity was opened and the liver was flushed of blood with 3-5 ml of RPMI 1640 and 5% FCS via the portal vein. This is done by inserting a syringe into the portal vein and injecting 3-5 ml of medium. The liver turns white once depleted of blood. The liver is mashed with a 5 ml syringe plunger through 70 µm nylon mesh Netwells (Co-Star). Single cell suspension was made and cells were pelleted and resuspended in 14 ml of 40% Percoll

solution. Using a 10 ml pipette, the 40% Percoll cell suspension was carefully laid over a 6 ml of 80% isotonic Percoll solution. The suspensions were centrifuged at room temperature for 20 min at 1000 x g with no brakes at termination. The resulting gradient consists of three layers: the liver fat on the top layer, followed by the 40% Percoll and the 80% Percoll containing hepatocytes and erythrocytes at the bottom. Hepatic lymphocytes reside at the 40-80% interface. The fat layer and parts of the 40% Percoll layer were aspirated away. The lymphocytes were carefully removed with an 1 ml pipette and washed 2-3 times with RPMI 1640 and 5% FCS before they were stained and analysed by FACS.

2.4.5 Harvesting lymphocytes from the bone marrow

Femurs were removed by cutting away the skin and neighboring muscle groups. Both ends of the femurs were cut and the bone marrows were flushed out into an 1.5 ml eppendorf tube by inserting an 1 ml syringe into one end of the bone. When all the marrow is flushed out, the bone will turn white. The bone marrow cells were spun at maximum speed for 30 seconds with a desk top centrifuge. Erythrocytes were lysed in 0.5 ml of 0.16 M ammonium chloride solution for 2 minutes and terminated with 1 ml of cold RPMI 1640. The cells were washed 2 times with RPMI1640 before they are stained for FACS analysis.

2.5 Flow cytometry

Solution:

I. FACS buffer: PBS, 2% FCS and 0.1% sodium azide.

Single cell suspensions (5 x 10^5 in 100-200 µl of FACS buffer) were obtained from the indicated tissues and preincubated for 30 min. at 4°C with anti-CD16/CD32 (clone 2.4G2) in FACS buffer to block FCγRII/III receptors. Cells were washed and stained with the following antibodies: FITC- or biotin-labeled anti-CD3 mAb (clone 2C11), PE-labeled mAb anti-NK1.1 (clone PK136), biotin-labeled anti-HSA (clone M1/69), PE-labeled DX5, FITC-labeled anti-CD45 (clone 16A), FITC-labeled anti-CD44 (clone IM7), anti-β1 integrin (clone Ha2/5), biotin-labeled anti-IL-2Rβ (gift of T. Tanaka and M. Miyasaka, Japan), biotin-labeled anti-CD69 (clone H1.2F3), FITC-labeled anti-CD16/CD32 (clone 2.4G2), FITC-labeled anti-Ly49D (clone 4E5), FITC-labeled anti-Ly49G2 (clone LGL-1) and biotinylated anti-FasL (clone MFL3). For samples stained with biotin-labeled antibodies, the primary antibodies were washed away with 500 µl of FACS buffer. Cells were spun down for 5 minutes at 1200 rpm before resuspended in 200 µl of FACS buffer. Biotinylated antibodies were visualized with streptavidin-Cy-chrome, streptavidin-APC or streptavidin-PerCP. Labeled cells were washed with 500 µl of FACS buffer and spun down for 5 minutes at 1200 rpm. The cells were resuspended in 500 µl of FACS buffer before analysed with FACScalibur (Becton Dickinson) and quantified with CellQuest software. Unless stated otherwise, all antibodies used for FACS analyses were purchased from Pharmingen.

2.6 *In vivo* injection of anti-CD3

Mice were intravenously injected with 2 µg of anti-CD3 antibodies in 500 µl of PBS. Ninety minutes after anti-CD3 treatment, splenocytes were harvested and resuspened in complete medium (RPMI 1640, 10% FCS, Glutamine, penicillin, streptomycin, 5 x 10^{-5} M 2-mercapto-ethanol). They were plated in triplicates (62.5 – 1000 x 10^4 cells per well) in 96 well plate for 1.5 hr. at 37°C. Supernatants (50 µl) were harvested and subjected to ELISA.

2.7 ELISA

Solutions:

I. **Coating buffer for IL4 and IFNγ:** 0.1 M Na_2HPO_4, adjust to pH 9.0 with 0.1 M NaH_2PO_4.

II. **Coating buffer for TNFα:** 0.1 M Na_2HPO_4, adjust to pH 4.35 with 0.1 M NaH_2PO_4.

II. **Blocking buffer:** PBS with 10% FCS and 0.1% sodium azide.

IV. **Washing buffer:** PBS with 0.05% Tween-20.

V. **Substrate solution:** 0.2 g/ml 4-nitrophenyl phosphate disodium salt (Serva) in AP-substrate buffer.

VI. **AP-substrate buffer:** 900 ml H_2O, 97 ml diethanoamine (Sigma), 0.1 ml 10% NaN_3, 0.1g $MgCl_2.6H_2O$, pH 9.8 (HCl).

Capturing antibodies for IL-4 (clone 11B11, Pharmingen, final concentration: 1 µg/ml), IFNγ (clone R4-6A2, Pharmingen, final concentration: 1 µg/ml) and TNFα (clone G281-2626, Pharmingen, final concentration: 2 µg/ml) were diluted in coating buffer. This solution (50 µl) were placed in ELISA plates (Nunc-Immuno^{TM} MaxiSorp^{TM}) overnight in the dark at 4°C. The coating solution was removed and the plates were incubated with 150 µl per well with blocking buffer for 1 hour at room temperature. The plates were washed three times with 0.2 ml/well of washing buffer before 50 µl of undiluted culture supernatants were applied to the plates and incubated at 4°C overnight in the dark. The supernatants were decanted and the plates were washed three times in washing buffer. Detecting antibodies (50 µl) diluted in blocking buffer were added (anti-IL-4, clone BVD4-1D11, final concentration 0.5 µg/ml; anti-IFNγ, clone XMG1.2, final concentration 0.666 µg/ml; and anti-TNFα final concentration 1 µg/ml). The detecting antibodies were incubated for 1 hour at room temperature and the plates were washed three times in washing buffer. 50 µl of streptavidin alkaline phosphatase (Amersham, dilution 1:2000) were added and incubated for an additional one hour. The plates were washed ten times in washing buffer and 100 µl of substrate solution was added. The reactions were developed in the dark at room temperature until the color turned yellow. The absorbance was measured at 405 nm on a Microplate Reader (Metertech). Standards for IL-4, IFNγ and TNFα were obtained from Biozol and diluted in blocking buffer. The standards were in duplicates and their concentrations ranged from 10 pg/ml to 8000 pg/ml. Background values were those wells coated with blocking buffer and contained no culture supernatant. Approximately 500 pg/ml of IL-4 and 7000 pg/ml of IFNγ were detected in the

supernatants from 10^7 stimulated splenocytes.

2.8 Cytotoxic assays

2.8.1 Spontaneous cytotoxicity

Splenocytes freshly isolated *ex vivo* can spontaneously lyse tumors without prior stimulation. This activity is mediated by NK cells. Cytototoxic activities can be greatly enhanced by culturing NK cells in IL-2 or IL-18 to generated LAK (lymphokine activated killer) cells (Trinchieri, 1989). This is likely due to the up-regulation of many death-inducing receptors during the culturing process (Kayagaki et al., 1999). To assay NK cytotoxicity, I used tumors targets purchased from ATCC, and one cell line, C4.4.25, from H. G. Ljunggren (Sweden). NK cells were either cultured in IL-2 for 6 days to generate LAK cells or they were directly used after the depletion of splenic $CD4^+$ and $CD8^+$ cells. FACS analysis confirmed the lack of $CD3^+$ T cells and $CD3^+$ $NK1.1^+$ NKT cells in freshly purified effector cell populations. NK cells were tested against tumor targets as indicated in the text. Target cells (1 - 5 x 10^6 in 200 µl of RPMI 1640) were labeled in a 15 ml tube with ^{51}Cr (100 µCi, Amersham) for 60 min at 37°C. Cells were washed four times with 10 ml of RPMI 1640 and resuspended in complete medium (RPMI 1640, 10% FCS, Glutamine, penicillin, streptomycin, 5 x 10^{-5} M 2-mercapto-ethanol) at a concentration of 10^5 cells/ml. 100 µl of this cell suspension were placed in each well of a V-bottom 96-well plate (Sarstedt). NK or LAK effectors were harvested and resuspended in complete medium. 100 µl of effectors were added into wells with 100 µl of

targets. For LAK effectors, the E:T (effector:target) ratios were 30:1, 10:1, 3:1. For freshly purified effectors, E:T ratios were 100:1, 50:1, and 25:1. Triplicates were carried out for each ratio value. The plates were placed in an incubator at 37°C for 4 hours. Spontaneous cell lysis and maximum cell lysis were determined with 10^4 cells in medium alone and 1% TX-100 respectively. After 4 hours at 37°C, samples were centrifuged for 5 minutes at 1200 rpm and 50 µl of the supernatants were transferred in a gamma counter to determine ^{51}Cr release. Percentage of specific lysis was calculated with the formula: % sample specific lysis = [(sample counts-spontaneous counts)/(maximum count-spontaneous counts)] x 100%

2.8.2 ADCC

CD16 (or FcRγII) on NK cells can mediate tumorcidal activity. In the presence of anti-tumor antigen antibodies, the Fc portion can ligate FcRγII on NK cells, leading to their activation and the subsequent lysis of tumor targets. To access CD16-mediated target lysis (ADCC), I used RMA cells as target cells. These cells are resistant to NK spontaneous cytotoxicity, but they can be readily lysed in the presence of antibodies against cell surface antigens such as Thy1.2. RMA cells were labeled with ^{51}Cr and washed as described in 2.8.1. Cells ($1x10^6$) were resuspended in 1 ml of RPM I640 and incubated with 0.5 µg anti-Thy 1.2 antibodies for 0.5 hour at 4°C. A control sample were incubated with medium. The cells were washed once with 10 ml of RPMI 1640 and resuspended in a concentration of 10^5/ml. 100 µl of this cell suspension were placed in each well of a V-bottom 96-well plate. The remaining steps were carried out as described in 2.8.1.

2.9 Cell stimulation

2.9.1 FcR mediated MAPK activation

FcγRIII (CD16) cross-linking in NK cells induces the activation of ERK, JNK, and p38 kinases (Milella et al., 1997; Trotta et al., 2000). To assess the integrity of these pathways, LAK cells were stimulated with anti-CD16. LAK cells (5×10^5) harvested from cultures described in 2.4.2. were incubated with 4 µg of anti-FcγR (2.4G2, Pharmingen) for 30 min at 4°C. Cells were washed with ice-cold PBS prior to incubation with 4 µg of rabbit anti-rat $F(ab')_2$ antibodies (Southern Biotechnologies) for another 30 min at 4°C to cross-link FcR receptors. Cells were washed again, pelleted by centrifugation, and subsequently resuspended with 200 µl pre-warmed serum-free RPMI 1640. This is denoted as the start of stimulation and the samples were incubated at 37°C for various time points as indicated in the text. Reactions were terminated by centrifugation and lysis in 20 µl of sample buffer (see solutions of 2.10). These were subjected to Western blot analysis described in 2.10.

2.9.2 Stimulation of NK cells with tumor targets

Tumor cells are rapidly lysed upon contact with NK cells. To assess marker upregulation and cytokine production during NK cell-tumor cell engagement, splenocytes depleted of $CD4^+$ and $CD8^+$ cells as described in 2.4.2. were incubated with YAC1 cell at a E:T ratio of 100:1

for the indicated time points at 37°C with conditions described in 2.8.1. Cells were subsequently harvested for FACS analysis described in 2.5. Supernatants from the culture were taken for ELISA as described in 2.7.

2.10 Western blot analysis

Solutions:

I. 2x sample buffer: 2.5 ml of 0.5 M Tris-HCl, pH6.8; 4 ml 10% SDS, 2 ml of glycerol, 0.5 ml of mercaptoethanol and 0.05% bromophenol blue in 10 ml.

II. 10x SDS PAGE running buffer: 29 g Trizma Base, 14 g Glycine, 10 g SDS in 1 L.

III. Transfer buffer: 3.03 g Trizma-base, 14.4 g glycine, 100 ml methanol in 1 L.

IV. Western blocking buffer: 5% BSA or 5% skim milk in 1x TBS-T.

V. 10x TBS-T: 48.4 g Tris, 160 g NaCl, 20 ml Tween-20, pH 7.6 in 2 L.

VI. Stripping buffer: 62.5 mM Tris-Hcl pH 6.7, 2% SDS, 100 mM 2-mercaptoethaneol.

Protein samples were boiled for 2 min and briefly spun down before they are loaded in acrylamide gel. The gels were run in 125 v for 1.5 hour with 1x SDS PAGE running buffer. The proteins were transferred onto Hybond^{TM}-P membranes (Amersham) with Semi-Dry Transfer cell (Bio-Rad). The blots were blocked 1 hour with blocking buffer and were washed three times with 1 x TBS-T. Primary antibodies anti-pERK and anti-ERK (Santa Cruz), anti-pJNK (NEB), anti-JNK (NEB), anti-pp38 (NEB), anti-p38 (NEB), anti-Vav-1 (UBI), and anti-

Lsc (Girkontaite et al., 2001) were incubated for an additional 1 hours followed by anti-mouse HRP or anti-rabbit HRP (Amersham), and detection by ECL (Amersham) in accordance with manufacturer's instructions. Western blots were stripped in 50 ml of stripping buffer at 50°C water bath for 30 min and were washed three times in 100 ml of washing buffer before re-probed with antibodies indicated in the text.

3. Results

3.1 Development of NK and NK T cells in Vav-1-deficient mice

Vav-1 is a critical regulator for T cell development and activation. Since T cells and NK cells share a common progenitor (Sanchez et al., 1994), I reasoned that Vav-1 could also be important for NK cell maturation. Therefore I stained various lymphocyte populations from Vav-1$^{-/-}$ mice with antibodies against CD3 and NK1.1 to detect NK and NK T cell populations. To eliminate non-specific background, I gated on mature cells which are HSA^{lo} (Allman et al., 1992; Crispe and Bevan, 1987). I observed no difference in relative and absolute numbers of bone marrow, splenic and hepatic NK cells between wild type and Vav-1$^{-/-}$ mice (Figure 3.1A and B). Similar results were obtained when I stained NK cells with DX5, another putative NK cell surface marker (Figure 3.1C). I examined surface expression of markers on splenic NK cells and found normal expression of CD45, CD44, β1 integrin, IL-2Rβ, CD69 and FcRγII/II in Vav-1$^{-/-}$ NK cells (Figure 3.1D). In addition to the severe paucity of thymic and peripheral T cells, however, I observed a gross reduction of thymic, bone marrow, splenic and hepatic NK T cells in Vav-1$^{-/-}$ mice (Figure 3.1A and B). A decrease in NKT cells in the thymus and the liver was also evident using DX5 as a NK marker (Figure 3.1C). However, in the spleen, there was only a marginal decrease in CD3^{+}DX5^{+} cells in mutant animals (Figure 3.1C). This might be due to that fact that only selective splenic NKT cells express DX5, and therefore

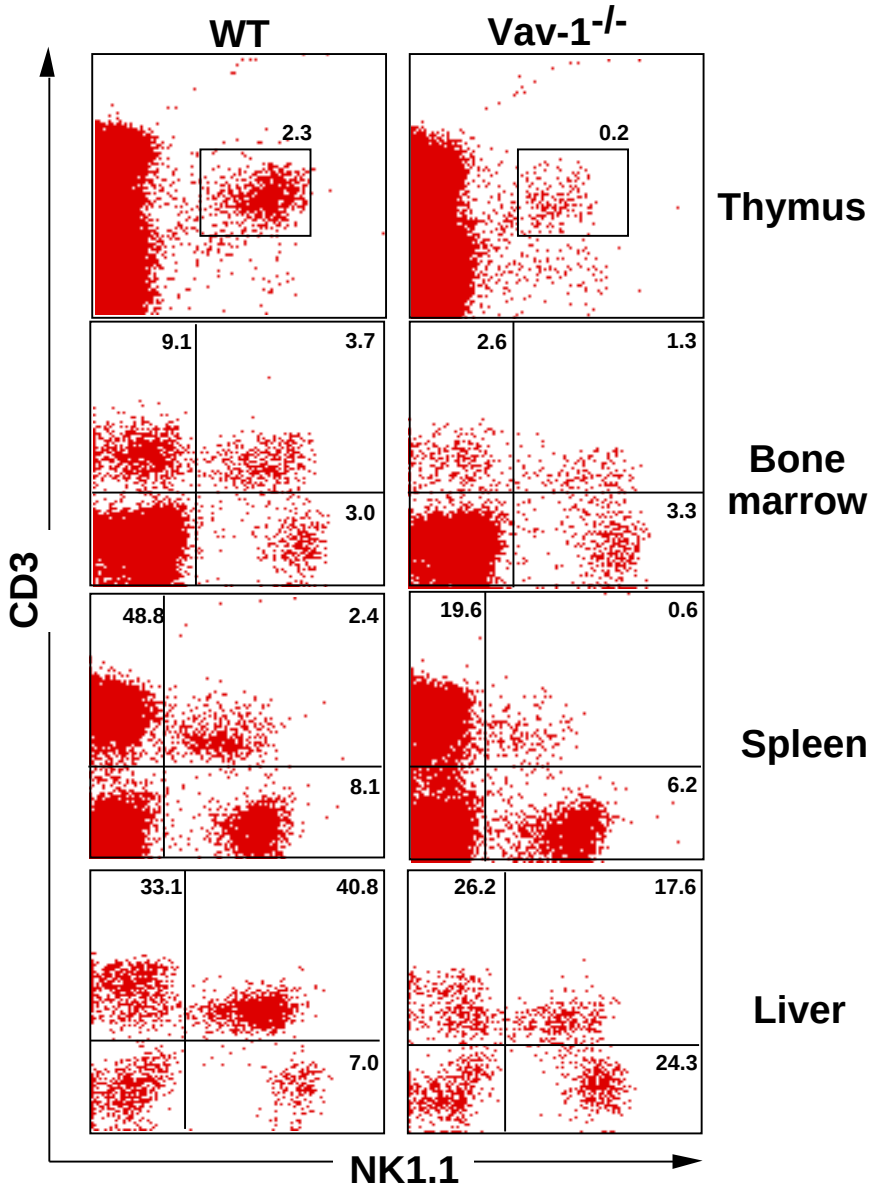


Figure 3.1 A. NK and NK T cell development in Vav-1$^{-/-}$ mice characterized by CD3 and NK1.1 surface expression. Immunofluorescence analysis of thymic, bone marrow, splenic and hepatic NK and NK T cells. Cells were isolated from 8-9 wks-old wildtype and Vav-1$^{-/-}$ mice and stained with anti-CD3-FITC, anti-NK1.1-PE and anti-HSA-biotin antibodies followed by Cy-chrome streptaviden to detect biotinylated antibodies. To visualize NK and NK T cells gates were set on HSA^{lo} cells. One result representative of 5 experiments is shown.

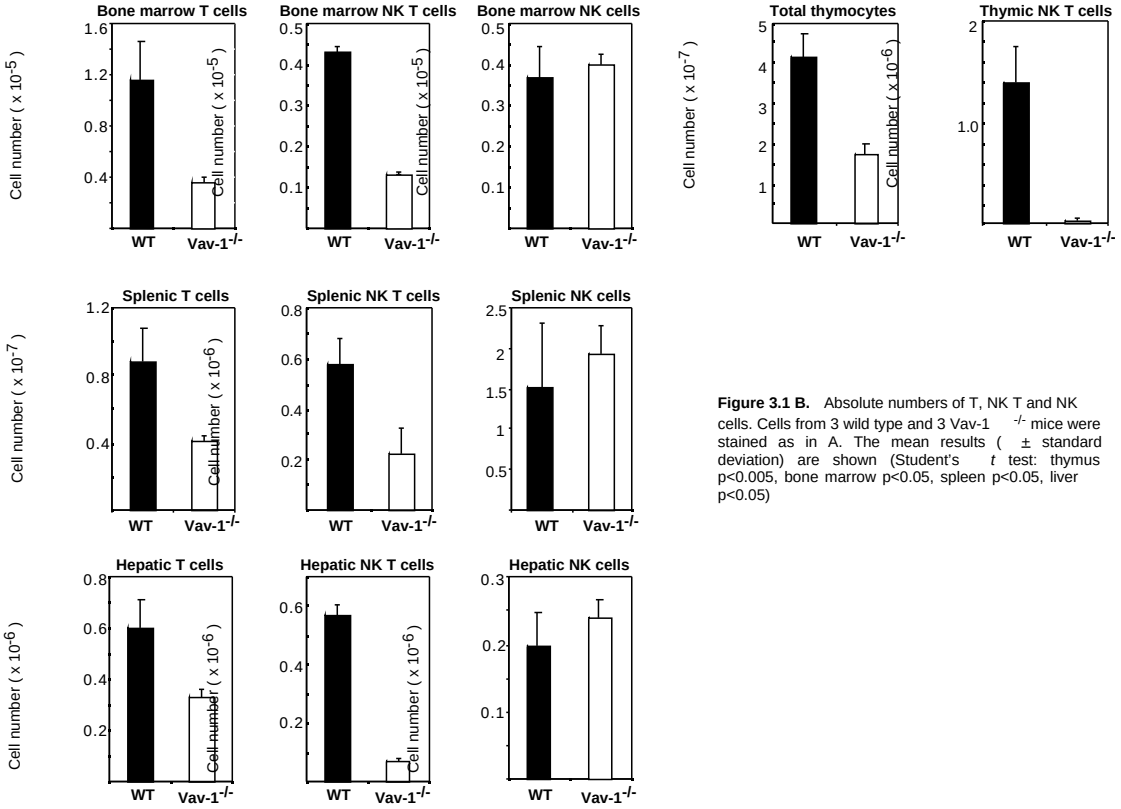


Figure 3.1 B. Absolute numbers of T, NK T and NK cells. Cells from 3 wild type and 3 Vav-1 $^{-/-}$ mice were stained as in A. The mean results (± standard deviation) are shown (Student's *t* test: thymus $p<0.005$, bone marrow $p<0.05$, spleen $p<0.05$, liver $p<0.05$)

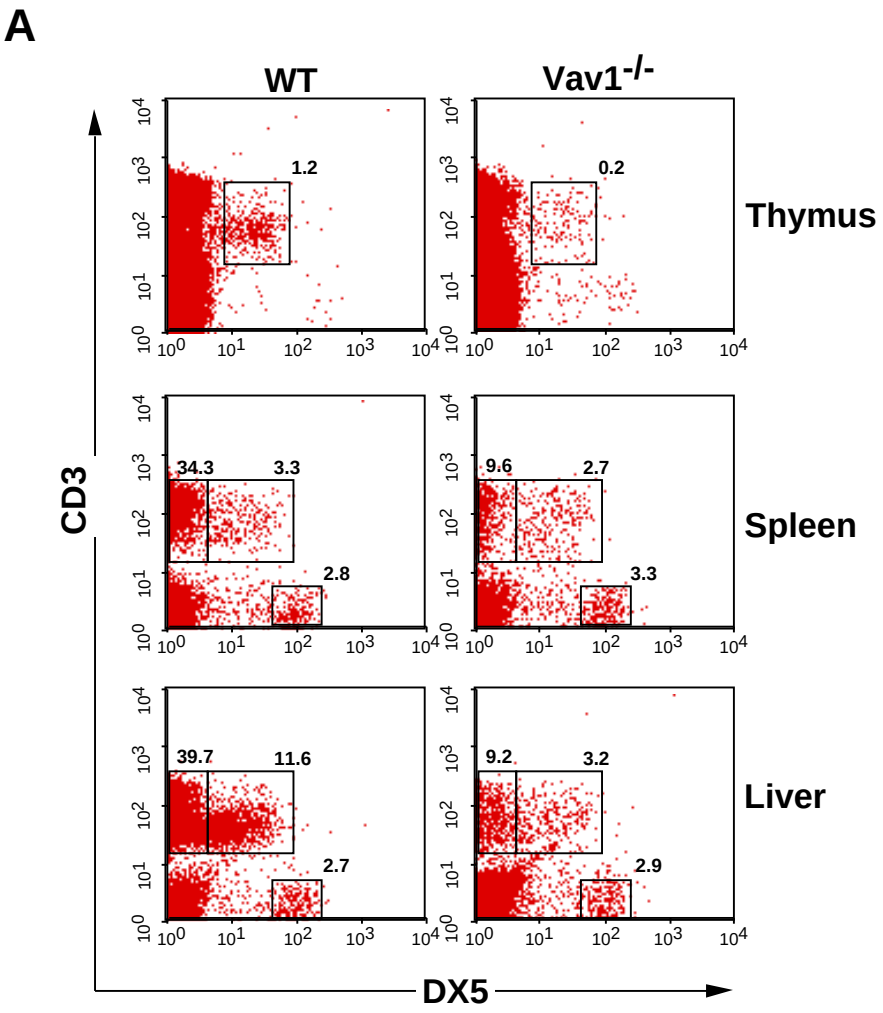


Figure 3.1 C. NK and NK T cell development in Vav-1$^{-/-}$ mice characterized by CD3 and NK1.1 surface expression. Immunofluorescence analysis of thymic, splenic and hepatic NK and NK T cells. Cells were isolated from 8-9 wks-old wildtype and Vav-1$^{-/-}$ mice and stained with anti-CD3-FITC, anti-DX5-PE and anti-HSA-biotin antibodies followed by Cy-chrome streptaviden to detect biotinylated antibodies. To visualize NK and NK T cells gates were set on HSA^{lo} cells. One result representative of 5 experiments is shown.

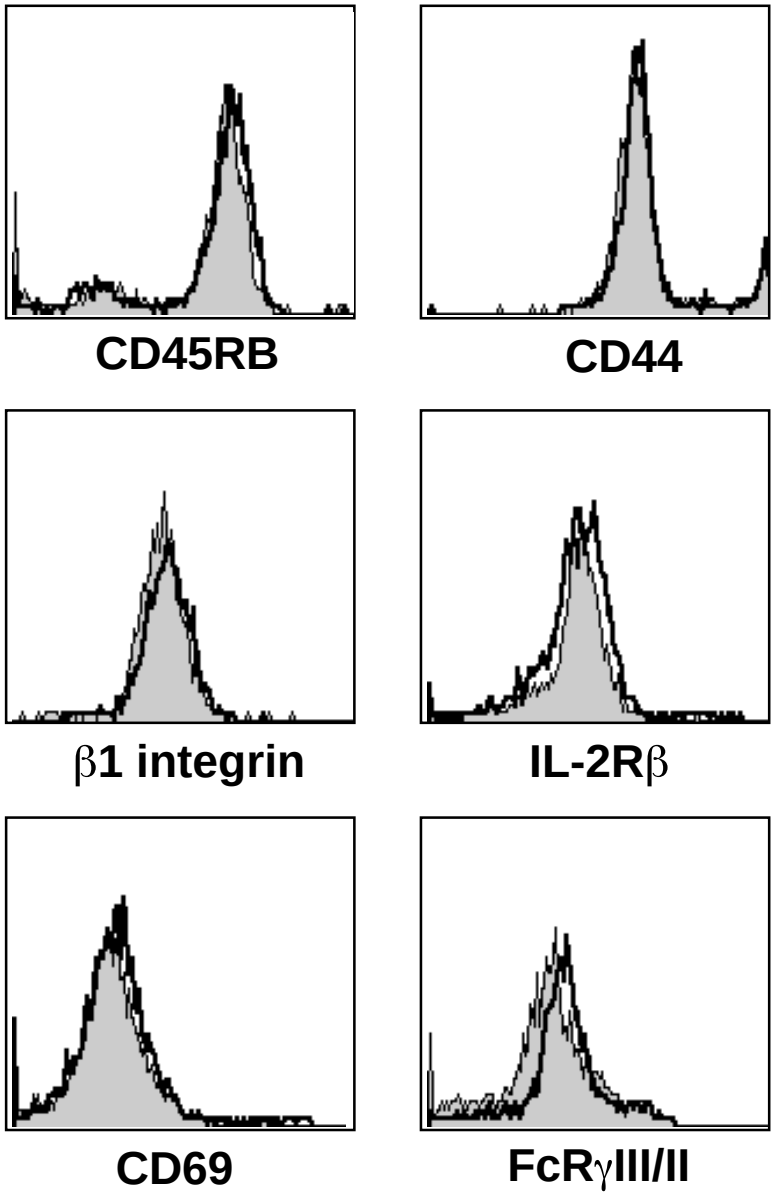


Figure 3.1 D. Expression of NK cell receptors on splenic NK cells. Splenocytes were stained with anti-CD3, anti-NK1.1, and antibodies against the indicated markers. Histograms for wild type (closed histogram) and Vav-1 $^{-/-}$ (opened histogram) NK cells were generated by gating on $CD3^{-}NK1.1^{+}$ populations. One result representative of 2 experiments is shown.

total numbers of NKT cells are under-represented by the $CD3^+DX5^+$ stain (Eberl et al., 1999b). Together, these data indicate Vav-1 is required for NKT cell development.

NKT cells are noted for their ability to rapidly secret large amount of IL-4 and IFNγ in response to *in vivo* administration of CD3 (Chen et al., 1997; Leite-de-Moraes et al., 1995; Mendiratta et al., 1997; Yoshimoto and Paul, 1994). To determine whether Vav-$1^{-/-}$ splenocytes retain the capability to produce these cytokines following CD3 activation, anti-CD3 antibodies were injected intravenously into wild type and Vav-$1^{-/-}$ mice and ninety minutes later splenocytes were seeded from injected mice at various densities and cytokine production was measured after a 90 min. incubation. I observed an approximate 90% reduction in IL-4 and IFNγ secretion by Vav-$1^{-/-}$ splenocytes compared to wild type (Figure 3.2). These data indicate that Vav-1 is not necessary for mainstream NK cell development, but is indispensable for NK T cells.

3.2 Vav-1 is not required for ADCC and CD16-mediated MAPK activation

NK cells use their low-affinity FcγRIIIA to form conjugates with antibody-coated cells. Once NK cells are bound to a target cell, they activate their ADCC responses. Ectopic expression of Vav-1 in human NK cells potentiates ADCC (Billadeau et al., 1998), while inhibition of Vav-1 expression with anti-sense oligonucleotides markedly reduced FcγR mediated cytotoxic activity (Galandrini et al., 1999). To determine whether Vav-1 is necessary for ADCC of

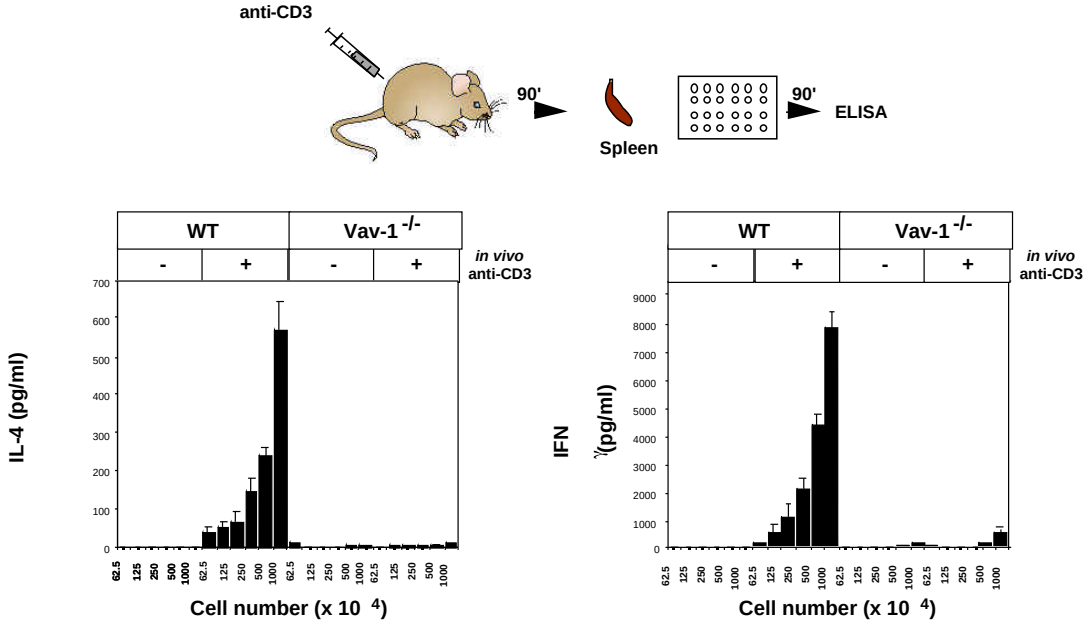


Figure 3.2. CD3-induced IL-4 and IFN γ production in wild type and Vav-1 $^{-/-}$ splenocytes. Mice were intravenously injected with PBS or 2 µg of anti-CD3. Splenocytes were harvested 90 min later and plated in 96-well plates at the indicated cell number for 1.5 hr. at 37°C. The amount of IL-4 (left) and IFN γ (right) released in the culture supernatants were determined by ELISA.

murine NK cells, I performed NK cell cytotoxicity assays. Since freshly isolated NK cells exhibit only minimal cytotoxicity against most target cells, I removed B cells, $CD4^+$ and $CD8^+$ T cells from splenic cell suspensions, and generated lymphokine activated killer (LAK) cells *in vitro* in the presence of IL-2. After culturing for 6 days, the purity of both wild type and Vav-$1^{-/-}$ NK cells ($CD3^-NK1.1^+$) cells was over 96% and no significant contamination of CD3 expressing NKT cells was observed as judged by FACS analysis (Figure 3.3A). Since LAK cells generated from mutant mice were comparable in both amount and purity to those of wild type, it is likely that Vav-1 is not necessary for NK cell proliferation. Vav-1 is present in wild type LAK cells, but it is absent in Vav-$1^{-/-}$ LAK cells (Figure 3.3B). I also detected Vav-1 expression in freshly purified *ex vivo* NK cells (Figure 3.3C). To determine whether Vav-1 is required for ADCC in murine NK cells, wild type and Vav-$1^{-/-}$ LAK cells were co-cultured with RMA target cells in the presence or the absence of anti-Thy 1.2 antibodies (Figure 3.4A). Vav-$1^{-/-}$ LAK cells exhibited a reduction in spontaneous cytotoxicity against RMA cells. The addition of anti-Thy 1.2 antibodies, however, led to the augmentation of cytotoxicity of both wild type and Vav-$1^{-/-}$ NK cells to the same degree, indicating that ADCC is independent of Vav-1 function.

Ligation of CD16 (FcRγIII) in human NK cells leads to rapid tyrosine phosphorylation of Vav-1 (Billadeau et al., 1998; Galandrini et al., 1999), implicating Vav-1 as a downstream target. CD16 cross-linking in NK cells also induces the activation of ERK, JNK, and p38 kinases

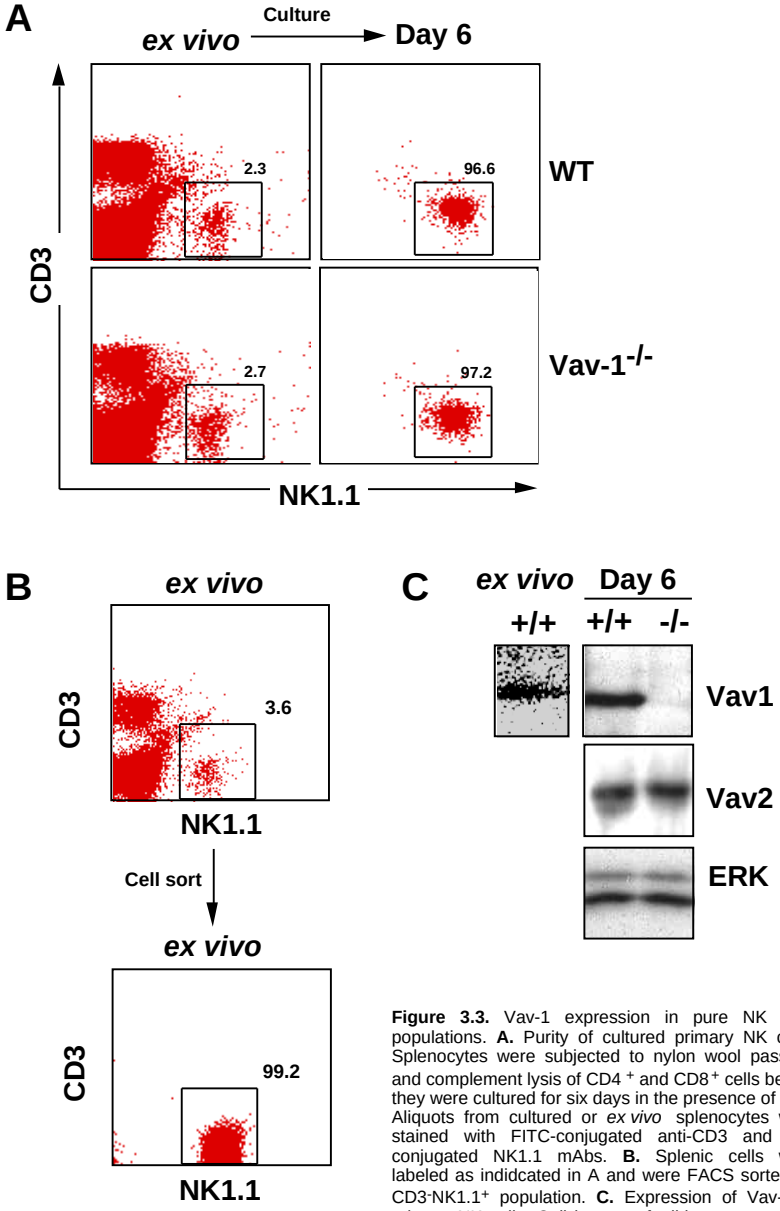


Figure 3.3. Vav-1 expression in pure NK cells populations. **A.** Purity of cultured primary NK cells. Splenocytes were subjected to nylon wool passage and complement lysis of $CD4^+$ and $CD8^+$ cells before they were cultured for six days in the presence of IL-2. Aliquots from cultured or *ex vivo* splenocytes were stained with FITC-conjugated anti-CD3 and PE-conjugated NK1.1 mAbs. **B.** Splenic cells were labeled as indidcated in A and were FACS sorted for $CD3^-NK1.1^+$ population. **C.** Expression of Vav-1 in primary NK cells. Cell lysates of wild type sorted NK cells and cultured NK cells from Vav-1 $^{+/+}$ and Vav-1$^{-/-}$ mice were subjected to Western blot analyses. Detection of protein expression was performed with anti-Vav-1, anti-Vav-2 and anti-ERK antibodies as a loading control.

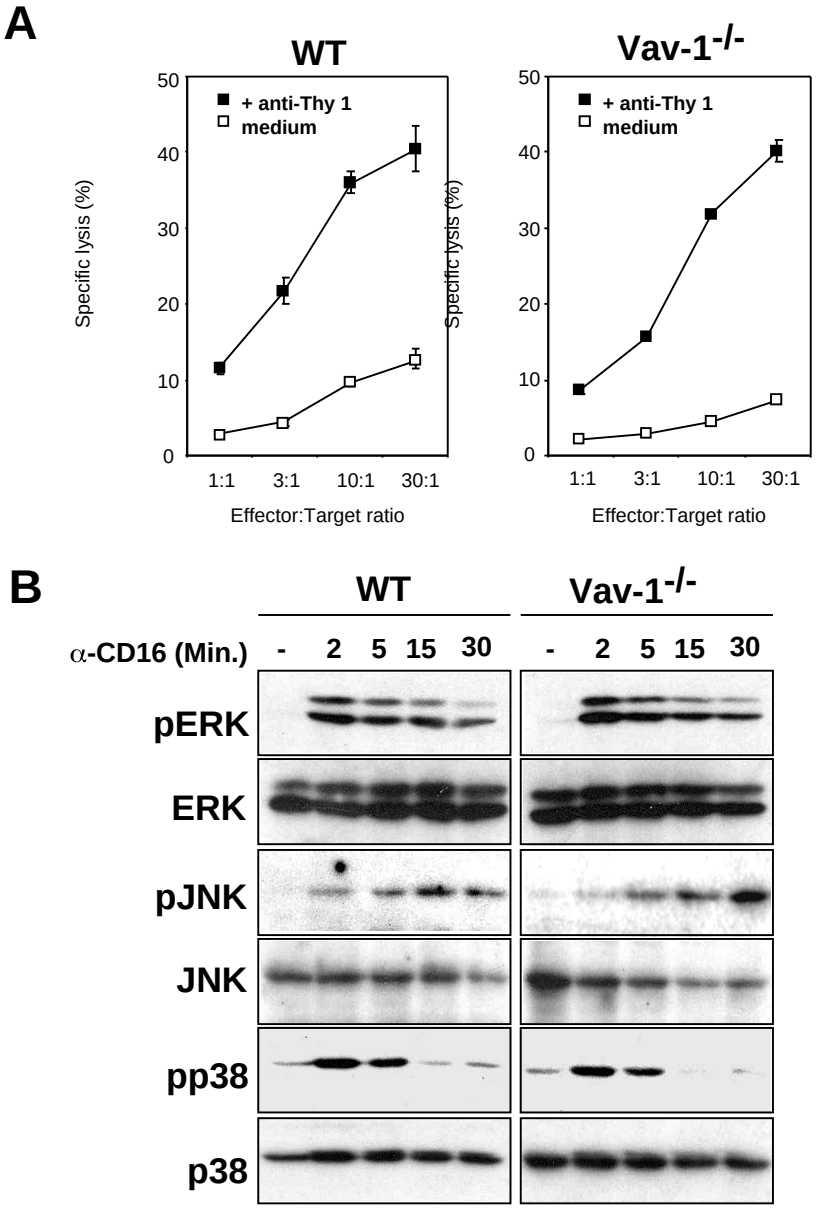


Figure 3.4. ADCC and CD16-mediated MAPKs activation of Vav-1$^{-/-}$ NK cells. **A.** ADCC. Cultured NK cells were distributed in triplicates into plates containing ^{51}Cr-labeled RMA targets at the indicated effector:target ratio. RMA cells were incubated for 1 hour at room temperature with anti-Thy1.2 culture supernatant or medium alone. Percentage of specific lysis was determined by the amount of chromium released at 4 hours. **B.** MAPK-activation. Cultured NK cells were stimulated with anti-CD16/CD32 and cross-linked with rabbit anti-rat antibodies. Cell lysates were resolved with SDS-PAGE and immunoblotted with the indicated antibodies. Results are representative of three independent experiments.

(Milella et al., 1997; Trotta et al., 2000). To determine whether Vav-1 plays a role CD16-mediated MAPK activation, Western blot analysis was performed with lysates from NK cells stimulated with anti-CD16 antibodies or with medium alone (Figure 3.4B). Consistent with published results, ligation of CD16 activates ERK, JNK, and p38. In both wild type and Vav-1$^{-/-}$ LAK cells, activation of ERK and JNK was sustained after 20-30 min, while the levels of active p38 reached a plateau at 2-5 min, and declined to background level by 15 min. The expression of CD16 was comparable between wild type and Vav-1$^{-/-}$ LAK cells (data not shown). These results indicate that Vav-1 is dispensable for CD16 mediated MAPKs activation in murine NK cells.

3.3 Spontaneous cytotoxicity is compromised in Vav-1$^{-/-}$ NK cells

NK cells exhibit spontaneous cytotoxicity towards some tumors cell lines. Since co-incubation of NK cells with target cells also leads to Vav-1 phosphorylation (Billadeau et al., 1998; Galandrini et al., 1999), Vav-1 is believed to play an important role in this process. Furthermore, human NK cell cytotoxic activity against K562 cells is potentiated by over-expressing Vav-1 (Billadeau et al., 1998), while reduction of Vav-1 expression by anti-sense oligonucleotides markedly reduces NK cell cytotoxic activity (Galandrini et al., 1999). To assess the role of Vav-1 in spontaneous cytotoxicity, I tested the ability of wild type and Vav-1$^{-/-}$ NK cells to lyse a panel of target cells. Freshly isolated NK cells were obtained from

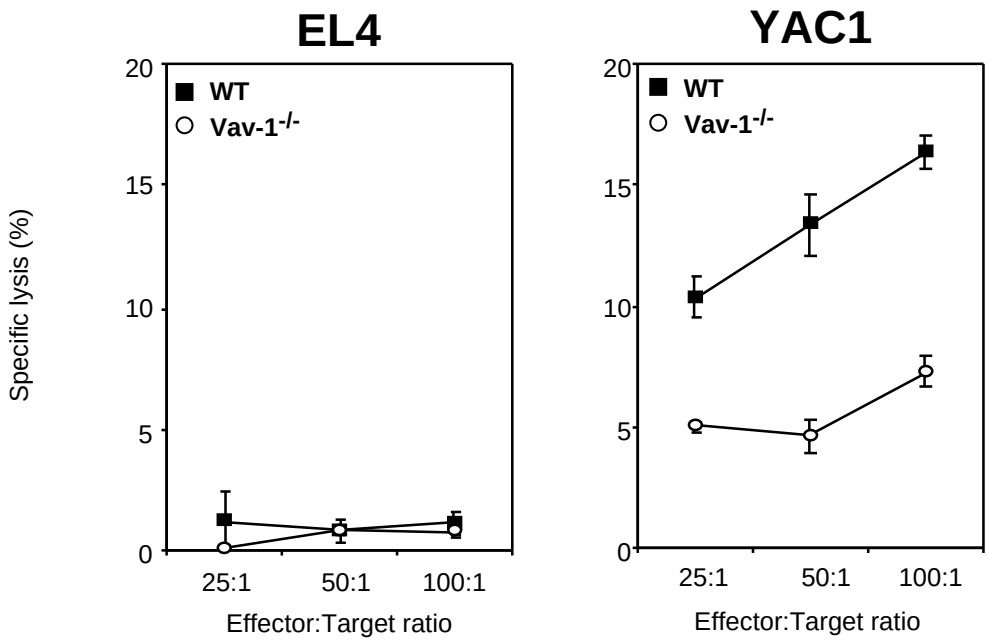


Figure 3.5. Impaired natural cytotoxicity of freshly isolated Vav-1$^{-/-}$ NK cells. Freshly purified splenocytes depleted of CD4^{+} and CD8^{+} were used as effectors against EL4 and YAC1 cells in a standard chromium release assay. Percentage of specific lysis was determined by the amount of chromium release at 4 hours. Results are representative of three independent experiments.

splenocytes depleted of $CD4^+$- and $CD8^+$-expressing lymphocytes. I found that Vav-$1^{-/-}$ NK effector cells displayed a consistently reduced ability to lyse YAC-1 cells (Figure. 3.5). Neither wildtype nor Vav-$1^{-/-}$ effectors showed cytotoxicity against syngenic targets such as EL4 cells and RMA and their respective MHC class I-deficient variants, C4.4.25 cells, and RMA/S cells (Figure. 3.5 and data not shown). In order to compare cytotoxic abilities of freshly isolated Vav-$1^{-/-}$ NK cells with those of LAK cells, I tested wildtype and Vav-$1^{-/-}$ LAK cell cytotoxicity against the same targets—EL4, C4.4.25, RMA and RMA/S cells. Whereas wildtype LAK cells developed cytotoxic capacity against these target cells, Vav-$1^{-/-}$ LAK cells failed to mount strong cytotoxic responses (Figure. 3.6). Interestingly, Vav-$1^{-/-}$ LAK cells were able to normally lyse YAC1 cells, indicating that freshly isolated NK cells and LAK cells have different requirements for Vav-1. Vav-$1^{+/-}$ and wildtype exhibited indistinguishable cytotoxic activity against all target cells (data not shown). Taken together, these data show that Vav-1 is important for NK cell spontaneous cytotoxicity.

3.4 Development of NK and NK T cells in Vav-2- and Vav-1/Vav-2 deficient mice

Although the above experiments have highlighted the importance of Vav-1 in many NK activation processes, many others such as CD16 mediated ADCC and MAPK activation seem to be independent of Vav-1. Furthermore, remnant spontaneous activity can be observed with Vav-$1^{-/-}$ effectors. These raise the possibility that other Vav family members, such as Vav-2 could compensate for Vav-1 function. Vav-2 is expressed in NK cells (Figure

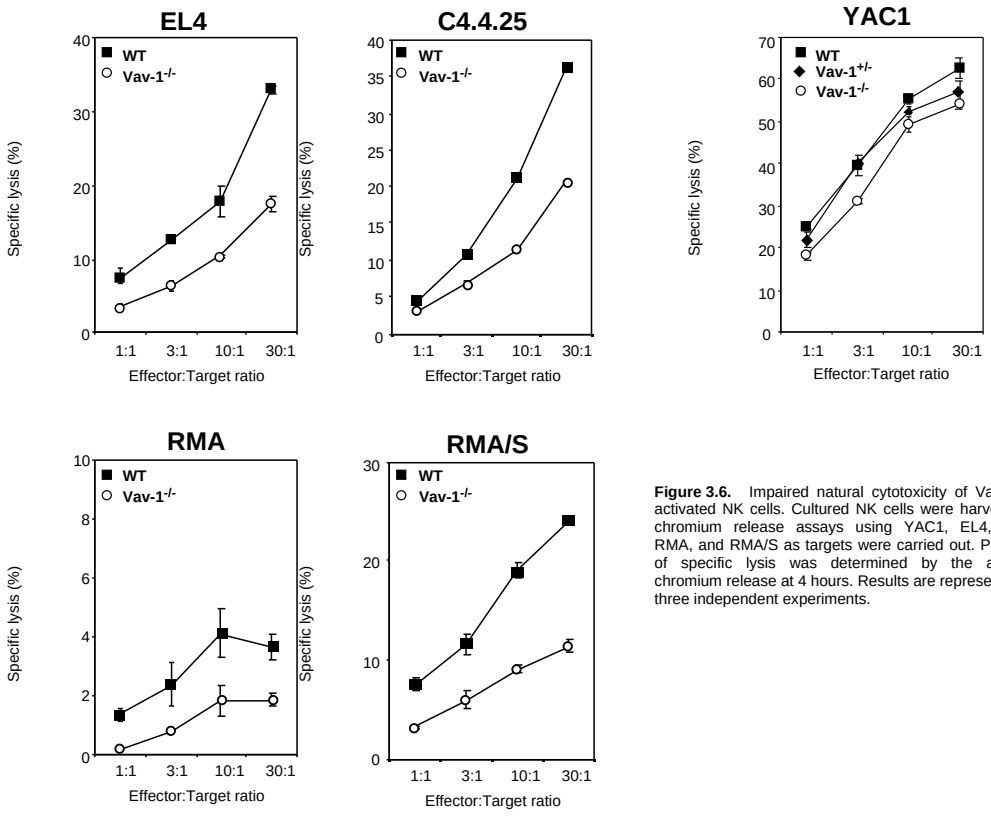


Figure 3.6. Impaired natural cytotoxicity of Vav-1 $^{-/-}$ IL-2-activated NK cells. Cultured NK cells were harvested and chromium release assays using YAC1, EL4, C4.4.25, RMA, and RMA/S as targets were carried out. Percentage of specific lysis was determined by the amount of chromium release at 4 hours. Results are representative of three independent experiments.

3.3) and it is structurally similar to Vav-1 (Bustelo, 2000). It was also implicated to participate in both NK cell natural cytotoxicity and ADCC since FcR ligation leads to Vav-2 phosphorylation and ectopic expression of Vav-2 increases NK cell natural cytotoxicity and ADCC (Billadeau et al., 2000). These studies however were done using human derived NK cell lines. The function of Vav-2 in murine NK cells has not been examined, and possible synergy of Vav-1 and Vav-2 remains to be investigated.

To determine whether Vav-2 is necessary for NK and NKT cell development, I examined these populations from lymphoid organs of Vav-2 deficient animals. I observed no difference in relative and absolute numbers of bone marrow, splenic and hepatic NK cells between wild type and Vav-$2^{-/-}$ mice (Figure 3.7 A and B). Unlike Vav-$1^{-/-}$ mice, Vav-$2^{-/-}$ mice displayed normal development of NKT cells, indicating that Vav-2 is not necessary for NK and NKT cell development.

It has been shown that Vav-1 and Vav-2 can compensate for each other in B cell development (Doody et al., 2001; Tedford et al., 2001). To determine whether such compensation is extended to the development of NK and NKT cells, I compared these populations from wild type and Vav-$1^{-/-}$/Vav-$2^{-/-}$ mice. Mice deficient for both Vav-1 and Vav-2 have normal numbers of NK cells, but a reduced numbers of NKT cell population similar to those observed in Vav-$1^{-/-}$ animals (Figure 3.7 A and B). Therefore, both Vav-1 and Vav-2 are

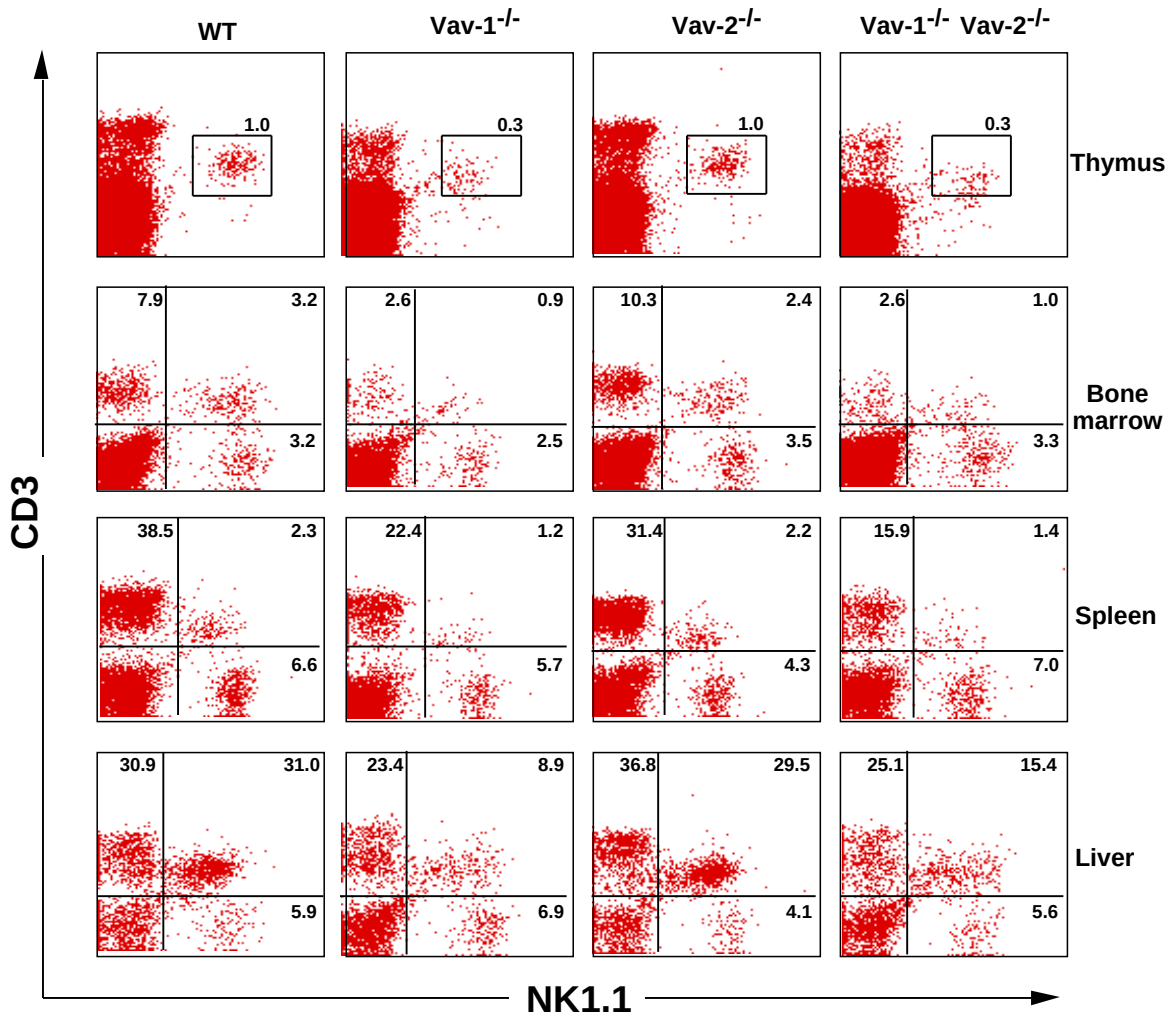


Figure 3.7 A. NK and NK T cell development in Vav-1$^{-/-}$,Vav-2$^{-/-}$ and Vav-1$^{-/-}$Vav-2$^{-/-}$ mice. Immunofluorescence analysis of thymic, bone marrow, splenic and hepatic NK and NK T cells. Cells were isolated from 8-9 wks-old wildtype and mutant mice and stained with anti-CD3-FITC, anti-NK1.1-PE and anti-HSA-biotin antibodies. To visualize NK and NK T cells gates were set on HSA^{lo} cells. One result representative of 4 experiments is shown.

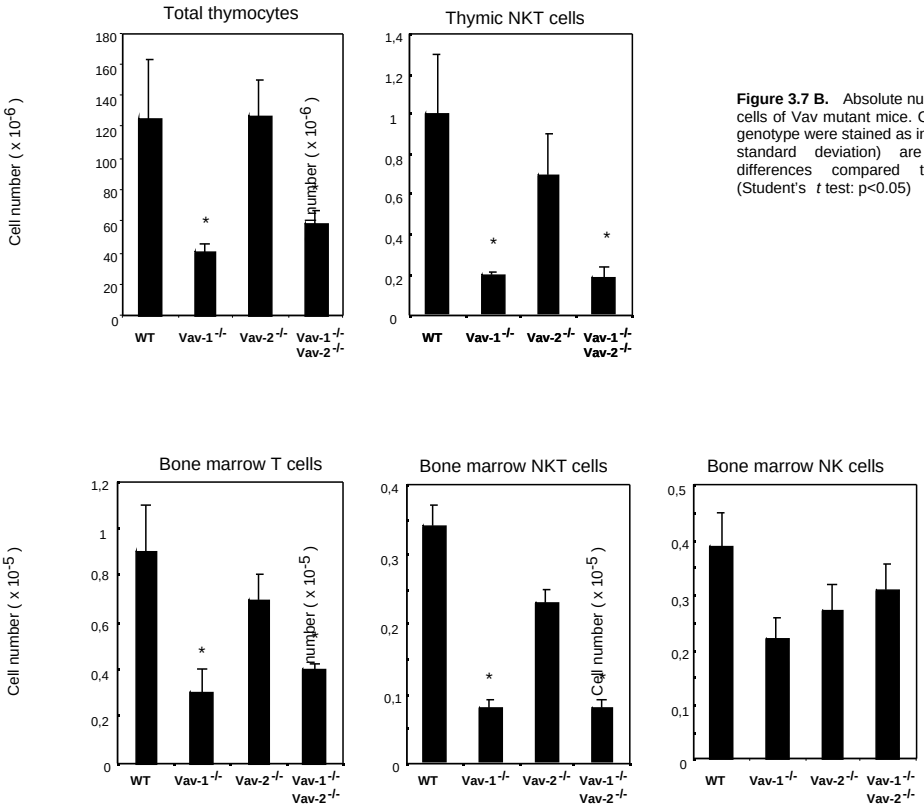
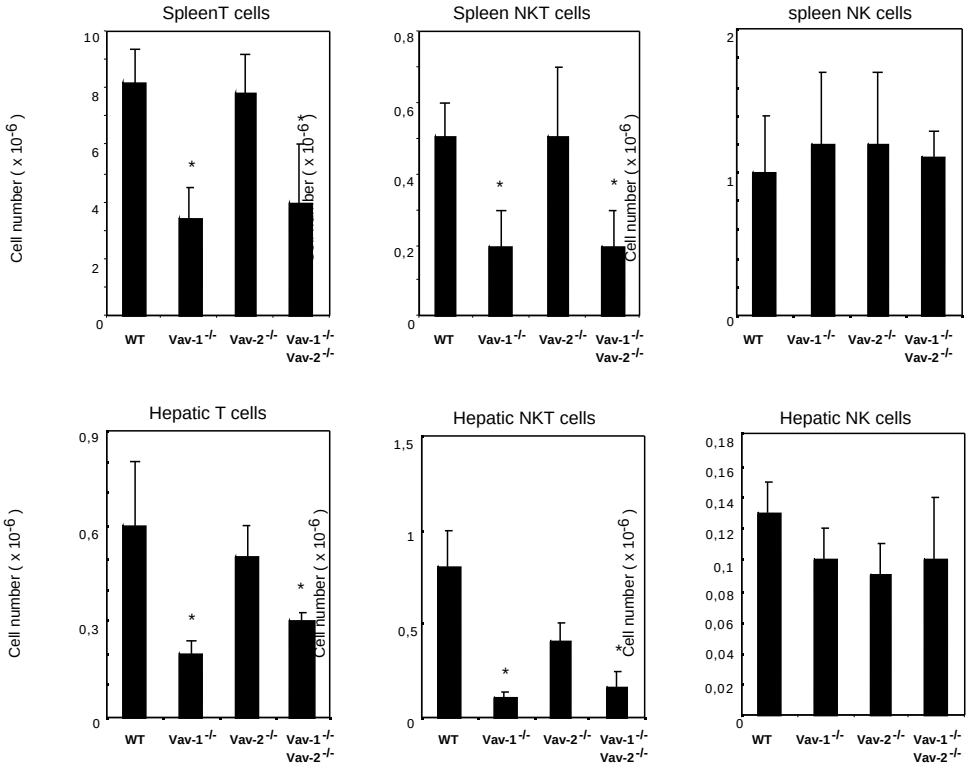


Figure 3.7 B. Absolute numbers of T, NK T and NK cells of Vav mutant mice. Cells from 3 mice of each genotype were stained as in A. The mean results (± standard deviation) are shown. *Significant differences compared to wild are indicated (Student's *t* test: p<0.05)


SpleenT cells
Spleen NKT cells
spleen NK cells
Hepatic T cells
Hepatic NKT cells
Hepatic NK cells
Cell number (x 10-6)
WT
Vav-1-/-
Vav-2-/-
Vav-1-/- Vav-2-/-

not mutual compensatory in the development of NK cells and the remnant presence of NKT cells in Vav-1$^{-/-}$ mice is not due signals mediated by the presence of Vav-2.

3.5 Vav-1 and Vav-2 are not required for ADCC and CD16-mediated MAPK activation

CD16 mediated ADCC and MAPK activation are normal in Vav-1$^{-/-}$ NK cells. Since Vav-2 is a close homologue of Vav-1, it might traffic signals from CD16. Indeed, in human NK cells FcR ligation leads to Vav-2 phosphorylation and ectopic expression of Vav-2 potentiates NK ADCC (Billadeau et al., 2000). To determine whether Vav-2 is required for ADCC in mouse NK cells, I performed cytotoxic assay using Vav-2$^{-/-}$ effectors. Wild type and Vav-deficient LAK cells were co-cultured with RMA target cells in the presence or the absence of anti-Thy 1.2 antibodies (Figure 3.8A). Both wild type and Vav-2$^{-/-}$ LAK cells exhibited low spontaneous cytotoxicity against RMA cells. The addition of anti-Thy 1.2 antibodies, however, led to the augmentation of cytotoxicity with all NK effectors. These data suggest that Vav-2 is not required for ADCC. To examine whether CD16 mediate MAPK activation is Vav-2 dependent, Western blot analysis was performed with lysates from wild type and Vav-2$^{-/-}$ NK cells stimulated with anti-CD16 antibodies (Figure 3.8 B). Consistent with normal ADCC, I found that ligation of CD16 on mutant cells leads to normal activation of MAPK. Therefore, Vav-2 is not required for CD16 mediated MAPK activation.

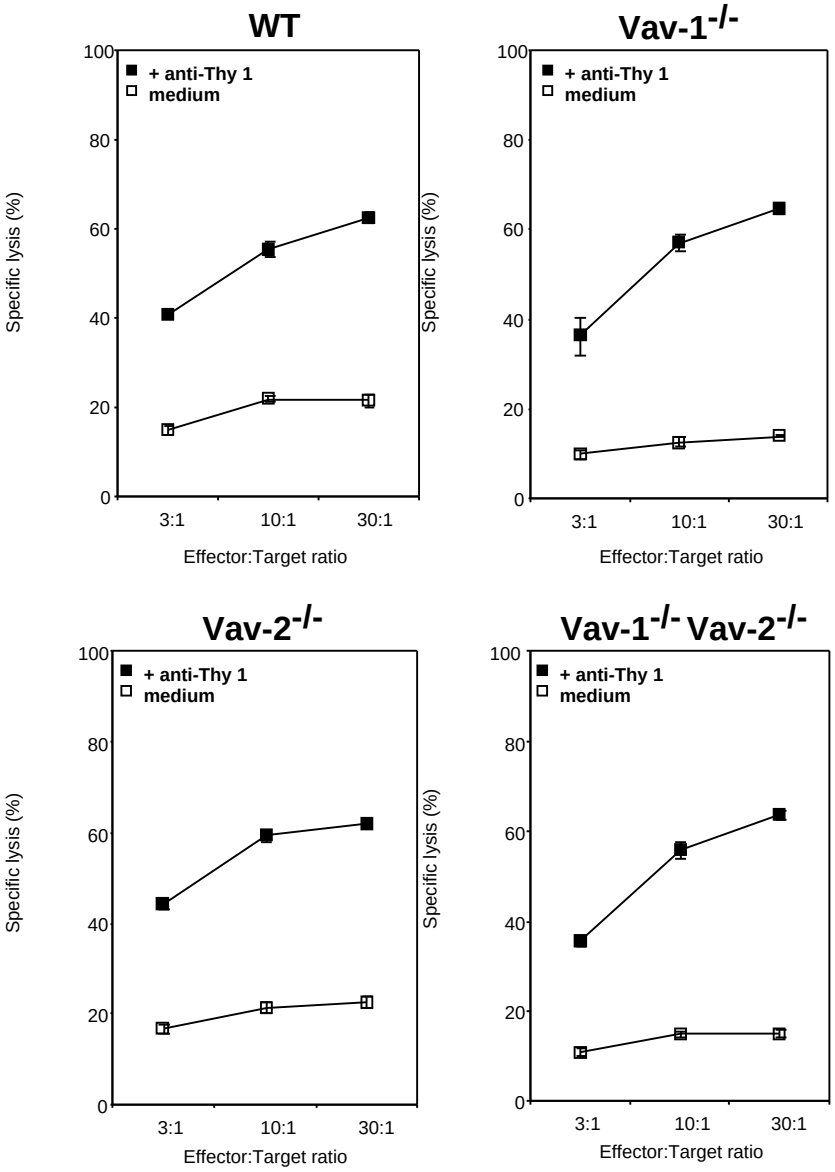


Figure 3.8. A. ADCC and CD16-mediated MAPKs activation of Vav deficient NK cells. ADCC. Cultured NK cells were distributed in triplicates into plates containing ^{51}Cr-labeled RMA targets at the indicated effector:target ratio. RMA cells were incubated for 1 hour at room temperature with anti-Thy1.2 culture supernatant or medium alone. Percentage of specific lysis was determined by the amount of chromium released at 4 hours. Results are representative of two experiments.

B

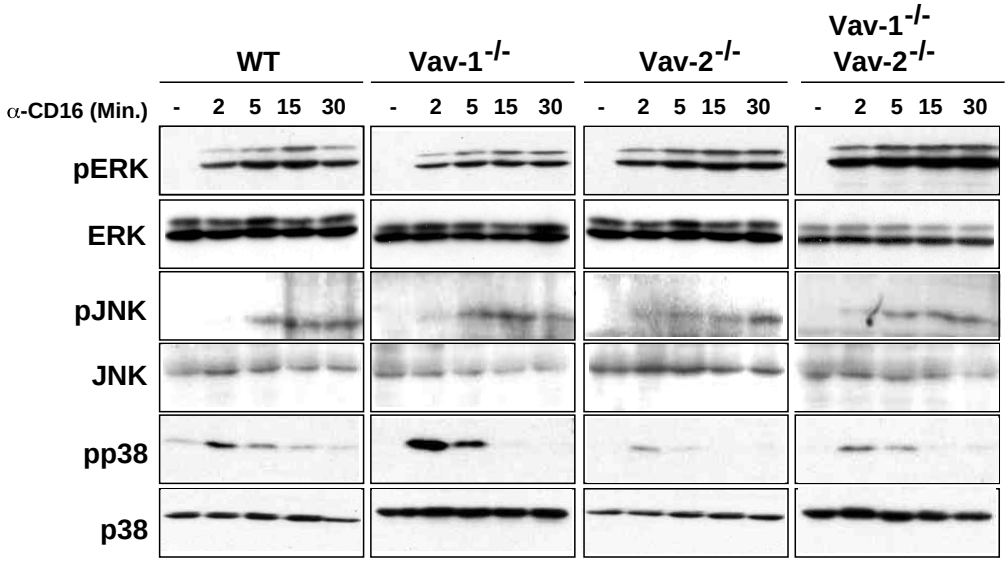


Figure 3.8. **B.** MAPK-activation of Vav mutant LAK cells. Cultured NK cells were stimulated with anti-CD16/CD32 and cross-linked with rabbit anti-rat antibodies. Cell lysates were resolved with SDS-PAGE and immunoblotted with the indicated antibodies. Results are representative of two independent experiments.

and Vav-2 are dispensable for CD16-mediated ADCC and MAPKs activation in murine NK cells and they do not assume mutual compensatory roles in these processes.

3.6 Spontaneous cytotoxicity in Vav-2 and Vav-1/Vav-2 deficient NK cells

Vav-1 is necessary in NK spontaneous cytotoxicity against a panel of tumors targets (Figure 3.5 and 3.6). To determine whether Vav-2 also share a similar role in this process, freshly isolated splenocytes cells from Vav-2 deficient animals were depleted of $CD4^+$- and $CD8^+$-expressing lymphocytes and the remaining cells were used in a cytotoxic experiment using YAC-1 as targets. I found both wild type and Vav-$2^{-/-}$ NK cells showed almost identical cytotoxic activity (Figure 3.9). I also generated LAK cells from Vav-2 deficients animals, and tested them against EL4, C4.4.25, and RMA/S cells (Figure 3.10). Both wildtype and Vav-$2^{-/-}$ LAK cells displayed similar cytotoxic responses against these target cells, indicating Vav-2 is not required for spontaneous cytotoxicity in murine NK cells.

The ability of Vav-$2^{-/-}$ NK cells to exhibit a normal cytotoxic response might be explained by the presence of Vav-1 in fulfilling a compensatory role for Vav-2. Therefore I examined spontaneous NK cytotoxicity of Vav-$1^{-/-}$Vav-$2^{-/-}$ NK cells. I found that Vav-$1^{-/-}$Vav-$2^{-/-}$ effectors exhibit reduced cytotoxicity against YAC-1 cells to the similar degree as those observed with Vav-$1^{-/-}$ effectors (Figure 3.9). Similarly, LAK effectors from Vav-$1^{-/-}$ and Vav-$1^{-/-}$Vav-$2^{-/-}$ mice

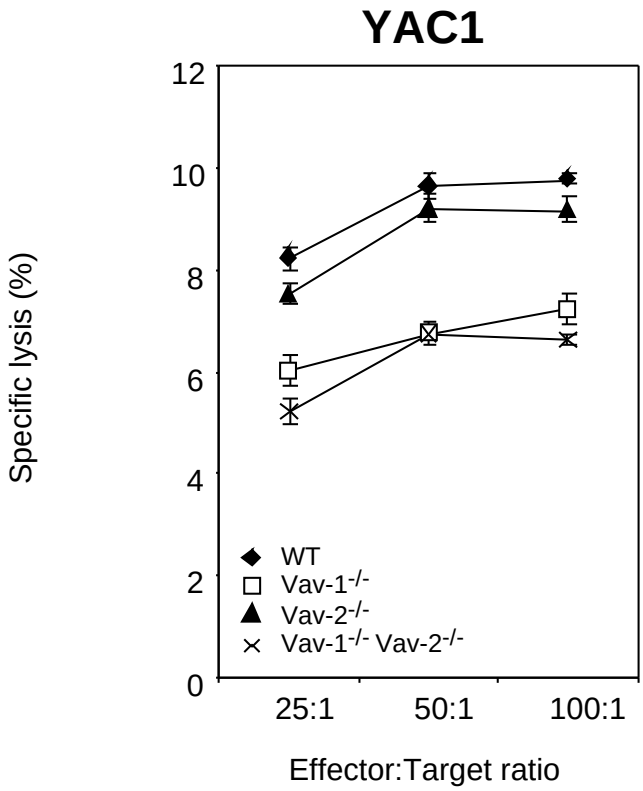


Figure 3.9. Natural cytotoxicity of freshly isolated $Vav^{-/-}$ NK cells. Freshly purified splenocytes depleted of $CD4^+$ and $CD8^+$ were used as effectors against YAC1 cells in a standard chromium release assay. Percentage of specific lysis was determined by the amount of chromium release at 4 hours. Results are representative of two experiments.

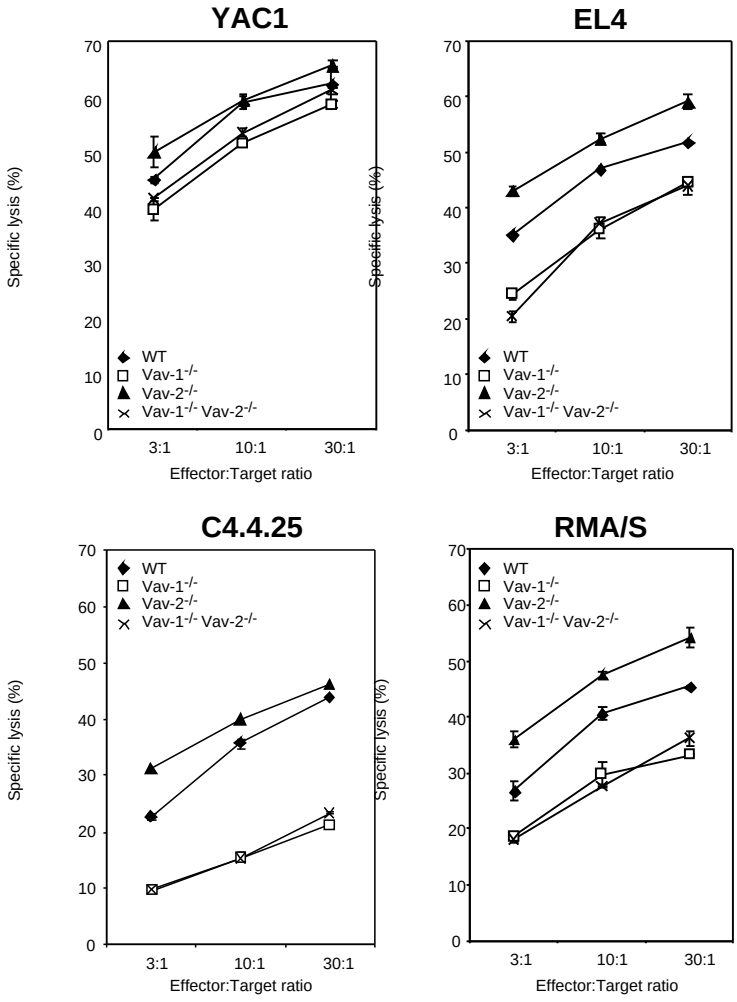


Figure 3.10. Natural cytotoxicity of Vav mutant LAK cells. Cultured NK cells were harvested and chromium release assays using YAC1, EL4, C4.4.25, and RMA/S as targets were carried out. Percentage of specific lysis was determined by the amount of chromium release at 4 hours. Results are representative of two experiments.

showed indistinguishable cytotoxic responses against EL4, C4.4.25 and RMA/S targets (Figure 3.10). All LAK cells were able to lyse YAC1 cells with similar effeciency. Taken together, these data show that Vav-1 but not Vav-2 is important for NK cell spontaneous cytotoxicity, and Vav-2 can not functionally compensate Vav-1 in these processes.

3.7 Analysis of NK cell function in Lsc$^{-/-}$ mice

Studies described above highlight the importance of Vav-1 in NK cell activation. Similar to Vav-1, Lsc is another Rho GEF that is expressed specifically in the hematopoietic system. It is also found in LAK cells, but its functions remain unknown (Girkontaite et al., 2001). Consistent with its presence in LAK cells, freshly purified CD3^{-}DX5^{+} NK cells also express Lsc (Figure 3.11A). To determine whether the absence of Lsc affects NK cell development, I stained NK cells from the spleens and the livers of wild type and Lsc$^{-/-}$ mice and found comparable numbers and ratios of these populations in both mice (Figure 3.11B). Mutant NK cells also express normal levels of Ly-49D, Ly-49G2, CD16, CD45, CD44, FasL, and CD69 (Figure 3.11C). These data indicate Lsc is not required for NK cell development. Furthermore, in Lsc$^{-/-}$ mice of the C57BL/6 background, CD3^{+}NK1.1^{+} splenic NKT cells were present in normal numbers and ratios as those from the wild type mice (data not shown), demonstrating Lsc is not required for the NKT cell development.

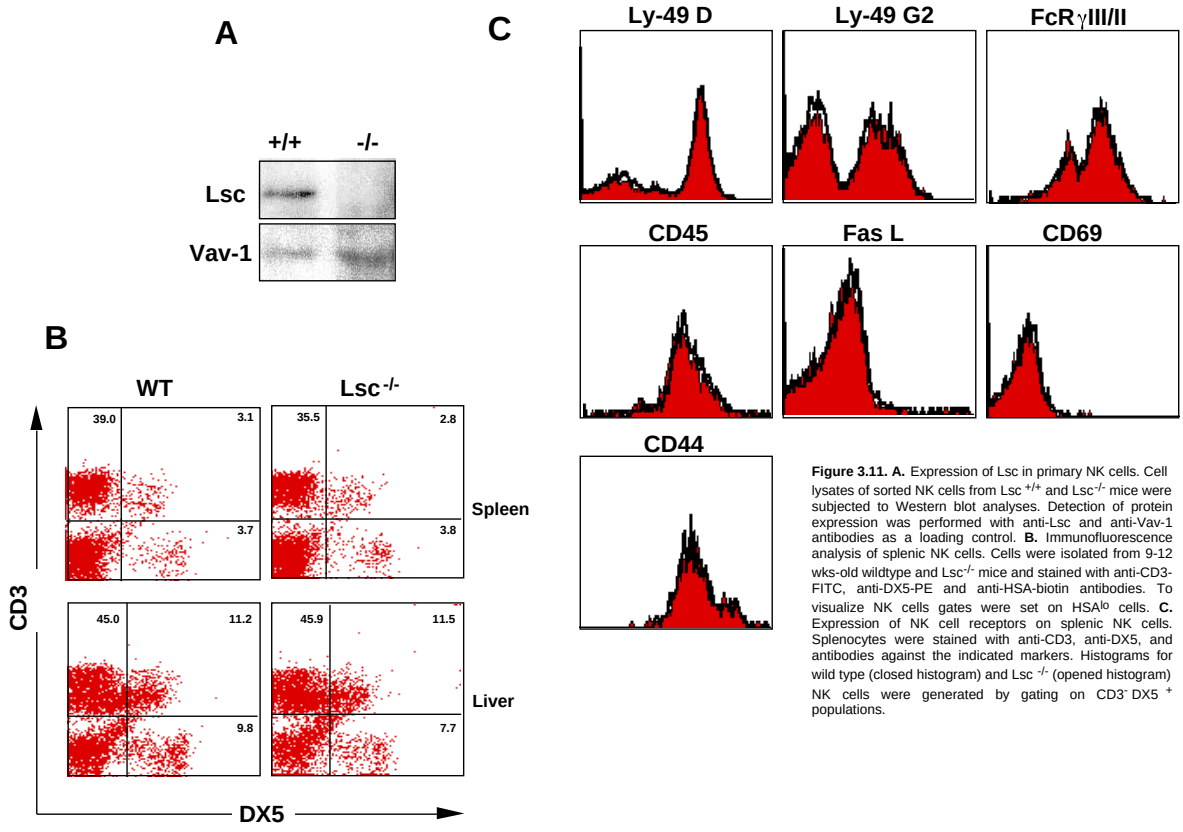


Figure 3.11. A. Expression of Lsc in primary NK cells. Cell lysates of sorted NK cells from Lsc $^{+/+}$ and Lsc$^{-/-}$ mice were subjected to Western blot analyses. Detection of protein expression was performed with anti-Lsc and anti-Vav-1 antibodies as a loading control. **B.** Immunofluorescence analysis of splenic NK cells. Cells were isolated from 9-12 wks-old wildtype and Lsc$^{-/-}$ mice and stained with anti-CD3-FITC, anti-DX5-PE and anti-HSA-biotin antibodies. To visualize NK cells gates were set on HSA^{lo} cells. **C.** Expression of NK cell receptors on splenic NK cells. Splenocytes were stained with anti-CD3, anti-DX5, and antibodies against the indicated markers. Histograms for wild type (closed histogram) and Lsc $^{-/-}$ (opened histogram) NK cells were generated by gating on CD3^{-} DX5 $^{+}$ populations.

To assess NK cell function, I performed cytotoxic assay using T cell depleted splenocytes from wild type and mutant mice against a panel of tumor targets. Wild type cells showed low level of cytotoxic responses towards YAC1 cells and almost no response against other targets (Figure 3.12). However, $Lsc^{-/-}$ splenocytes elicited a greater cytotoxic activity against YAC1, C4.4.25 and RMA/S compare to wild type cells (Figure 3.12). Both wild type and mutant effectors showed no tumorcidal responses against EL4. This is likely due to a rich presence of MHC class I on the surface of these cells. $Lsc^{+/-}$ and wildtype NK cells exhibited indistinguishable cytotoxic activity against all target cells (data not shown). Effectors used in the above experiments were T cell depleted splenocytes containing NK cells, B cells and monocytes. In order to rule out possible influence of contaminating cells, cytotoxic assays were performed with highly purified NK cells as effectors. To this end, $CD3^{-}DX5^{+}$ cells were FACS sorted from both wild type and mutant spleens, yielding NK cells with purity around 96-98%. These were used to kill YAC1 tumors. Consistent with results obtained from T cell depleted splenocytes, $Lsc^{-/-}$ NK cells showed higher tumorcidal activities compare to wild types cells (Figure 3.13). Taken together, these data indicate Lsc is a negative regulator of NK cell cytotoxic activity.

NK mediated cytotoxicity against YAC1 has been shown to be potentiated by the release of TNFα from NK cells (Baxevanis et al., 2000). To determine whether enhancement of cytotoxicity in mutant NK cells due to a concomitant increase in TNFα production, YAC-1 cells were co-incubated with T cell-depleted splenocytes from wild type and $Lsc^{-/-}$ mice. The

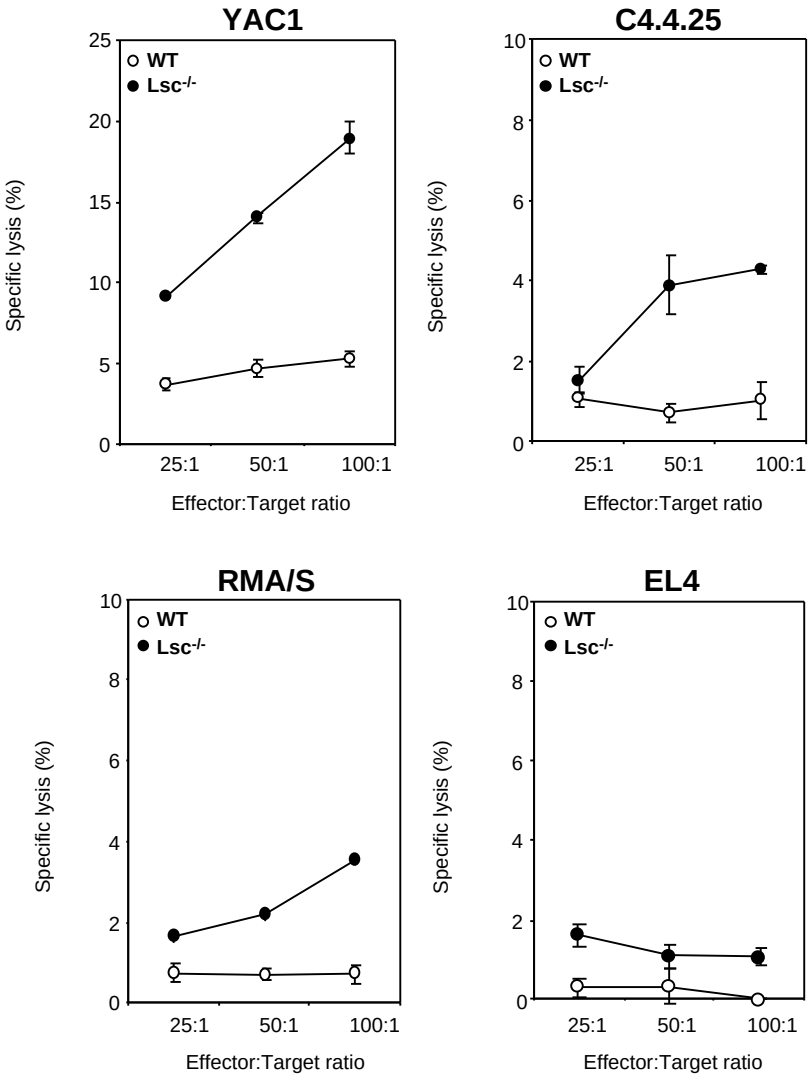


Figure 3.12 . Enhanced natural cytotoxicity of freshly isolated Lsc-1$^{-/-}$ NK cells. Freshly isolated splenocytes depleted of CD4^{+} and CD8^{+} were used as effectors against YAC1, C4.4.25, RMA/S and EL4 cells in a standard chromium release assay. Percentage of specific lysis was determined by the amount of chromium release at 4 hours. Results are representative of three experiments.

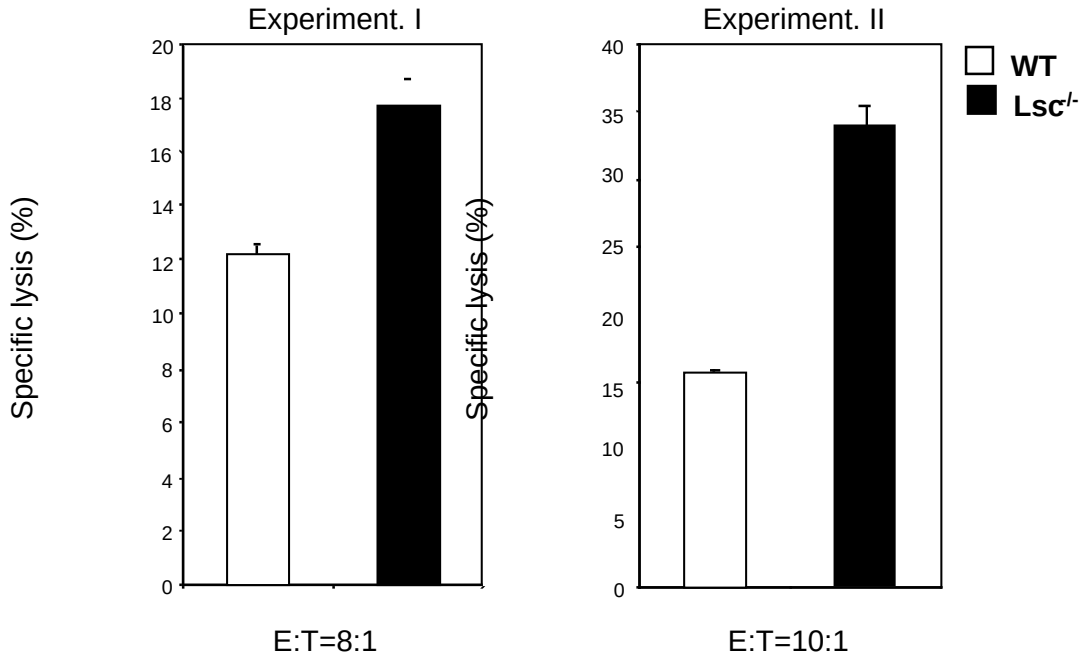


Figure 3.13. Enhanced natural cytotoxicity of freshly isolated Lsc-1$^{-/-}$ NK cells. Freshly purified splenocytes depleted of CD4^{+}, CD8^{+} and B220^{+} cells befored they were sorted for CD3^{-}DX5^{+} NK cells. These were used as effectors against YAC1 cells in a standard chromium release assay. Percentage of specific lysis was determined by the amount of chromium release after 4 hours.

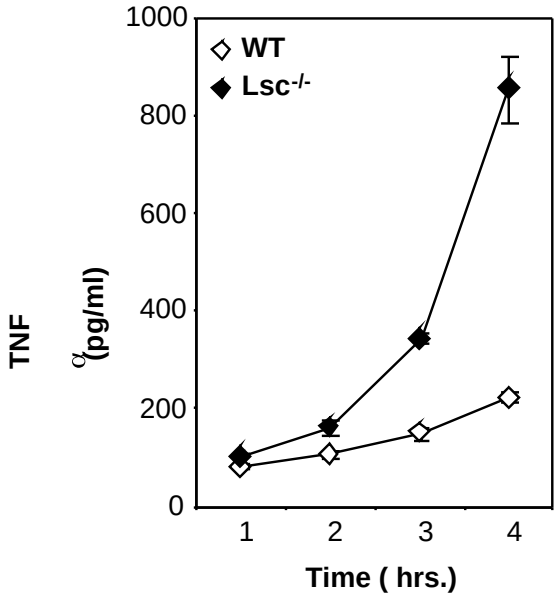


Figure 3.14. YAC1-induced TNF α production in wild type and Lsc $^{-/-}$ splenocytes. CD4 $^{+}$ and CD8 $^{+}$ depleted splenocytes were incubated with YAC1 tumors at a E:T ratio of 100:1 for the indicated time points. TNF α released in the supernatant were determined by ELISA.

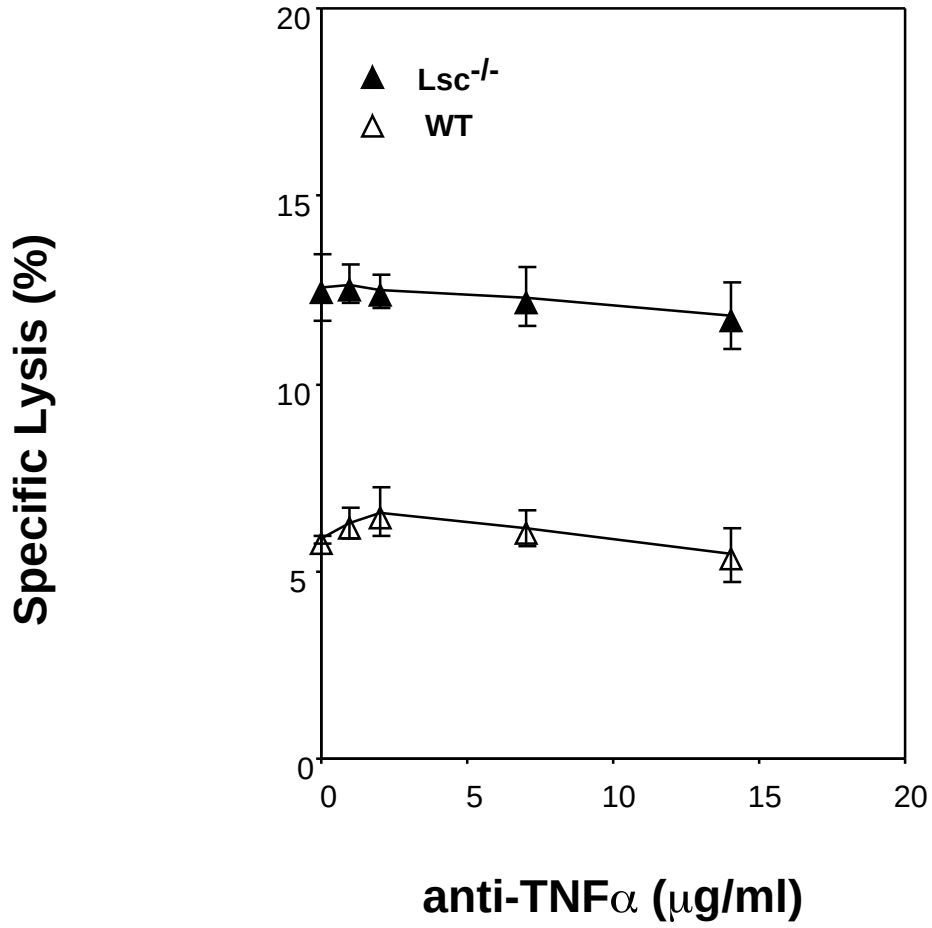


Figure 3.15. Neutralizing TNFα does not block cytotoxic responses against YAC-1 tumors. $CD4^+$ and $CD8^+$ depleted splenocytes were incubated with YAC1 tumor cells at a E:T ratio of 100:1 for the indicated time point with anti-TNFα antibodies. Cytotoxic responses were measured after 4 hours.

supernatants were harvested after 1, 2, 3, and 4 hours and the concentrations of TNFα were determined by ELISA. As shown in Figure 3.14, mutant splenocytes displayed a marked increase in TNFα secretion compared to wild type NK cells. To determine whether elevated TNFα secretion causes enhance tumorcidal activities in mutant cells, cytotoxic assays were carried out in the presence of neutralize anti-TNFα antibodies (Figure 3.15). I found that anti-TNFα antibodies had no effects on the ability of wild type and mutant NK cells in killing tumor targets. Furthermore, mutant cells consistently exhibited higher cytotoxic responses, even in the presence of 15-20 μg/ml of anti-TNFα antibodies. These data argue that the mechanism of hyper-responsive mutant NK cells is not due to the enhance secretion of TNFα and NK cytotoxicity is likely independent of TNFα secretion. Indeed, preliminary evidence derived from intracellular FACS analysis indicated that TNFα was produced by $CD3^{-}B220^{-}DX5^{-}Mac1^{+}$ cells, suggesting NK cells do not release of TNFα in response to YAC1 stimulation (data not shown). Future experiments will determine which mutant populations produce TNFα, and whether this burst of TNFα is a secondary response to hyper-active mutant NK cells or that target cells directly stimulate $Mac1^{+}$ cells to produce TNFα.

4. Discussion

4.1 Vav-1 regulates the development of NKT cells and the activation of NK cells

This study shows that Vav-1$^{-/-}$ mice exhibit a marked reduction in NKT cells, highlighting Vav-1 as an essential regulator of both T cell and NKT cell development. αβ cells have a polyclonal TCR repertoire, whereas NK T cells are biased for CD1d restricted TCR usage (Godfrey et al., 2000). Thus, the requirement for Vav-1 in selection events maybe similar between the two lineages. Indeed, some key signaling molecules that act downstream of TCR/CD3 are noted to play critical roles in development of NK T cells. Mice carrying mutations in the genes of src-kinases *lck* and *fyn* also exhibit compromised NKT cell development (Gadue et al., 1999). In T cells, Vav-1 is implicated as a downstream effector molecule of Lck and/or Fyn (Han et al., 1997; Huang et al., 2000). Therefore Vav-1, as a GTP-exchange factor for Rho-GTPases, may form part in a signaling cascade linking Src-kinase activity to the activation of Rho-GTPases in NKT cells. As T cells were also reduced in Vav-1$^{-/-}$ mice, further experiments are required to determine whether the defect in NKT cell ontogeny in Vav-1$^{-/-}$ mice is cell-intrinsic or partially due to secondary effect of accessory cells in the microenvironment. My observations are in line with those made by another study which also demonstrated a gross reduction of NKT cells in lymphoid organs of Vav-1$^{-/-}$ mice (Colucci et al., 2001).

Splenic NKT cells produce a large amount of IL-4 and IFNγ in response to *in vivo* CD3 stimulation. This response was largely ablated in Vav-1$^{-/-}$ mice, which exhibit a 10- to 12-fold reduction in IL-4 and IFNγ production. Since the absolute number of Vav-1$^{-/-}$ NK T cell was reduced 3- to 4-fold, the defect in IL-4 and IFNγ production cannot be only due to diminished numbers of NK T cells. Thus, my results highlight a possible signaling defect in peripheral NKT cells in Vav-1$^{-/-}$ mice and support the notion that Vav-1 acts downstream of CD3 in NKT cells.

The loss of CD3 mediated IL-4 and IFNγ production was also observed in NKT cell deficient CD1d$^{-/-}$ mice, consistent with the model that CD1d restricted NKT cells are involved in producing these cytokines in response to CD3 challenge *in vivo* (Mendiratta et al., 1997). It is interesting that wild type splenocytes produce 10 fold more IFNγ production than IL-4 in response to *in vivo* CD3 administration (Figure 3.2 and (Mendiratta et al., 1997)). In contrast, *in vitro* CD3 stimulation of purified thymic and splenic NKT cells produce large amount of IL-4 but only moderate amount of IFNγ (Hammond et al., 1999). Differences between the two models are likely due to the presence of by-standing cells that can secrete IFNγ in splenocytes. Indeed, Carnuad et al (1999) showed *in vivo* challenge of mice with αGalCer, a specific activator of NKT cells, leads to IFNγ production. This triggers further secretion of IFNγ by NK cells. Since NK cells are capable of producing large amount of IFNγ, a loss of CD3-induced IFNγ production in Vav-1$^{-/-}$ mice could be attributed to a NK cell defect. This is seem unlikely, since it was shown that Vav-1$^{-/-}$ NK cells produce normal levels of IFNγ in

response to anti-2B4, anti-NK1.1, anti-CD16 and IL-12 stimulation (Colucci et al., 2001). Taken together, the loss of NKT cells in Vav-1$^{-/-}$ mice leads to a gross reduction of IFNγ and IL-4 upon CD3 challenge, which could impinge on Th_1 and Th_2 differentiation in these mutant mice. Hence, it might be interesting in the future to graft wild type and Vav-1$^{-/-}$ NKT cells in CD1$^{-/-}$ mice and examine Th_1 and Th_2 responses to antigen challenge *in vivo*.

Despite the importance of Vav-1 in the development of T cells and NKT cells, NK cell development was normal in Vav-1$^{-/-}$ mice (Figure 1 A , B, C and D). In fact, both Vav-1 and Vav-2 were dispensable for maturation of NK cells (Figure 3.7). However, Colucci et. al. (2001) showed an increase in NK cells in the spleen and the bone marrow of Vav-1$^{-/-}$ mice (Colucci et al., 2001). It is difficult to reconcile these discrepancies. One possible explanation might be that Colucci and co-workers used mice of B10.BR background, whereas I used mice of C57BL/6 background.

Vav-1 has been shown to be important in eliciting CTL responses, since Vav-1$^{-/-}$ mice have reduced LCMV-induced footpad swelling reactions, a process mediated by CD8^{+} CTLs, and are unable to clear viruses efficiently 8 days after infection (Penninger et al., 1999). In order to examine Vav-1$^{-/-}$ NK cytotoxic responses, but at the same time avoid the influences contributed by the presence of defective CD8^{+} CTL and NKT cells in Vav-1$^{-/-}$ splenic culture, pure LAK cells were generated from both wild type and mutant mice. Cytotoxic assay using pure LAK effectors indicated that Vav-1$^{-/-}$ NK cells exhibited reduced efficiency in killing EL4 and RMA target cells (Figure 3.6). I also observed a reduction of cytotoxic activity against

C4.4.25 and RMA/S, MHC class I-deficient variants of EL4 and RMA cells, respectively. Furthermore, using freshly isolated Vav-1$^{-/-}$ NK cells, I found a marked reduction in cytotoxicity against YAC1 cells. My data demonstrate that Vav-1 is essential for NK spontaneous cytotoxicity and that Vav-1 may act in an activation process that is independent of MHC class I. In contrast to freshly purified NK cells, Vav-1$^{-/-}$ LAK cells exhibit normal cytotoxic activities against YAC1 cells. One possible explanation for this altered degree of tumorcidal activity could be the upregulation of death receptors such as FasL and TRAIL in LAK cells (Kayagaki et al., 1999). A change in the expression pattern of receptors could overcome the requirement for Vav-1 leading to efficient lysis of YAC1 cells. Alternatively, subsets of Vav-1 dependent activating receptors involved in the lysis of YAC1 cells down-regulated in LAK cells. Together, my data are consistent with another study on Vav-1$^{-/-}$ LAK cells which also showed abated spontaneous cytotoxicity against YAC-1, RMA/s and β2m$^{-/-}$ Con A blasts. Furthermore, NK cells activated by injection of poly(I:C) *in vivo* also displayed reduced spontaneous cytotoxicity aginst YAC-1 and IC-21 (Colucci et al., 2001).

Spontaneous cytotoxicity of NK cells can be enhanced by ligation of CD16 (or FcγRIII). This process, known as antibody directed cell mediated cytotoxicity (ADCC), remained intact in the absence of Vav-1 (Figure 3.4 A). In fact CD16-mediated ERK, JNK, and p38 activation in Vav-1$^{-/-}$ LAK were also normal (Figure 3.4 B). These findings are inconsistent with the previous notion that Vav-1 is a downstream effector of CD16 and a mediator of ADCC in human NK cells (Billadeau et al., 1998; Galandrini et al., 1999). One explanation could be that other Vav proteins such as Vav-2 and Vav-3 can compensate for Vav-1 function in Vav-

$1^{-/-}$ NK cells. The expression of Vav-2 in NK cells and its reported participation in human NK ADCC further illustrated Vav-2 as potential candidate capable of taking over Vav-1 function (Billadeau et al., 2000). This possibility is unlikely in the murine model since Vav-$2^{-/-}$ NK cells mediate normal ADCC (Figure 3.8). In fact, ADCC and CD16-induced MAPK activation are also normal in Vav-$1^{-/-}$Vav-$2^{-/-}$ NK cells (Figure 3.8 A and B). These data indicate that neither Vav-1 nor Vav-2 act downstream of CD16. Alternatively, Vav-3 could overtake the functions Vav-1 and Vav-2 in their absence. However, it is not known whether Vav-3 is expressed in NK cells. Interestingly, another study demonstrated reduced ADCC activity mediated by Vav-$1^{-/-}$ NK effectors (Colucci et al., 2001). However, the target cells used by Colucci and co-worker were EL4 cells and the mice were of different genetic background. The combination of these factors could explain some of the discrepancies.

Vav-1 catalyse the activation of Rho GTPases, such as Rac1, Cdc42, and RhoA (Bustelo, 2000). Given that both Vav-1 and Rac1 are implicated in NK cytotoxicity (Billadeau et al., 1998; Galandrini et al., 1999), it is likely that Vav-$1^{-/-}$ NK cell defect results from a failure to activate Rac1. Interestingly, Rac2, a member of the Rac subfamily is expressed strictly in hematopoietic lineage (Reibel et al., 1991). Furthermore, $Rac2^{-/-}$ and Vav-$1^{-/-}$ mice share many striking similarities. T cells from both mice show reduced TCR-mediated proliferation, ERK activation, and Ca^{2+} mobilization (Yu et al., 2001). TCR-induced actin polymerization and TCR capping are also reduced in both mutant T cells (Yu et al., 2001). Although the functions of Rac family members have not been examined, it is likely that Rac1 and Rac2 are downstream targets of Vav-1 in NK cells.

Vav-1 functions not only as a GEF for Rho GTPases, but can also serve as an adaptor in recruiting many molecules to the signaling complexes downstream of activated receptors. Interestingly, some of these Vav-1 binding proteins can also mediate NK cytotoxicity. For example, Syk has been shown to bind to Vav-1 (Deckert et al., 1996), and it is known to be recruited to DAP12 upon NK activation (Lanier and Bakker, 2000). However, in $Syk^{-/-}$ mice, NK natural cytotoxicity is normal, while CD16-mediated ADCC and IFNγ production are reduced (Colucci et al., 1999). This is different from $Vav\text{-}1^{-/-}$ NK cell which show a decrease in spontaneous cytotoxicity but normal ADCC and CD16-mediated IFNγ production (Figure 2.4, 2.5, 2.6 and data not shown). These results suggest that Vav-1 and Syk are involved in distinct signaling pathways in NK cells.

ZAP-70, another Syk family kinase, can also bind to Vav-1 (Katzav et al., 1994). $ZAP\text{-}70^{-/-}$ NK cells exhibit normal cytotoxicity (Iwabuchi et al., 2001), suggesting that either ZAP-70 is not essential for NK cell cytotoxicity, or that the Syk can compensate for the loss of ZAP-70 function in $ZAP\text{-}70^{-/-}$ NK cells. However, a recent report showed that $Syk^{-/-}ZAP\text{-}70^{-/-}$ NK cells are still capable of killing a panel of tumors cells (Colucci et al., 2002), thus raising a possible involvement of an unidentified kinase in compensating the loss of both Syk and ZAP-70.

SLP-76 is another adaptor molecule that can associate with Vav-1 in T cells (Onodera et al., 1996; Wu et al., 1996). However, NK cells derived from $SLP\text{-}76^{-/-}$ mice show normal cytokine

secretion and cytotoxicity, thus ruling out a functional coupling of Vav-1 and SLP-76 in NK cells (Peterson et al., 1999).

Biochemical evidence showed that the Src family kinase Lck can phosphorylate Y174 in Vav-1 (Crespo et al., 1997; Han et al., 1997), thus relieving autoinhibition by exposing the DH domain, leading to an enhanced GEF activity towards Rho GTPases (Aghazadeh et al., 2000). Such observations would predict Src family kinases lying upstream of Vav-1. However, in $Lck^{-/-}$, $Fyn^{-/-}$ and $Lck^{-/-}Fyn^{-/-}$ mice, NK cytotoxicity is normal (van Oers et al., 1996), suggesting these kinases are not involved in regulating Vav-1 activation in NK cells. It is known that another Src family kinase, Hck, is expressed in human NK cells (Biondi et al., 1991), raising the possibility that it is involved in Vav-1 phosphorylation and activation.

One of the earliest events of NK cell activation is the release of perforin and serine proteases such as granzyme B (Henkart et al., 1984; Young et al., 1986). This event is dependent on the activation of ERK (Trotta et al., 1998; Wei et al., 1998). Interestingly, Vav-$1^{-/-}$ T cells are unable to activate ERK in response to TCR stimulation (my unpublished observation) (Costello et al., 1999). It is therefore likely that a reduced cytotoxic activity in Vav-$1^{-/-}$ NK cells is due the loss of Vav-1 mediated ERK activation, leading to the reduction of cytotoxic granule release. Consistent with this model, Colucci et al (2001) showed a marked reduction of granyzme release in Vav-$1^{-/-}$ NK cells upon stimulation with YAC-1 cells (Colucci et al., 2001). I have shown that CD16 mediated ERK activation and cytotoxicity are normal in the absence of Vav-1 (Figure 3.4), indicating that in NK cells, ERK could be activated by both

Vav-1 dependent and independent pathways. CD16 can mediate ERK activation independent of Vav-1, leading to granzyme release and tumor cell lysis.

Since the presence of Vav-1 is crucial in NK spontaneous cytotoxicity, it might mediate signals of stimulatory NK receptor(s). Although several receptors have been implicated in NK natural cytotoxicity, most of these studies were done with human cell lines and it is not known whether all these receptors are conserved in the murine model (Moretta et al., 2001). Vav-1 was shown to be a downstream target of Ig family receptors such as TCR, BCR, CD19 and FcεRI. Homologous to these are the human NK receptors NKp44, NKp46 and NKp30 belonging to the Ig superfamily. They also associate with co-receptors bearing ITAM motifs, and were shown to be key stimulatory receptors for NK cell activation (Moretta et al., 2001). It is therefore likely that Vav-1 can act downstream of NKp44, NKp46 and/or NKp30. To date, only the NKp46 mouse analogue has been identified but the intracellular signals mediated by NKp46 remain unknown (Biassoni et al., 1999).

Upon engagement of NK cells and target cells, membrane lipid rafts become polarized to the site of cell-cell contact, a process partially dependent on the activation of both Src and Syk family kinases (Lou et al., 2000). The polarization of lipid rafts is sensitive to signals mediated by inhibitory receptors ligation and SHP-1 activation (Fassett et al., 2001; Lou et al., 2000). Vav-1 is required for TCR mediated lipid raft clustering since in Vav-1$^{-/-}$ T cells raft aggregation is severely reduced in response to CD3-CD28 stimulation (Krawczyk et al., 2000). Furthermore, when mutant T cells are stimulated with peptide-loaded antigen

presenting cells (APCs), lipid rafts did not translocate to the immunological synapse (Villalba et al., 2001). Similarly, introducing dominant negative mutant of Vav-1 to Jurkat cells lead to a failure of raft polarization at the site of cell-cell conjugation (Villalba et al., 2001). These observations suggest that Vav-1 also mediates raft aggregation at the NK cell-target cell synapse. A possible scenario could involve ligation of stimulatory NK cell receptors leading to Vav-1 and Rho GTPases activation which results in polarization of rafts at the site of cell-cell contact. This lead to the displacement of NK inhibitory receptors from the signaling complexes at the lipid raft scaffolds (Fassett et al., 2001; Lou et al., 2000). The removal of inhibitory receptors allows sustained stimulatory signaling in NK cells, leading to the activation of ERK, which mediates cytotoxic granule release resulting in the lysis of target cells (Henkart et al., 1984; Young et al., 1986) (Figure 4.1). Future experiments are necessary to define a more precise mechanism of raft polarization and how Vav-1 might take part in this process.

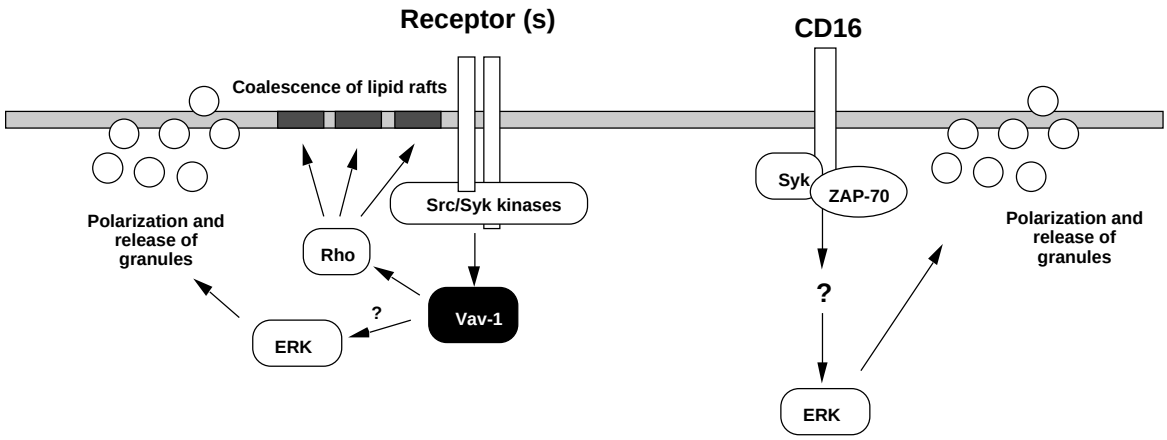


Figure 4.1. A possible scenario Vav-1 in NK activation could involve ligation of stimulatory NK receptors leading to Vav-1 activation. Vav-1 catalyzes Rho GTPases activation results in polarization of rafts at site of cell-cell contact, and the displacement of inhibitors such as Ly49 family members. This leads to a sustained activation signal in NK cells and the activation of ERK and cytotoxic granule release which results in the lysis of target cells

4.2 Lsc is a negative regulator of NK cell cytotoxicity

Experiments on Vav-1$^{-/-}$ NK cells highlight the importance of GEFs in NK cell activation. Since many Rho GTPase-mediated processes are normal in Vav-1 deficient NK cells, it is likely that other GEFs are also involved in NK cell activation. Lsc is another Rho GEF that is expressed specifically in the hematopoietic system including LAK cells and freshly purified CD3^{-}DX5^{+} NK cells (Figure 3.11A (Girkontaite et al., 2001)). Data presented in this manuscript show that Lsc is not required for NK cell development (Figure 3.11). However, NK cells derived from Lsc mutant mice show enhanced cytotoxicity responses against three different tumor targets (Figure 3.12 and 3.13). This may be due to a loss of inhibitory signals in mutant cells. Lsc acts downstream Gα12 and Gα13 coupled receptors (Kozasa et al., 1998) (Mao et al., 1998b) (Majumdar et al., 1999) (Hart et al., 1998). Although Gα12 is express in LAK cells, no function has been ascribed (Al-Aoukaty et al., 1997). There is also no evidence that G-protein coupled receptors are involved in NK cell cytotoxicity. Indeed, all major NK cell stimulatory and inhibitory receptors belong to either the lectin-like family or Ig superfamily (Moretta et al., 2001).

The GTPase Rho is known to promote NK cell cytotoxicity (Lou et al., 2001). As Lsc is a known activator of RhoA (Hart et al., 1998; Hart et al., 1996), one would have predicted that NK cell cytotoxic responses are abated in the absence of Lsc. This is contrast to the enhanced cytotoxic activity of Lsc$^{-/-}$ NK cells. Since expression of cell surface markers on both wild type and mutant cells are nearly identical (Figure 3.11), they suggest that Lsc

mutant NK cells are not pre-activated *in vivo*, but they elicit aberrant activation signals upon engagement with tumor targets. It is possible to envisage that negative regulation of Gα12/Gα13 is lost in the absence of the RGS domain of Lsc, leading to the hyper-activation of these G-proteins. This in turn could result in a constitutive activation of downstream signaling cascades, and ultimately hyper-reactive NK cells.

Lsc traffics signals derived from Gα12 and Gα13 coupled receptors (Kozasa et al., 1998). Interestingly, a lymphoid-expressed G-protein coupled receptor (GPCR) G2A has been identified as functionally associates with Gα13 (Kabarowski et al., 2000) and regulates lymphocyte homeostasis (Le et al., 2001). This raises the possibility that Lsc acts downstream of G2A. Genetic evidences seem to support this notion, since $G2A^{-/-}$ and $Lsc^{-/-}$ mice have similar phenotypes such as an enlargement of lymph nodes and spleen as the mice age, and hyperactive T cells ((Le et al., 2001) and Girkontaite, I., unpublished data). The identification of receptors upstream of Lsc warrants further investigation.

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During the course of this study, I observed that co-incubation of $Lsc^{-/-}$ T cell depleted splenocytes together with YAC-1 cells led to an increase in TNFα secretion by mutant cells (Figure 3.14). This enhanced production of TNFα was not responsible for the increase in cytotoxicity as neutraling anti-TNFα antibodies did not dampen tumorcidal activities of both wild type and Lsc mutant cells (Figure 3.15). Preliminary evidence indicated that the population secreting TNFα is $DX5^{-}CD3^{-}B220^{-}Mac1^{+}$ (data not shown), suggesting these cells

are not T cells, B cells and NK cells. The expression of Mac1 on these cells suggests that they are monocytes, macrophages, or dendritic cells. Recently, it was observed that dendritic cells (DCs) can produce TNFα in the presence of activated NK cells (Ferlazzo et al., 2002; Gerosa et al., 2002; Piccioli et al., 2002). As $Lsc^{-/-}$ NK cells were hyperactive, they might trigger neighboring DCs to produce TNFα. To determine whether secretion of TNFα is dependent on the presence of activated NK cells, future experiments will involve depleting $DX5^{+}$ NK cells from splenocytes and examine whether the burst of TNFα is concomitantly ablated.

In summary, the data presented in this thesis provided the first genetic evidence that Rho GEFs are critical regulators of NK cell activation and function. Importantly, Rho GEFs participate in both stimulatory and inhibitory functions as exemplified by Vav-1 and Lsc respectively. These data also show for the first time a RGS domain containing GEF in down-modulating NK cytotoxic responses. Significantly, these observations highlight the possibility that in addition to known cell surface receptors such as the lectin and Ig family members, GPCRs are also involved in regulating NK activation.

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Publications and Abstracts

Publications:

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