THE ROLE OF CASP IN NATURAL KILLER CELL IMMUNE FUNCTION

by

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Dedication Page

This thesis is dedicated to my loving mother and father Helen and Robert Cormier.

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Abstract

Natural Killer (NK) cells are highly mobile, specialized sub-populations of lymphocytic cells that survey their host to identify and eliminate infected or tumor cells. They are one of the key players in innate immunity and do not need prior activation through antigen recognition to deliver cytotoxic packages and release messenger chemicals to recruit immune cells. Cytohesin associated scaffolding protein (CASP) is a lymphocyte specific adaptor protein that forms complexes with vesicles and sorting proteins including SNX27 and Cytohesin-1. In this study I show that by using stably integrated shRNA, CASP has a direct role in the secretion of messenger cytokines including IFN-γ, and impedes NK cell motility and ability to kill tumor cells. I also show that CASP is post-translationally modified by ubiquitination and cleavage by granzyme B. CASP is an essential and multi-faceted protein, which has a very diverse role in NK cell specific immune functions.

List of Abbreviations Used

ADP	adenosine diphosphate
APC	antigen presenting cell
ARF	ADP-ribosylation factor
ARNO	ARF nucleotide-binding site opener
BSA	bovine serum albumin
bp	base pair
CASP	cytohesin-associated scaffolding protein
CC	coiled-coiled motif
ccRCC	clear-cell renal carcinoma cell
CD	cluster of differentiation
cDNA	complementary DNA
CTL	cytotoxic T lymphocyte
CXCR	cxc chemokine receptor
Cybr	cytohesin binder and regulator
CYTIP	cytohesin interacting protein
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
EE	early endosome
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
GDP	guanosine diphosphate
GEF	guanidine nucleotide exchange factor
GRASP	GRP1-associated scaffolding protein
GrB	Granzyme B
GRP1	general receptor for phosphoinositides
GTP	guanosine triphosphate
GTPase	GTP phosphatase
HRP	horseradish peroxidase
IP	Immunoprecipitation
kb	kilobases
kDa	kilodalton
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IL	interleukin
IS	immunological synapse
LAMP	lysosomal-associated membrane protein
LB	lysogeny broth
LFA-1	lymphocyte function-associated antigen 1
LSM	laser scanning microscope
MTOC	microtubule organizing center
mRNA	messenger RNA
MHC	major histocompatibility complex

miRNA	micro RNA
NCBI	national center for biotechnology information
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PDZ	PSD-95/Dlg/ ZO-1
PDZbm	PDZ binding motif
PEI	polyethyleneimine
PH	pleckstrin homology domain
PI	phosphphatidylinositol
PMA	phorbol 12-myristate 13-acetate
PSCDBP	pleckstrin homology Sec7 and coiled coil domain-binding protein
PVDF	polyvinylidine fluoride
PX	phox homology domain
RE	recycling endosome
RNAi	RNA interference
RPMI	Roswell Park Memorial institute
RT-PCR	real-time polymerase chain reaction
SDF-1	stromal-cell derived factor
SDS	sodium dodecyl sulfate
SE	sorting endosome
Sec7	domain with homology to yeast Sec7 protein
shRNA	short hairpin RNA
siRNA	short interfering RNA
SNX	sorting nexin
SOCS-1	suppressor of cytokine signaling 1
TBS	tris-buffered saline
Th	T helper cell
TNF	tumor necrosis factor

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Chapter 1 Introduction

Natural killer (NK) cells are a sub-population of white blood cells of the immune system known as lymphocytes. They are lymphocytes on the basis of their morphology, their expression of many lymphoid markers, and their origin from the common lymphoid progenitor cell in the bone marrow. They are close relatives of the key players of adaptive immunity, T cells, B cells and dendritic cells. Adaptive immunity is a slower, more robust and highly specialized targeting of infection. NK cells, however, are generally considered to be components of innate immune defense because they lack antigen-specific cell surface receptors and can act almost immediately. NK cells have been shown in humans and mice to participate in the early control against virus infection, especially herpesvirus (Biron et al, 1999), and in tumor immunosurveillance. They are highly mobile, and must traffic the various tissues and microenvironments present in an individual in order to be effective in prevention of disease. NK cells are named 'natural' since they do not require previous activation by chemokine messengers and previous exposure to antigen to illicit a kill response, however, they do possess regulatory capabilities mediated by the secretion of cytokines. NK cells display regulatory capabilities mediated by various cytokines. These cytokines are released upon engagement of different triggering NK receptors or upon signaling by other cytokines. This is particularly relevant during the early phases of immune responses.

In order to engage tumours or virally infected cells, NK cells must be able to migrate. The process of migrating NK cells is a highly structured and fluid process. The organization and distribution of proteins, lipids, and organelles within an individual cell

are in a constant state of flux as the cell travels throughout an organism and encounter other cells with which they interact and communicate. Migration requires a specific orientation of intercellular machinery which is called polarization (Sa & Angel, 1999). Polarization allows for the asymmetrical distribution of proteins and organelles within the cell and allows for directionality, so that the cell may move within the microenvironment in a defined direction. This occurs with the formation of a lamellipodium at the leading edge and a uropod at the trailing edge (Sa & Angel, 1999). This cell shape allows them to convert cytoskeletal forces into net cell-body displacement. Polarization and migration is stimulated in NK cells through chemoattractants, such as chemokines. Chemoattractant receptors, integrins and other adhesion molecules, cytoskeletal proteins and intracellular regulatory molecules change their cellular localization during cell polarization to restructure and move the cell toward the stimulus (Angus & Griffiths, 2013; Smyth et al., 2005).

NK cells are critical for immune responses against virus infections and cell transformation. Cytotoxic function occurs when NK cells recognize target cells through target cell down-regulation of self-antigen presenting receptors. Natural Killer cells then release granules containing perforin and granzyme that causes apoptosis of the target cell (Krzewski & Coligan, 2012). After binding to a target cell, secretory lysosomes relocate along microtubules to the microtubule-organizing center that polarizes towards the target cell. The centrosome and associated secretory lysosomes relocate together to the site of contact between the target cell and the NK cell, known as the Immunological Synapse (IS) (Trapani & Smyth, 2002). The IS is made up of a large complex where cell adhesion molecules make up the periphery, while signaling proteins are in the center. It is here that

the secretory lysosomes dock and fuse, delivering their cytotoxic contents into the target cell, inducing signaling cascades that activate apoptosis. NK cells are also a critical bridge between innate and adaptive immunity through their release and secretion of chemokines and cytokines that recruit other lymphocytes to the site of infection, as well as self-stimulate to cause further proliferation (Alter, Malenfant, & Altfeld, 2004). NK cells are the primary cell that secretes IFN-γ, an important activator of macrophages and other lymphocytes, its role in inhibition of viral replication (Schoenborn & Wilson, 2007).

Cytohesin associated scaffolding protein (CASP a.k.a CYBR, CYTIP, PSCDBP, B3-1) is a lymphocyte specific adaptor protein that was first identified and cloned by the Pohajdak laboratory in the early 1990's from human natural killer cells, and named about a decade later (Mansour, Lee, & Pohajdak, 2002a). CASP is involved in the recruitment of protein complexes involved in intracellular trafficking and signaling. CASP is characterized by its lack of catalytic domains, and presence of three protein-protein interaction domains. These domains are essential for the assembly of larger protein complexes. The 40 kDa protein has a N-terminal PDZ domain, a coiled-coil motif (leucine zipper) and a C-terminal PDZ binding motif (PDZbm) (Fig 1.1).

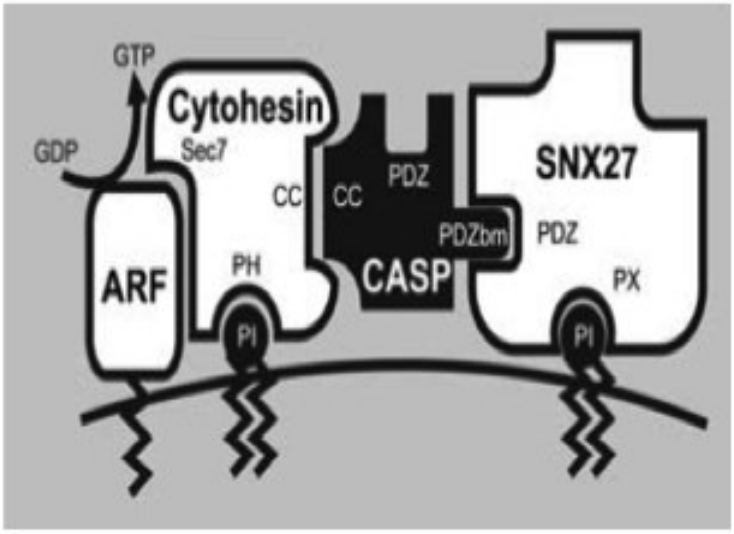

Figure 1.1 Model depicting the binding partners and involvement of CASP as an adaptor protein in vesicle initiation and endosome transport. SNX27 binds the PDZ binding motif, while the coiled-coiled domain interacts with cytohesin family proteins. The PDZ domain still has no known binding partner. Cytohesin-1 contains a Sec7 domain that binds a guanine nucleotide exchange factor, as well as a pleckstrin homology (PH) domain that is a lipid binding domain. SNX27 contains a phox homology (PX) domain that also is involved in lipid binding. Adapted from MacNeil & Pohajdak, 2009.

There is a homologue of CASP known as GRP1-associated scaffolding protein (GRASP or Tamalin). GRASP has the same domain and motif distribution as CASP. It contains a N-terminal PDZ domain, a central coiled-coil motif and a C-terminal PDZbm. GRASP is a neuronal specific adaptor protein that comes from the same family as CASP (Macneil & Mceachern, 2008). GRASP is primarily expressed in the brain but has been shown to be expressed in low-levels in lymphocytes (Coppola et al., 2006a). In the brain, GRASP acts in plasma membrane receptor signaling and recycling predominantly in neurons (Venkataraman et al, 2012). Due to the differences of the PDZ domains and the

overall size of both CASP and GRASP, it is not suspected that GRASP is interchangeable with CASP function in lymphocytes (Macneil & Mceachern, 2008).

CASP has been found to directly interact with two proteins (Fig 1.1). Most of what is known about CASP's molecular function is attributed to its first binding partner Cytohesin-1. CASP interacts with cytohesin-1 through the coiled-coil motifs of both proteins. Cytohesin-1 is an ADP ribosylation factor (ARF) guanidine exchange factor (GEF) that was first cloned and characterized in the Pohajdak laboratory. Cytohesin activates GEFs which insert and recruit protein to membranes in the cell. CASP is also able to bind through this motif to other members of the cytohesin family (ARNO/cytohesin-2 and ARNO3/ GRP1/cytohesin-3) (Mansour, Lee, & Pohajdak, 2002).

The PDZbm of CASP interacts with Sorting Nexin 27 (SNX27) a molecule that mediates PDZ-directed sorting from endosomes to the plasma membrane (Lauffer et al., 2010). This interaction was recently discovered by Dr. Adam MacNeil (MacNeil, Mansour, & Pohajdak, 2007) (Fig 1.1) also from our laboratory. Of particular interest to the focus of my study is the fact that SNX27 polarizes to the IS during tumor cell engagement. Although this occurs, it has been found that SNX27 is found in a distinct compartment adjacent to cytotoxic granules containing perforin and granzymes hinting that CASP may not have a direct role in cytotoxic granule shuttling. (MacNeil & Pohajdak, 2007).

Similar to CASP, GRASP also binds members of the cytohesin/ARNO family through its coiled-coil motif, however proteins interacting with the PDZ domain and PDZbm of GRASP are neuron-specific proteins. GRASP is not suspected to bind similar proteins as CASP in this domain (Macneil & Mceachern, 2008).

The objective of the following research at its inception was to discover and characterize the role of CASP in the specific functions of cells in the immune system by searching for loss of functions in NK cells when CASP is knocked down. This was completed by knocking down CASP stably by means of lentiviral transduction of interfering shRNA to trigger degradation and thus impair CASP mRNA translation. There was some difficulty in identifying CASP knockdown through conventional methods such as western blotting, and as a side project, we explored and identified that CASP is post-traslationally modified by ubiquitination and Granzyme B cleavage.

After the stable CASP knockdown line was cloned, the phenotypic changes in NK cell function were investigated. In this study we show a successful method of knocking down CASP by means of lentivirally delivered shRNA. We then show that migration and adhesion of NK cells is impaired when CASP is knocked down, and that NK cell secretion of cytokine IFN-γ is reduced indicating CASP involvement in activation of immune cells. Lastly, NK cell cytotoxicity is also significantly reduced in the absence of CASP, potentially due to the fact that early granules containing perforin and Granzyme B possibly fail to mature and reach the target cell.

Chapter 2 Developing a CASP Knockdown

2.1 Introduction

RNA interference is a biological process in which RNA transcripts inhibit protein translation through the destruction of mRNA. It has quickly become the primary means to elucidate molecular pathways by observing loss-of-function phenotypes (Mao, Lin, Hung, & Wu, 2007). It also holds the potential for genetic disease therapies. This gene knockdown effect is caused through site targeted double stranded RNA that subverts the protective biochemical mechanisms of the host to convert it to either: small interfering RNA (siRNA) molecules that bind to targeted mRNA sequences along with cleavage complexes, or to microRNA (miRNA) that also binds to complexes that prevent ribosomal translation of targeted mRNA transcripts (Zamore, Tuschl, Sharp, & Bartel, 2000).

Choosing short interfering RNA is an empirical process, as the rules that govern efficient siRNA-directed silencing are still unknown. There are several parameters that must be considered, in addition to sequence considerations. Any region of an mRNA transcript can be targeted, however a desirable siRNA construct is difficult to predict, and trial and error is the best method to date. The only real criteria is the target is specific to the desired gene, for any transcript that shares partial sequence identity could have undesired off-target effects.

The delivery of the RNAi is also a factor that affects the outcome of the overall protein translation of the targeted gene. Simply injecting siRNA or miRNA into a cell will give temporary and transient silencing of the desired gene, until the mRNA is

consumed or eliminated. A permanent or stable silencing of a gene is best achieved by integrating the gene that expresses the RNAi into the host genome (Gentner et al., 2009). There are several methods of achieving this, from electroporation to retroviral derived gene delivery vehicles.

Lentiviruses are a class of retrovirus, but they have two distinct characteristics that make them more effective for gene delivery than their retrovirus counterparts. Unlike oncoretrovirus vectors, HIV-1-based lentivirus vectors can infect both actively dividing and non-dividing, post-mitotic cells. In addition, oncoretroviruses undergo proviral silencing during development, which leads to decreased or abrogated gene expression. Lentivirus based vectors are resistant to this silencing and therefore can be used to generate stable transgenic cell lines or organisms (Rubinson et al., 2003).

In this chapter, we have created and confirmed a stable CASP knock-down in natural killer cell line NK-92.

2.2 Materials and Methods

2.2.1 Cells

Human cell line NK-92 were a gift from Dr. D. Burshtyn, (University of Alberta). NK-92 was grown in RPMI 1640 supplemented with 12.5% FBS, 12.5% horse serum (Gibco), 100 units/mL of IL-2 (PeproTech), and antibiotics. YTS and K562 cells were cultured in RPMI 1640 (Gibco) with 10% FBS and antibiotics.

2.2.2 Cloning and isolation of shRNA plasmids

Competent XL-1 Blue *Eschericia coli* (*E. coli*) cells were prepared by growing in A medium (LB containing 0.2% glucose and 10 mM $MgSO_4$) with 0.00125% tetracycline to an optical density of 0.3. They were chilled on ice (10 minutes), then transferred to a

solution of 14% A medium, 57% 2xTSS (LB containing 20% PEG 6000 and 100 mM MgSO4), and 23% glycerol in water. This preparation was immediately frozen at -80°C until needed. Plasmids cloned and isolated are listed in Table 1. These plasmids were inserted into the above cells by mixing plasmid DNA and competent cells in a 1:20 ratio on ice for 30 minutes, then heat shocked (42°C, for two minutes) and left to cool (5 minutes), then grown on agar plates to amplify the plasmid. Plasmid DNA was isolated by performing a EndoFree plasmid prep (Qiagen). Briefly, plasmid DNA was isolated first by alkaline lysis. Cells were pelleted by centrifugation, then resuspended and left in one volume of glucose solution (50 mM glucose, 10 mM EDTA, and 25 mM Tris-Cl, pH 8) for 5 minutes at room temperature. Two volumes of lysis buffer (0.2 M NaOH and 1% SDS) were added, and the whole solution was kept on ice for 5 minutes. Finally, 1.5 volumes of potassium acetate solution (3 M potassium, 5 M acetate, pH 4.8) were added and again kept on ice for 5 minutes. All insoluble material was removed from this solution by centrifugation (4,800 g for 30 minutes). To remove proteins from the plasmid DNA, we added an equal volume of phenol/chloroform, mixed, and retained the supernatant. This was repeated, using pure chloroform, to remove any trace phenol. Precipitation was performed by adding two volumes of 95% ethanol and letting the solution stand at -20°C for 30 minutes, followed by centrifugation at 17,000 g for 30 minutes.

Precipitated DNA was dried, dissolved in sterile irrigation-grade H_2O, then brought up to 12 mL volume by adding buffer P3 from the EndoFree system. From here, we followed the EndoFree instructions as though the DNA sample was a bacterial lysate (Qiagen, 2005). The resulting plasmids were digested using SalI and EcoRI restriction

endonucleases and analyzed on a 1% agarose gel to ensure presence and cleanliness of the proper plasmid in the preparation.

2.2.3 Lentivirus production and infection

For lentivirus production, HEK 293T cells were plated to 30-40% confluency in a 6 cm tissue culture plate (BD Falcon). 3.3 μg of cargo plasmid (Table 2.1), together with the packaging plasmids psPAX (2 μg) and pMP-2G (1 μg) were suspended in 500 μl of Optimem media (Gibco, Life Sciences). 18 μl of polyethalamineimine (PEI) was also added to a separate tube containing 500 ul of Optimem. The DNA and PEI were incubated for 5 minutes, then mixed together, and incubated for 15 minutes. The HEK293T cells were quickly washed with PBS, and then 7 ml of serum free DMEM was added to the cells. The DNA transfection media was then added dropwise evenly throughout the plate and the plate was gently mixed. The cell were then incubated at 37 °C for 6 hours and the media was replaced with 10 ml of DMEM supplemented with 10% FBS. The supernatant was removed from the cells 48 hours post infection, and syringe filtered through a 0.45 micron filter. The virus harvested was then aliquotted and kept frozen at -80°C.

For infection, 2.5 x 10^5 cells NK92 cells were incubated in 1 ml of IL-2 media supplemented with 100 mM Hepes buffer, and 8 ug/ ml of polybrene. 1 ml of the previously harvested viral particles containing the desired cargo plasmid (Table 2.1) was then added to the cells in a 6 well plate (BD). The plate was then centrifuged at 2000 rpm for 1 hour for spinoculation. After infection, the cells were then allowed to incubate for 24 hours before the media was replaced with 10 ml of IL-2 media. After 48 hours post-infection, the cells were submitted to antibiotic selection with 2 μg /ml of puromycin.

After 14 days cells grown under puromycin selection were deemed stable transformed cells. Cells were then isolated by dilution and cloned from single cell populations.

2.2.4 Lysates

Once a sufficient number of cells were grown (~1 x 10^7 cells/mL) in the cell lines, they were then lysed with lysis buffer which contained 50 mM Tris (pH 7.5), 5 mM EDTA, 300 mM NaCl, 1% Triton X-100, and a cocktail of protease inhibitors (Roche). Cells were sheared using a 23 guage syringe and were then sonicated. The sample was then centrifuged at 10,000 RPM for 10 minutes and the supernantant was then removed.

2.2.5 Western blot

NK92 protein lysate (30 μg and 60 μg) were used from the NK92 CASP and CASP knockdown cell lines. Loading dye was then added to the samples and then boiled for 5 minutes. The BioRad protein ladder, and various NK92 samples were then loaded separately into a 15% polyacrylamide gel. The gel was electrophoresed at 200 V for approximately 1 hour. Once completed, the gel was then transferred to a blotting apparatus. Protein samples were transferred to a polyvinylidene fluoride (PVDF) Hybond-P paper (Amersham Bioscience) at 100 volts for one hour. Next, the paper was stained in Ponceau to confirm protein transfer to the PVDF paper. The PVDF was then washed with PBS to remove the Ponceau stain. The PVDF was then blocked with 5% QuickBlocker Membrane Blocking Agent (Chemicon International) containing 0.2% sodium azide overnight at 4 °C. The next day, the PVDF was rinsed 3 times with tris-buffered saline (TBS) with tween (TBST). The anti-CASP primary antibodies (1:2000 Aviva anti-PSCDBP, 1:5000 Genetex anti-PSCDBP or 1:5000 PDZ2F9 rat anti-sera) were then added to the blot and shaken for 2 hours. The PVDF was then rinsed 5 times

using TBST. The PVDF was then immersed in secondary antibodies (conjugated to horse-radish peroxidase [HRP]) diluted in TBST (1:5000 – 1:1000) and incubated for 1 hour. The PVDF was then washed another 5 times with TBST. SuperSignal West femto maximum sensitivity substrate (Thermo Scientific) was then added to the PVDF and exposed onto Lumi-film detection film paper (Roche) for 3 minutes. The film was then developed (Kodak). Protein levels were then quantified using densitometry with the program imageJ.

2.2.6 CASP mRNA detection

RNA was extracted using RNEasy spin kit. 1 μg of RNA was then used to generate cDNA with SuperScript III reverse transcriptase kit (Life). For qPCR, I used DNA primers qCASP- S2 (5 – AGA TCG GGA AAC CTG CT- 3') and qCASP AS2 (5'- GGG GCA ATT AGC TGC ATC ACC – 3') to detect CASP mRNA expression levels. GAPDH was used as a target housekeeping control gene for reference and analysis using Rotogene 6 software. Three replicates of samples were run for each sample.

2.3 Results

2.3.1 Identification of an effective shRNA delivery vehicle

The generation of stably transformed CASP shRNA NK92 cell lines by electroporation or nucleofection using linear or plasmid retroviral and lentiviral vectors targeting different CASP mature mRNA sites (Table 2.1) (Fig 2.1) proved unsuccessful.

CASP mRNA sequence

GTTGTACTTTTAGCTTCCCCCATCCTGCAAGGCCACTCAACCATGTGCTAGCTGGAGTGATCTTTATTCAC
AATGTCTTTACAAAGGCTCCTGCAACACAGCAGCAATGGCAATTTGGCGGACTTCTGCGCTGGGCCAGCGT
ATAGCTCTTACTCCACACTCACCGGCAGCCTTACGATGGACGATAATAGAAGGATTCAAATGCTAGCAGAC
ACGGTGGCTACTCTGCCTCGGGGACGAAAGCAG|CTTGCTTTGACCAGATCAAGTTCTTTAAGTGACTTTT
CCTGGTCTCAAAG|AAAGCTTGTTACTGTGGAGAAGCAGGATAATGAAACATTTGGATTTGAAATTCAG|T
CTTACAGGCCCCAGAATCAGAATGCCTGCTCCTCGGAAATGTTCACTTTGATATGCAAAATACAGGAGGAC
AGCCCAGCTCACTGTGCTGGCCTGCAAGCTG|GTGATGTCCTTGCAAATATCAATGGTGTGAGCACAGAAG
GTTTTACCTACAAACAAGTCGTTGACCTGATCAGATCGTCCGGAAACCTGCTAAC|GATAGAGACTCTTAA
TGGAACAATGATTCTGAAAAGAACGGAGCTTGAAGCAAAGCTGCAGGTTTTAAAG|CAAACTTTGAAACAA
AAATGGGTGGAGTACAGATCTCTGCAGTTACAGGAACATCGTCTGCTTCATG|GTGATGCAGCTAATTGCC
CCAGTTTGGAAAACATGGACTTGGATGAATTGTCTTTGTTTGGACCCCTGCCTGGGCCAGGCCCAGCCCTT
GTGGACCGGAATCGATTATCCAGTGAGAGCAGCTGTAAGAGCTGGCTGAGCTCCATGACGATGGACAGTGA
AGATGGCTACCAGACGTGTGTGTCTGAGGACTCCAGCAGGGGTGCCTTCAGTCGGCAGACGAGTACAGATG
ATGAGTGCTTTATCCCCAAGGAGGGGGATGATTTTCTGAGGAGGTCATCTTCAAGGAGGAACCGGAGCATC
AGTAACACCAGCAGCGGATCCATGTCTCCCTTGTGGGAGGGCAACTTATCAAGCATGTTTGGGACCCTGCC
CCGGAAGAGCAGAAAGGGAAGTGTCCGAAAGCAACTCTTGAAATTTATCCCTGGCCTTCATCGTGCTGTGG
AAGAGGAAGAAAGTCGCTTTTGA|CGGATTGTGGTGTCCTTTCAAATTAGCTTATTTCACAAATATCTCTA
GACTCACCCAGATCCCAGCTTGGTGGGAAAGTGCAGAAGAATTGCAAAACTGACATCCCATTTCACAGCAA
TAGTGACCTTTATTTAAATTGTTGTGTTATAGTTTATGCTTCTTAAATCATTTTTCAACCTAAACAGCCAA
TTTCTAAGCAGACAGGAAAACTAAATAATAAGTTAATTAATATAACAAAGATGCAGGTTCCTGCTCATTCC
AGTAATGTCTTTGAAAGCAAAACTAATATTTATTTTCTAGATTATCCCTGTGAATAATTGAGAACTTTTTG
GAGTCAAGTATGAATAAAGGTGTGGCAGGATAATTGCTGGGTTATAAAATCTTAGGTTTGCTTATGCCCAG
TAGCTCCTGCGGAGGCTTAATAATAGGCAATTTTGAATTTGTTCAAACCTGTAATGGCTTGTAAACAAAGA
TGACCATCAGCTGTTTCTCACATCTATAGTGACAATAAAGCGGGAAGTATAAGATTTAATAGGAGGGGTTA
AGGTTCATGAGAACCATGGAAAGATGTGGTCTGAGATGGGTGCTGCAAAGATCATAATAAAGTCATTTTTA
TAGACAGTCTAAACAAAATGGGTGGGGATGTCGTGTTTTTTGCCCAATTCAGCTTTTGTTCTGCCTGAACA
TTAATGGCAAGTCTAGAACTCTCCGAATCCTACAGCTTTGTAATTTTTTTTCTACAAATGTCTAACATCCA
AAACTGAGGGTTGGGAAAAGGACTTCCCTCCTGTAGTTTTTTTCATATTACTTCTCACTTTATATCTTATA
TTCTAAATAGCTATCACCTCAGCAGTCTTTTGCCTATTGGTTATGTTAGTATCACATTACTTCTAGCCTTT
CAATTACTCCATGTTTTATTTAATATCCATTGAAGTCTATGAATTCTCTGTTCTGGTGGCACAGCTATTCA
TAACCTATATTCTAGAGTAGACAATCTGGACTATGTAATAAATAGTCTGCTGATTTTAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 2.1 Mature mRNA transcript of CASP. Highlighted sequences correspond to the target sites of shRNA lentiviral vectors found in Table 2.1. Exons are separated by | and are ordered sequentially.

Table 2.1 Information on the various shRNA viral vector plasmids targeting CASP mRNA.

ID #	Virus type	Vector	Target sequence	Target Area on mCASP
v2lhs_239901	Lenti	pGFP-V-RS	TTATAACCCAGCAATTATC (antisense)	3' untranslated
v2lhs_27755	Lenti	pGFP-V-RS	AAATAAAGGTCACTATTGC (antisense)	3' untranslated
v3lhs_337712	Lenti	pGFP-V-RS	TGTAGGTAAAACCTTCTGT (antisense)	Exon 5
TRCN0000122741	Lenti	pLKO.1	CCGGAATCGATTATCCAGTGA	Exon 8
TRCN0000141641	Lenti	pLKO.1	CAGAAAGGGAAGTGTCCGAAA	Exon 8
GI340505	Retro	pGIPZ	TCAGAATGCCTGCTCCTCGGAAATGTTCA	Exon 4
GI340506	Retro	pGIPZ	GCAGTTACAGGAACATCGTCTGCTTCATG	Exon 7
GI340507	Retro	pGIPZ	TGCCTTCAGTCGGCAGACGAGTACAGATGATG	Exon 8
GI340508	Retro	pGIPZ	GGACAGTGAAGATGGCTACCAGACGTGTG	Exon 8

Each specific shRNA target plasmid was then grown in viral particles, and the virus was infected into the natural killer cell line NK92. Cells were determined to be stably transfected if they survived under selective pressure with puromycin or displayed GFP expression for 2 weeks. Nine different CASP mRNA targets were made, as well as 3 non-specific (mamalian genome non-specific) shRNA lines. Cargo vectors containing the P-GFP-RS plasmid were stably expressing GFP after 2 weeks as well (Fig 2.2).

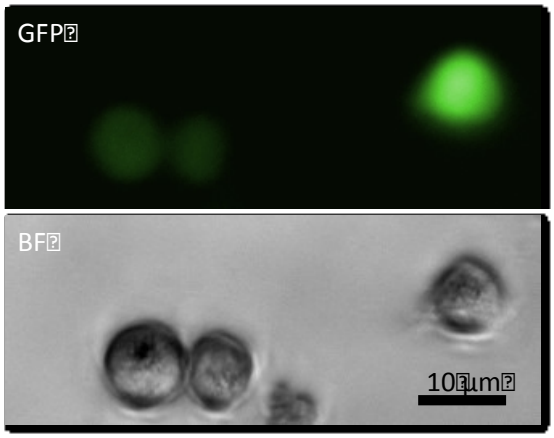

Figure 2.2 Representative view of stably transduced NK-92 cell lines with lentiviral vector pGFP-V-RS. Cells transduced with the virus expressed GFP, and if were viable after 14 days under puromycin antibiotic selection were determined to be stable. GFP picture was taken under 63x magnification with excitation of 480 nm.

2.3.2 CASP mRNA Detection

After determining the cells were stably transduced, each cell line was harvested for their mRNA translation. RT-PCR shows that the majority of CASP mRNA targets were unsuccessful in knocking down the transcript (Fig 2.3). In one case, the retroviral shRNA GI340505 increased CASP expression by 20%. Non-specific shRNA controls showed no difference in expression compared to wild-type NK cell line NK92, and cytotoxic T-cell lines YT and YTS. The tumour cell line K562 was used as a control for a non-lymphocyte line that does not express CASP.

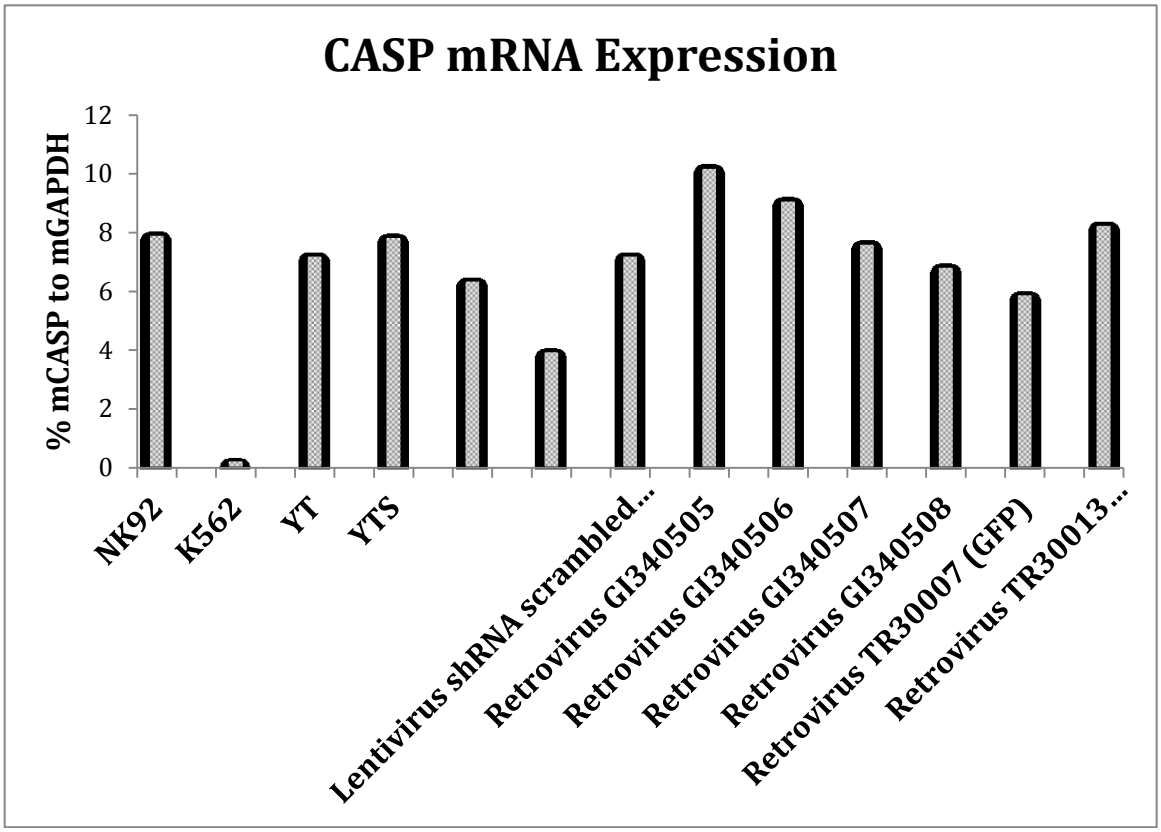

Figure 2.3 Comparison of CASP mRNA expression in several transformed and wild-type cell lines. CASP mRNA expression is normalized to constitutively expressed GAPDH mRNA in wild-type NK92, K562, cytotoxic T-cell lines YT and YTS and several different stably transduced shRNA CASP mRNA targets. These stably transduced cell lines showed insignificant knockdown of CASP mRNA expression when compared to the wild-type NK92 cell line.

There were two promising transformed cell lines. pLKO.1 lentivectors containing CASP shRNA targets TRCN0000141641 and TRCN0000122741 showed modest reduction in CASP expression 14 days post infection (not shown). The cell lines were then sub-cloned from individual cells, and TRCN0000122741 showed a 20% knockdown of CASP expression while TRCN0000141641 showed a promising 70% reduction in CASP mRNA expression when compared to wild-type NK92 and non-specific shRNA control (Fig 2.4).

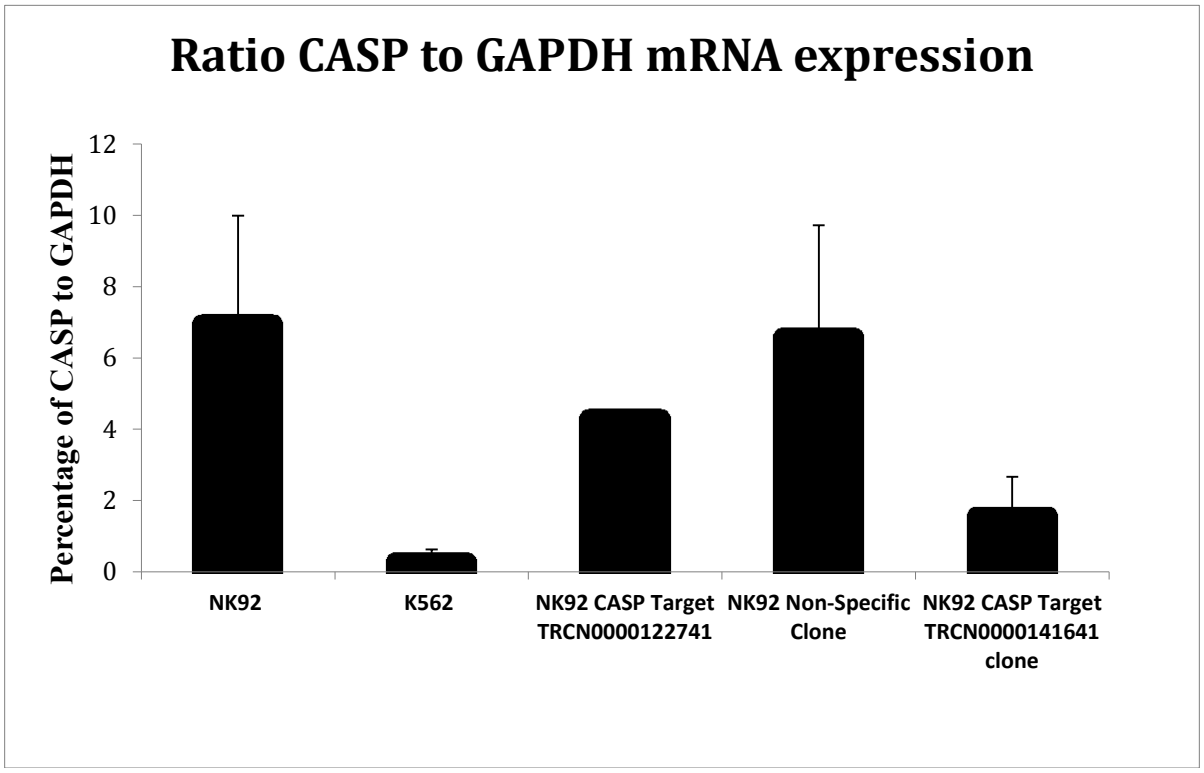

Figure 2.4 CASP mRNA expression normalized to constitutively expressed GAPDH mRNA in wild-type NK92, K562 (non-expressing cell line) and lentiviral shRNA transformed NK92. shRNA clone id TRCN0000122741 and TRCN0000141641 are sub-cloned populations of transduced NK92 cells. NK92 CASP knockdown 2 clone has approximately 70% reduction in mRNA expression compared to wild-type NK92 cells. Experiment was replicated three times with similar results.

2.3.3 CASP protein knock-down confirmation

After CASP mRNA expression was confirmed to be knocked down in the pLKO.1 vector cloned lines a western blot was performed to confirm reduction in protein expression. When compared to wild-type cell line NK92, cloned shRNA NK92 transformed cell line TRCN0000122741 showed a 40% reduction in CASP protein levels. The best knockdown however was seen with the cloned TRCN0000141641 line, giving an 80% reduction in CASP protein expression when compared to the wild-type (Fig 2.5).

The pLKO.1 plasmid shRNA CASP targets have also been shown to effectively knockdown CASP in mouse kidney tumor cell lines (Vanharanta et al., 2012).

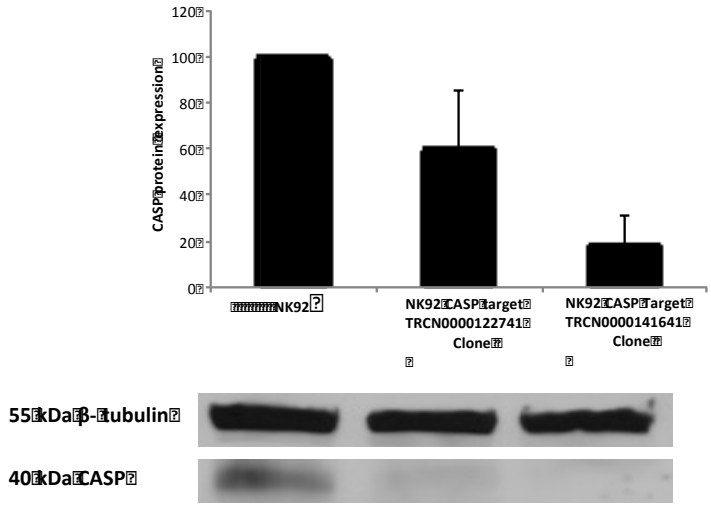

Figure 2.5 CASP protein expression in NK92 after transduction with two different shRNA CASP targets using lentiviral pLKO.1 vector. shRNA targets against CASP come from the lentiviral pLKO.1 vector that was reported to work in renal cell carcinoma cells (Vanharanta et al., 2012) CASP expression was normalized to tubulin expression in each sample. NK92 CASP knockdown shRNA id TRCN0000141641 clone has approximately 80% knockdown of CASP protein compared to wild-type NK92 cells ($P<0.05$). This experiement was repeated three times with similar results.

2.4 Discussion

After several attempts, a stable CASP shRNA knockdown line was cloned from the pLKO.1 lentiviral vector with the target sequence TRCN0000141641. The cloned line had a 70% and 80% decrease in CASP mRNA and protein levels, respectively. This result is satisfactory, due to the fact that CASP expression is highly regulated and an 80% knockdown in protein abundance should show large phenotypic change in further studies of natural killer cell function (Dykxhoorn et al, 2003).

Natural killer cells are noted as being extremely difficult to stably transform (Becknell et al., 2005; Burshtyn et al., 1999; Chiorean et al., 2003; Guven et al., 2005; Imai et al, 2005; Jiang et al., 2013; Karimi et al., 2013; Kim et al., 2005; Schroers et al., 2004; Trompeter et al, 2003). That being said, we did have great difficulty in getting any type of stably integrated shRNA vector through electroporation or nucleofection (data not shown), since cells would die within a few days of antibiotic selection. It was then decided to move to viral transduction for an attempt at a stable CASP knock down in NK92. Initially, we attempted to use the GFP expressing shRNA plasmids pGFP-V-RS. Expression of GFP would take effect within 24 hours of transformation, giving a transient expression of the CASP knockdown shRNA. However, in every case, 72 hours post infection, the cells would die when submitted to antibiotic selection.

In addition, a stable expressing GFP NK cell CASP knock down was not desirable due to the limitations it would cause with later analysis with immunofluorescence. The pLKO.1 non-GFP shRNA vectors were then decided to be the most desirable transformation vehicle.

The viral means of stable transformation was the most effective method reported in the literature (Becknell et al., 2005; Karimi et al., 2013; Lee et al., 2009; Purdy & Campbell, 2010) and thus was the vehicle of integration of choice. Again, an effective infection was difficult, and I had several attempts at each specific shRNA virus before I had stable NK92 transformants. NK cells by nature target and kill virally infected cells, and are extremely resistant to retrovirus infection giving reason to why they are so difficult to transform. In addition, NK cells are IL-2 stimulation obligates in order to continue proliferating and are extremely sensitive to almost any change or stress in culturing conditions (Becknell et al., 2005).

Also, it was thought that a reason why it was so difficult to use shRNA to knock down CASP is the fact that NK cells are the primary means of the innate immune system to combat viral infection. Double-stranded RNA is the hallmark characteristic of reovirus, and NK cells contain toll-like receptors (TLR2) that activate immune responses (Szomolanyi-tsuda et al, 2006). This could effectively cause NK cells to attempt to kill themselves in culture. However, the pLKO.1 is a third generation lentiviral vector system that uses a U6 promoter that does not produce a significant interferon response (Yu et al, 2002).

Chapter 3 Post-Translational Modification of CASP

3.1 Introduction

Cytohesin associated scaffolding protein (CASP a.k.a CYBR, CYTIP, PSCDBP, B3-1) is a lymphocyte specific adaptor protein that was first identified and cloned by the Pohajdak lab in the early 1990's from natural killer cells by subtractive hybridization (Mansour et al., 2002a). The CASP gene is approximately 29 kilobases (kb) in size, and includes 8 exons and 7 introns encoding a 40 kDa (359 aa) final protein product. It is an adaptor protein, so it lacks any catalytic domains but it does contain three protein-protein interaction domains. These domains are essential for the assembly of larger protein complexes which are suspected to be involved in intracellular trafficking and signaling (MacNeil & Pohajdak, 2007; Mansour et al., 2002a). The 40 kDA protein has a N-terminal PDZ domain, a coiled-coil motif (leucine zipper) and a C-terminal PDZ binding motif (PDZbm) (Fig. 1.1).

CASP has been found to have direct binding interactions with two proteins (Fig 1.1). Most of what is known about CASP's intracellular function is attributed to its first binding partner Cytohesin-1. CASP interacts with cytohesin-1 through the coiled-coil motifs of both proteins. Cytohesin-1 is an ARF-GEF that was first cloned and characterized in the Pohajdak laboratory. Cytohesin binds to ARF's through its plekstrin homology (PH) domain, changing their conformation by allowing GDP to be switched for GTP to allow the ARF to bind to membranes at sites that are rich in phophoinositols. CASP is also able to bind through the coiled-coil motif to other members of the cytohesin family (ARNO/cytohesin-2 and ARNO3/ GRP1/cytohesin-3) (Mansour et al., 2002a). The PDZbm of CASP interacts with Sorting Nexin 27 (SNX27) a molecule that mediates

PDZ-directed sorting from endosomes to the plasma membrane (Lauffer et al., 2010). This interaction was recently discovered by Dr. Adam MacNeil (MacNeil & Pohajdak, 2007) (Fig 1.1) also from our laboratory.

Granzyme B is a major constituent of NK cell granules, promoting apoptosis through proteolysis of a target proteins. Granzymes have a crucial role for cell caspase activation and DNA fragmentation. Granzyme B is characterized as an aspase because of its preference for substrate cleavage after aspartic acid residues (Cullen & Martin, 2008). There appears to be two main pathways to granzyme B-induced killing, one involving direct activation of caspases and the other mediated through granzyme B-initiated promotion of mitochondrial permeabilization (Adrain et al., 2006).

It has also been shown that CASP is ubiquitinated, a fact that was published while we were researching the same topic (Grabher et al, 2013). It was determined that the suppresor of cytokine signaling 1 (SOCS-1) is another binding partner for CASP. It acts by bringing proteins to the proteasome for degradation. However, in the study they show by yeast 2 hybrid and co-immunoprecipitation with CASP that SOCS-1 is present, however they fail to mention through which domain or how CASP binds and interacts with SOCS-1. Here we present our findings on the ubiquitination of CASP and its targeting to the proteosome for degradation through two lysine residues that are potential sites for ubiquitination.

3.2 Methods

3.2.1 Lysates

Once a sufficient number of cells were grown (~1 x 10^7 cells/mL) of the cell lines they were then lysed with lysis buffer which contained 50 mM Tris (pH 7.5), 5 mM

EDTA, 300 mM NaCl, 1% Triton X-100, and a cocktail of protease inhibitors (Roche). Cells were sheared using a 23 guage syringe and were then sonicated. The sample was then centrifuged at 10,000 RPM for 10 minutes and the supernatant was recovered and protein concentration determined.

3.2.2 Granzyme B inhibition

One x 10^7 cells/ ml of NK92 were suspended in a 6 well plate (BD) in 1 ml of Il-2 media supplemented with 2 μM Granzyme B inhibitor II (Z-AAD-CMK) (in DMSO) (Calbiotech). The cells were incubated at 37°C, 5% CO_2 for 3 hours. After the incubation, the cells were then washed in serum free media and harvested as per lysate protocol found above. Granzyme B target sites were predicted with GraBCas online software.

3.2.3 Western blot

NK92 protein lysate (30 μg and 60 μg) were prepared from the NK92 CASP and CASP knockdown cell lines. Loading dye was added and samples were then boiled for 5 minutes. The BioRad protein ladder, and various NK92 samples were then loaded separately into a 15% polyacrylamide gel. The gel was electrophoresed at 200 V for approximately 1 hour. Once completed, the gel was then transferred to a blotting apparatus. Protein samples were transferred to a polyvinylidene fluoride (PVDF) Hybond-P paper (Amersham Bioscience) at 100 volts for one hour. Next, the paper was stained in Ponceau to confirm protein transfer to the PVDF paper. The PVDF was then washed with PBS to remove the Ponceau stain. The PVDF was then blocked with 5% QuickBlocker Membrane Blocking Agent (Chemicon International) containing 0.2% sodium azide overnight at 4 °C. The next day, the PVDF was rinsed 3 times with tris-buffered saline (TBS) with tween (TBST).The anti-CASP primary antibodies (1:2000

Aviva anti-PSCDBP, 1:5000 Genetex anti-PSCDBP or 1:5000 PDZ2F9 rat anti-sera) were then added to the blot and shaken for 2 hours. The PVDF was then rinsed 5 times using TBST. The PVDF was then immersed in secondary antibodies (conjugated to horse-radish peroxidase [HRP]) diluted in TBST (1:5000 – 1:1000) and incubated for 1 hour. The PVDF was then washed another 5 times with TBST. SuperSignal West femto maximum sensitivity substrate (Thermo Scientific) was then added to the PVDF and exposed onto Lumi-film detection film paper (Roche) for 3 minutes. The film was then developed (Kodak).

3.2.4 Co-Immunoprecipitation (C0-IP)

Cells were lysed with the co-immunoprecipitation (CO-IP) lysis buffer, which contained 50 mM/L Tris (pH 7.5), 5 mM/L EDTA, 300 mM/L NaCl, 1% Triton X-100, 1 mM/L PMSF, 10 μg/mL aprotinin, 1 μg/mL leupeptin, Tyr phosphatase cocktail I, and Ser/Thr phosphatase cocktail II. Cells were sheared by brief sonication, and cellular debris was removed by centrifugation at 10 000g for 10 min. Aliquots (1 mg/ml) of lysates were incubated with anti-PDZ 2F9 overnight at 4°C, and then for 2 h at 4°C with 20 μL Protein A/G PLUS-Agarose. Immune complexes were collected by centrifugation and washed three times with ice-cold phosphate-buffered saline (PBS). After a final wash, the supernatant was discarded and the pellet was resuspended in SDS lysis buffer, and then boiled in 4× SDS loading dye for 5 minutes. Protein was separated by SDS–PAGE and transferred on PVDF membranes. Membranes were then probed with anti-Ub (P4D1)(1:2000, Santa Cruz).

3.3 Results

In trying to confirm if CASP protein was indeed knocked down by the shRNA, we needed to find an antibody that had high specificity. After testing every commercially available antibody, both mono and polyclonal, we never had a single Western blot that had one single band at the 40 kDa range. There were always many bands that appeared, most consistently (with most all antibodies) at 95, 70, 40, 29 and 10 kDa. Even with monoclonal antibodies targeting the N-terminus of the protein (PSCDBP from Epitomics), we had multiple bands that would appear in NK92 lysates (Fig 3.1). These other bands had never been reported in the literature, most likely due to the fact that it potentially implies non-specific binding of antibody. For any probe for CASP protein expression authors would only show and point at one band at the 40 kDa range that was CASP (Boehm et al., 2003; Coppola et al., 2006; Theodoridis et al, 2011).

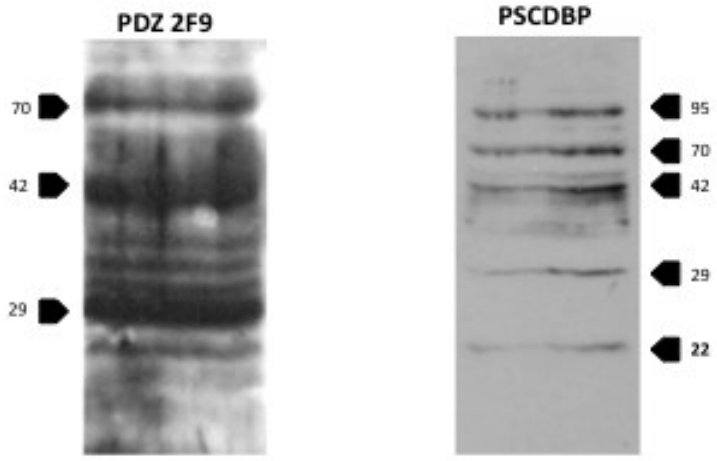

Figure 3.1 Typical western blot banding when probing for CASP in NK-92 cell line lysates. PDZ 2F9 is a polyclonal rat antisera. PSCDBP is a comercially available rabbit monoclonal antibody. In each blot there are consistant bands that appear at 70, 42, and 29 kDa.

However, the consistency of the multiple signals seen with antibodies targeting diferent sites on the CASP protein lead us to believe that there had to be post-translational modifications that were occurring. The larger sized CASP residues could be explained by ubiquitination, whereas the smaller sized bands would be due to cleavage events.

3.3.1 CASP is ubiquitinated and targeted to the proteasome for degradation

Ubiquitin is a ubiquitously expressed protein which typically labels proteins for degradation by the proteasome (Hicke, 2001). To demonstrate that CASP is indeed ubiquitinated, NK92 cell lysates were probed with the polyclonal rat anti-PDZ 2F9 anti sera and brought down with protein A/G coated agarose beads. Any proteins that bind,

interact or form a complex with CASP would also be brought out of the lysate. The beads were then electrophoresed and probed for ubiquitin (Fig 3.2)

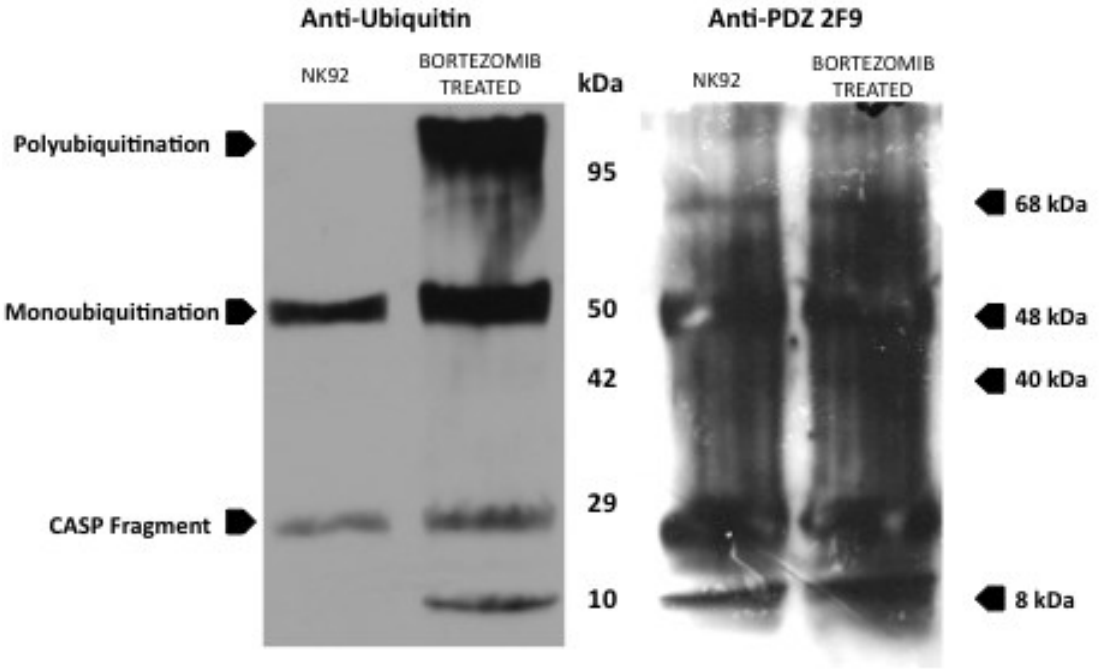

Figure 3.2 CO-IP of CASP from NK92 lysates in untreated conditions and with proteasome inhibition by bortezomib. Anti-ubiquitin probing shows large banding when treated with bortezomib, indicating the buildup of CASP for degradation. The 10 kDa band is indicative of a single ubiquitin monomer (about 8.5 kDa). The 50 kDa band seen in the untreated NK92 lysate corresponds to a potential multiubiquitination of CASP (potentially 2 subunits resulting in 48 kDa). PDZ 2F9 antisera shows multiple banding typical of the 68, 48, 40 and 29 kDa forms of CASP in both treated and untreated NK lysates.

When probed for ubiquitin, a band at the 48 kDa appear, indicating a ubiquitination of CASP by potentially 2 ubiquitin subunits. The 29 kDa band could be due to cross reactivity of the secondary antibody with the PDZ 2F9 rat anti-sera, because the ubiquitin antibody was raised in mouse. The blot was then stripped and probed again for CASP, bands at 68 kDa, and two or three additional minor bands between 95 kDa and 50 kDa (Fig 3.2) were apparent. This suggests that CASP is found with 1, (around 44 kDa), 2 (48

kDa), 4 (68 kDa) or more ubiquitin subunits attached. Bioinformatic analysis for ubiquitination motifs on mature CASP protein sequences (Fig 4) show two potential sites, at lysine residues 247 and 288 (Fig 3.3) (Table 3.1).

MetSLQRLLQHSSNGNLADFCAGPAYSSYSTLTGSLTMDDNRRIQMLADTVATLPRGRK
QLALTRSSSLSDFSWSQRKLVTVEKQDNETFGFEIQSYRPQNQNACSSEMFTLICKIQE
DSPAHCAGLQAGDVLANINGVSTEGFTYKQVVDLIRSSGNLLTIETLNGTMILKRTELE
AKLQVLKQTLKQKWVEYRSLQLQEHRLLHGDAANCPSLENMDLDELSLFGPLPGPGPAL
VDRNRLSSESSC**K**SWLSSMTMDSEDGYQT**CVSEDSS**RGAFSRQTSTDDECFIP**K**EGDD
FLRRSSSRRNRSISNTSSGSMSPLWEGNLSSMFGTLPRKSRKGSVRKQLLKFIPGLHRA
VEEEESRF

Figure 3.3 Mature protein sequence of CASP showing possible sites of ubiquitination and granzyme B cleavage. Predicted lysine residues that are targets for ubiquitination indicated in red. Residues are located at positions 247 and 288. Sites were predicted using UbPred bioinformatics prediction tool. Cleavage site of granzyme B is indicated by bolded residues.

Table 3.1 Possible sites for ubiquitination on mature CASP protein sequence according to UbPred bioinformatics prediction tool. Residues 247 and 288 are determined to be potential sites for ubiquitin attachment.

Residue	Score	Ubiquitinated
57	0.29	No
76	0.49	No
82	0.43	No
113	0.37	No
145	0.41	No
170	0.39	No
177	0.35	No
182	0.46	No
186	0.36	No
188	0.42	No
247	0.74	Yes Medium confidence
288	0.77	Yes Medium confidence
331	0.11	No
334	0.17	No
339	0.15	No
343	0.13	No

Label	Score range	Sensitivity	Specificity
Low confidence	0.62 ≤ s ≤ 0.69	0.464	0.903
Medium confidence	0.69 ≤ s ≤ 0.84	0.346	0.950
High confidence	0.84 ≤ s ≤ 1.00	0.197	0.989

Degradation of CASP is also inhibited when treated with the proteasome inhibitor Bortezomib (Velcade) (Fig 3.2). The boron atom of Bortezomib binds with high specificity to catalytic site of the 26S proteasome, preventing targeting of proteins that have been ubiquitinated. The large smear seen in the anti-ubiquitin lane of the bortezomib treated cell population indicates the accumulation of multi-ubiquinated CASP residues that are not degraded due to the fact that the proteasome cannot cleave and recycle the protein due to inhibition (Fig 3.2). This result is consistent with the recently reported result by Grabher *et al*.

3.3.2 CASP is cleaved by Granzyme B

When NK92 were treated with granzyme B inhibitor (Z-AAD-CMK) prior to lysis, the predominant band at 29 kDa was not present by Western blotting. In previous unpublished data by Adam MacNeil, he had shown that recombinant purified CASP was cleaved by purified GrB *in vitro*. It was also found that the cleavage activity (i.e. GrB) was in certain cytotoxic cells (NK92, YT and YTS) but not in other cell types (Jurkat, K562, Raji).

The GrB cleavage site in CASP was found using the bioinformatics tool (GraBCas) (Backes et al, 2005). The predicted site is CVSED / SS about 268 aa into the 359 aa protein, facilitating the removal of a 10 kDa fragment from the carboxy terminus, effectively removing the PDZ binding motif (Fig 3.4).

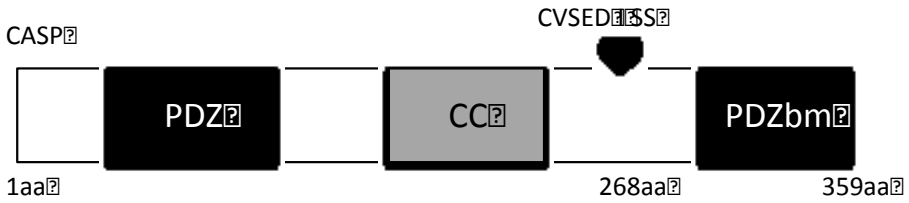

Figure 3.4 Predicted CASP proteoltic cleavage site by granzyme B (GrB). Bioinformatics tool GraBCas predicts a significant ($p<0.05$) site for CASP at amino acid 268, CVSED / SS. This is consistent with experimental findings using recombinant CASP as a target for pure granzyme B and purified CASP that was performed and adapted from the unpublished research of Dr. Adam MacNeil.

This smaller fragment corresponds to the remaining 30 kDa that appears in Western blot with the PSCDBP monoclonal antibody that targets the n-terminal end of CASP (Fig 3.5). When NK92 cells were treated with the GrB inhibitor prior to harvesting and lysis, the 29 kDa band disappears (Fig 3.5).

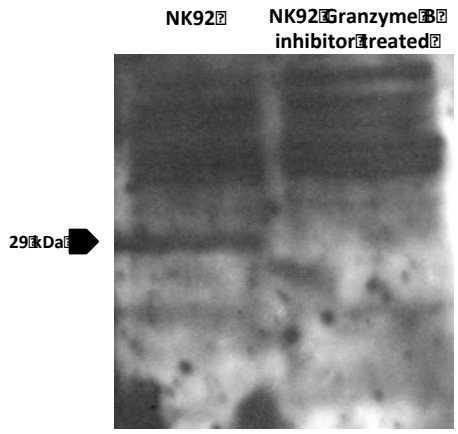

Figure 3.5 Western blot depicting the proteolytic cleavage of endogenous CASP by Granzyme B. The blot was probed with the monoclonal PSCDBP antibody (Epitomics) targeting the n-terminal region of mature CASP protein. In the Granzyme B inhibitor treated sample, the 29 kDa residue is not present, indicating that CASP is cleaved by endogenous Granzyme B.

3.4 Discussion

The multiple banding seen in NK cell lysates for CASP is indicative of several post translational modifications, and here we focused on ubiquitination and cleavage events. The larger CASP residues are due to the ubiquitination of the protein, whereas the residues smaller than the natively described 40 kDa are due to GrB cleavage.

In addition to targeting proteins to the proteasome for degradation by polyubiquitination, monoubiquitination has been shown to have a role in the endocytosis of membrane bound proteins. Ubiquitin comprises a three dimensional internalization signal that can be appended to proteins destined for downregulation. This is interesting, because CASPs binding partner SNX27 also has a role in the recycling of receptors from the plasma membrane (Lauffer et al., 2010)

An emerging subject of interest in molecular immunology is deciphering the role played by proteins of the endocytic pathway in polarized events such as active maintenance of the immunological synapse in cytotoxic lymphocytes, as well as other polarized events such as migration (Krummel & Macara, 2006). Recycling of receptors involved in both activation and adhesion are central to maintaining polarity both as lymphocytes navigate their microenvironment and in communication with antigen-presenting cells. The ubiquitin internalization signal located on a membrane bound protein may recruit an adaptor protein such as CASP, that would promote endocytosis. CASP could have a role in the localization of endocytic cargo (such as recycled effector molecules like perforin and granzyme) into subdomains of the plasma membrane competent for vesicle budding or might be part of the vesicle budding machinery through its interactions with cytohesins and SNX27. If CASP is indeed monoubiquinated, the

ubiquitin internalization signal could target endocytic cargo and CASP would be involved in assembling a complex that is necessary for the budding of primary endocytic vesicles (Hicke, 2001).

Our laboratory has reported on the active polarization of endosomal SNX27 during lymphocyte migration and tumor cell engagement, indicating that this CASP binding partner plays a role during several polarized events in lymphocytes (MacNeil & Pohajdak, 2007). The highly limited and regulated expression of CASP in immune cells, coupled with its role in reducing cell adhesion and interaction with the polarizing protein SNX27, suggests an even more diverse role of CASP endocytic pathways associated with lymphocytes.

Bortezomib induces apoptosis in cells when there is a buildup of non-degraded proteins. While multiple mechanisms are likely to be involved, proteasome inhibition may prevent degradation of pro-apoptotic factors, permitting activation of programmed cell death in neoplastic cells dependent upon suppression of pro-apoptotic pathways (Bonvini et al., 2007). This, however, is irrelevant to the harvesting of protein lysates, we are lysed the cells well before the exposure to bortezomib induces and execute apoptotic pathways.

The cleavage caused by GrB facilitates the removal of a 10 kDa fragment from the c-terminus of CASP, eliminating CASP's interaction with SNX27 through the PDZbm. The effect of this cleavage may eliminate the recruitment of CASP to endosomes. This disruption of CASP function may be important in apoptosis of hemopoietic cells and/or the destruction of lymphomas by NK cells. It could also act as a fail-safe mechanism. If granzyme is released accidentally into the cytoplasm, it would cleave CASP and prevent

the release, recycling or maturation of cytotoxic granules, due to the fact that the cell has become compromised.

The two modifications may be regulating each other as well. If CASP is cleaved by granzyme, it also loses a potential site of ubiquitination (Fig 3.3, 3.4). This could facilitate the regulation of CASP by being targeted to the proteasome for degradation. CASP would lose ability to bind to SNX27, but would still be able to interact with cytohesin through its coiled coil domain. Conversely, ubiquitination of CASP at the tail-end ubiquitination site could inhibit CASP cleavage by granzyme B.

Inhibition of CASP expression is also induced by herpes simplex virus (HSV) infection of dendritic cells, with consequences on their ability to migrate and to interact with the interstitial space and T cells. Theodoridis *et al.* (2011) found that CYTIP is rapidly degraded in dendritic cells infected with HSV leading to impaired migration of dendritic cells and impaired immune responses. This is thought to be a novel viral evasion mechanism from immune responses (Theodoridis et al., 2011). This process could also be a key defensive mechanism against NK cells, the immune systems primary defense against initial HSV infection. These mechanisms to eliminate CASP indicate that CASP has an important role in specialized immune cell functions. Further, the diverse ways that CASP is post-translationally modified show that it has a very dynamic role in the mechanics of natural killer cell function.

Chapter 4 CASP is Essential for NK Cell Migration *ex vivo*

4.1 Introduction

Natural Killer (NK) cells are highly motile cells that patrol lymphoid and peripheral organs and tissue, ready to react to stimulus by either inflammatory situations or infection. Migration is important for both invasion into tissue from the blood, and tumor or infected cell engagement. Immune cell migration is a critical and complex polarized event. Cells must navigate many stimulus-laden microenvironments en route to their destination and will encounter and engage with scores of cells along the way, making the process very dynamic and rapidly controlled at the molecular level (MacNeil & Pohajdak, 2007).

CXC chemokine receptor 4 (CXCR4) is a G-protein coupled receptor protein specific for stromal derived factor-1 (SDF-1), a molecule that has a potent chemotactic activity for lymphocytes. SDF-1 is known to have a role in hematopoietic stem cell quiescence and homing to the bone marrow, and is thought to have an overall role in immune surveillance of lymphocytes migrating in the periphery. CXCR4 is almost predominately expressed in lymphocytes, and there are only two identified ligands for the receptor, being SDF-1 and ubiquitin (Bleul, 1996).

Recent studies into the cause of metastasis of renal cancers in mice has found that in migrating metastatic clear cell renal carcinoma cells (ccRCC) CASP and CXCR4 gene transcription is significantly increased (Vanharanta et al., 2012). When CASP is knocked down in ccRCC, there is complete inhibition of lung colonization, indicating a role in tumor metastasis, which fits with CASP's potential role in cellular migration. According

to Vanharanta et al. (2012), the epigenetic expansion in ccRCC of CXCR4 allows for chemotaxis while CASP promotes survivability.

There have been two mouse CASP knockout models that have shown little effect on immune function (Coppola et al., 2006b; Watford et al., 2006). Minimal effects included limited migration and reduced lymph node enlargement when challenged against Moloney murine sarcoma virus. However, this study focused on myeloid cells as opposed to lymphocytes as well as small overall effects on lymphocytes in the knockout mice. NK cells only make up approximately 3-5% of the overall lymphocyte population, and their functions may not be evident in a whole animal model. In addition, CASP has a lower overall expression in mice as compared to humans (Watford et al., 2006) which would also explain the minimal effect on overall lymphocyte migration. This gives more reason to pursue the role of CASP in human NK cell migration.

We have shown previously that SNX27 polarizes toward the leading edge of migrating NK cells and is associated with the early endosome (MacNeil & Pohajdak, 2007). In the study, CASP was shown to co-localize with SNX27 in resting NK cells, however it has not been shown that CASP polarized toward the leading edge in actively migrating cells. In this study, CASP is shown to polarize toward the leading edge of migrating NK cells, associating in the same pseudopod as the chemokine receptor CXCR4, and when CASP is lacking, NK cell migration is impeded.

4.2 Methods

4.2.1 Cells and antibodies

Human cell line NK-92 was a gift from Dr. D. Burshtyn, (University of Alberta). NK-92 was grown in RPMI 1640 supplemented with 12.5% FBS, 12.5% horse serum

(Gibco), 50 Mb- mercaptoethanol (Sigma), 100 units/mL of IL-2 (PeproTech), and antibiotics. SNX27 antiserum (a gift from Dr. T. Nishikawa, Tokyo Medical and Dental University, Japan) was used at 1:200 while subcellular marker antibodies used were: anti-CXCR4 1:200 (R&D Systems); rat PDZ- 2F9 anti-sera; anti-beta tubulin (Genetech).

4.2.2 Immunocytochemistry

For immunocytochemistry, 2–5 x 10^5 cells were allowed to adhere to poly-L-lysine coated slides (Lab Scientific) for 15 min at 37 °C. Cells were then fixed with 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 in PBS (PBS–TX) for 5 min. Slides were blocked with 1% BSA in PBS–TX and primary antibodies/antisera were incubated at room temperature for 30 min. Slides were then washed before applying Cy3 and Alexa 488 conjugated secondary antibodies (Jackson ImmunoResearch and Molecular Probes). Finally, slides were washed extensively before application of VectaShield mounting medium (Vector Laboratories).

4.2.3 Migration assay

Two point five x 10^5 cells per well of each cell line (NK92, NK92 Scrambled, and NK92 CASP knockdown) were aliquotted in triplicate. Cell were washed in serum-free RMPI 1640 (Life Technologies) and then starved for a half hour in 300 μl /well Serum-Free RPMI 1640 supplemented with 0.1% BSA. 650 μl Serum-Free RPMI 1640 supplemented with 0.1% BSA and 200 ng/ml recombinant human SDF-1α (CXCL12) (PeproTech) were then aliquotted into separate wells of a 24 well Corning Costar plate. Cells were then transferred to a Transwell 5 μm permeable support (Costar) and placed

into the wells containing the media supplemented with the SDF-1α chemo-attractant (Fig 4.1).

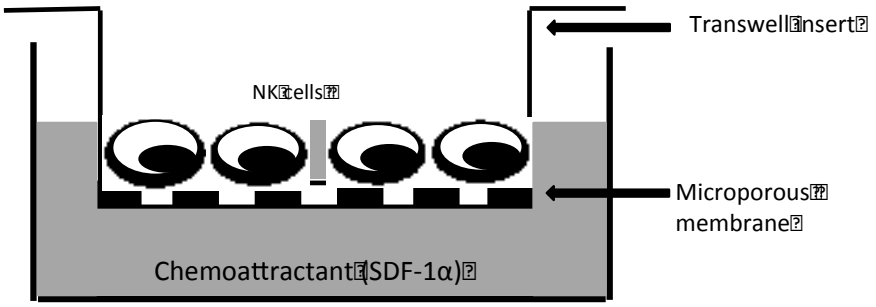

Figure 4.1 Migration assay setup. Transwell inserts with a size selective membrane (5 μm) were loaded into a 24 well plate that contained RPMI media supplemented with 0.1% BSA and 200 ng/ml of chemoattractant SDF-1α. NK cells (either NK92, NK92 non-specific or NK92 CASP knockdown) were then added into the inserts with serum-free RPMI media. Cells would then under go chemotaxis and migrate through the membrane. The inserts were then removed and the cells that migrated through were collected and quantified by WST-8 proliferation dye.

Cells were incubated for 6 hours and then permeable supports were washed twice with PBS. The supports were emptied of liquid and replaced into the respective wells. The plates were then centrifuged for 4 mins at 250 g to recover cells that may be stuck in the pores of the insert. Supports were removed and the remaining media was harvested. The media was centrifuged at 400 g for 5 mins to recover cells. The cells were then resuspended in 100 μl IL-2 supplemented media containing 10% WST-8 Cell

proliferation assay dye (Cayman Chemical) in a 96-well plate. Cells were incubated for 2 hours and then readings were taken at 450 nm on a SpectraMax plus 384.

4.3 Results

4.3.1 CASP's role in natural killer cell migration

When submitted to starvation conditions to induce chemotaxis and challenged with a size selective membrane, chemotacting NK cells migrate to the chemoattractant SDF-1α. NK cells lacking CASP are significantly impeded (Fig 4.2). Non-specific shRNA expressing NK92 cells were used as a control to demonstrate that the lentiviral transduction method had no effect and that phenotypic change was due to CASP knockdown.

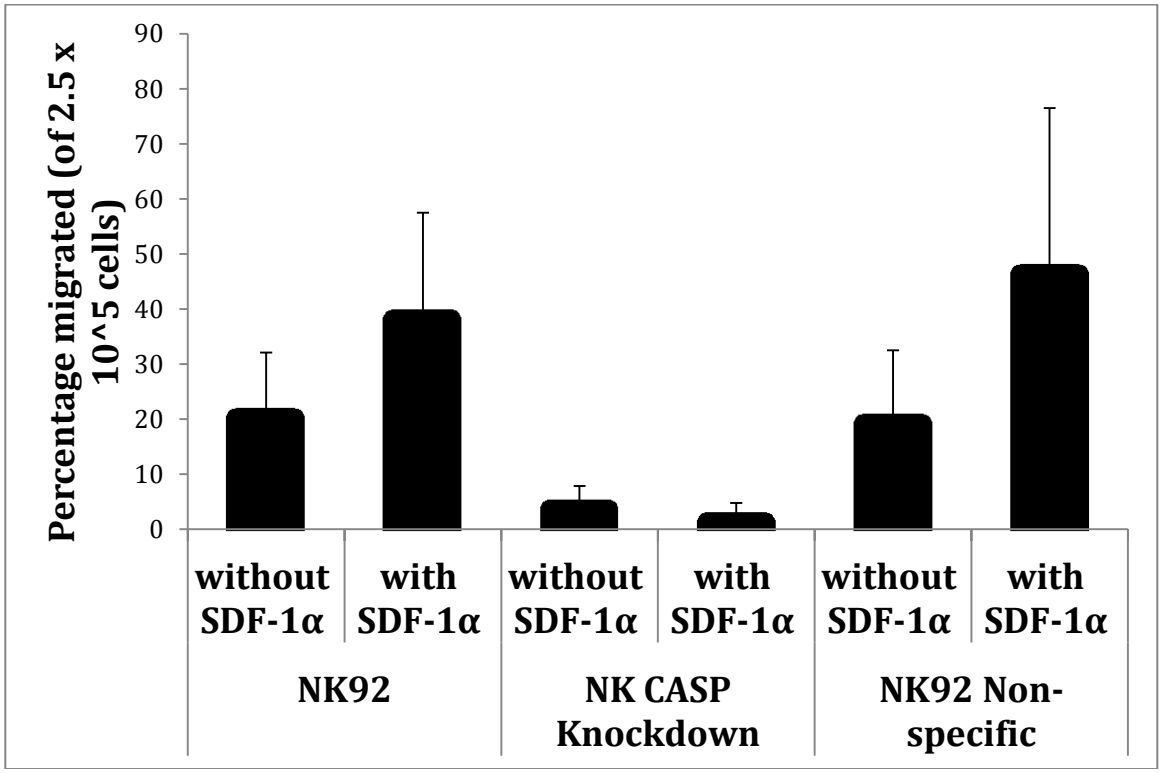

Figure 4.2 Migration of NK cell lines through a size selective membrane when stimulated by the chemoattractant SDF-1α. Wild-type cell line NK92 migrates through the membrane when there is chemoattractant located on the other side of the barrier. Stable CASP shRNA interference proves to have a

significant impairment of migration when SDF-1α is present (P= 0.026) compared to NK92 cell line. Non-specific shRNA control line shows similar results to the wild-type NK92 cell line. This figure is representative of three separate experiments that were averaged and was repeated three times. Each experiement yielded similar results.

4.3.2 CASP does polarize to the leading edge in NK cells.

Confocal microscopy shows that CASP polarizes at the site of adherence to slides coated with poly-l-lysine when subjected to induced migration conditions (Fig 4.3).

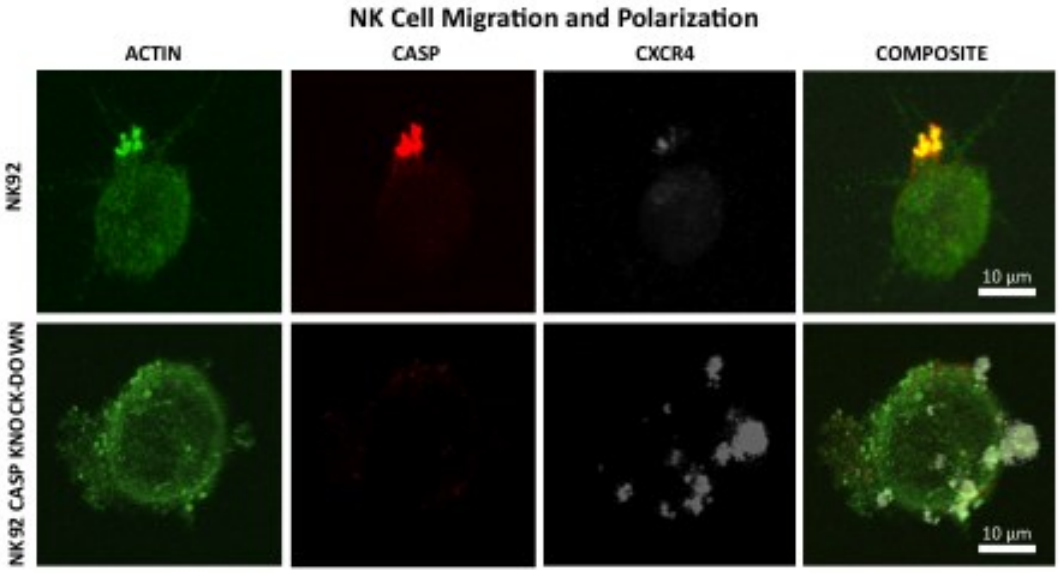

Figure 4.3 CASP cellular localization in migrating NK cells. NK cell migration phenotypes viewed under 63x magnification with a Zeiss 510 laser scanning microscope. Wild-type NK92 cells demonstrate pseudopod formation and establishment of a polarized leading edge (CXCR4) when migrating under starvation conditions and stimulated with the chemoattractant SDF-1α. PDZ 2F9 is antisera against CASP. NK cells lacking CASP demonstrate no formation of pseudopods nor a distinct leading edge as denoted by the chemokine receptor CXCR4. These figures are representative of 10 different cells (for each line) that were subjected to the same parameters.

The leading edge of migrating and chemotacting NK cells is denoted by the polarization of the chemokine receptor CXCR4. CASP does appear to be present in the

area of adhesion of the attached migrating cell, as well as locating in the same area pseudopod as CXCR4. When CASP is knocked down, CXCR4 does not appear to polarize uniformly in a single pseudopod in front of the migrating cell (Fig 4.3), and appears to localize in an extension of the plasma membrane that is distinct from the body of the cell (Fig 4.3 and Fig 4.4).

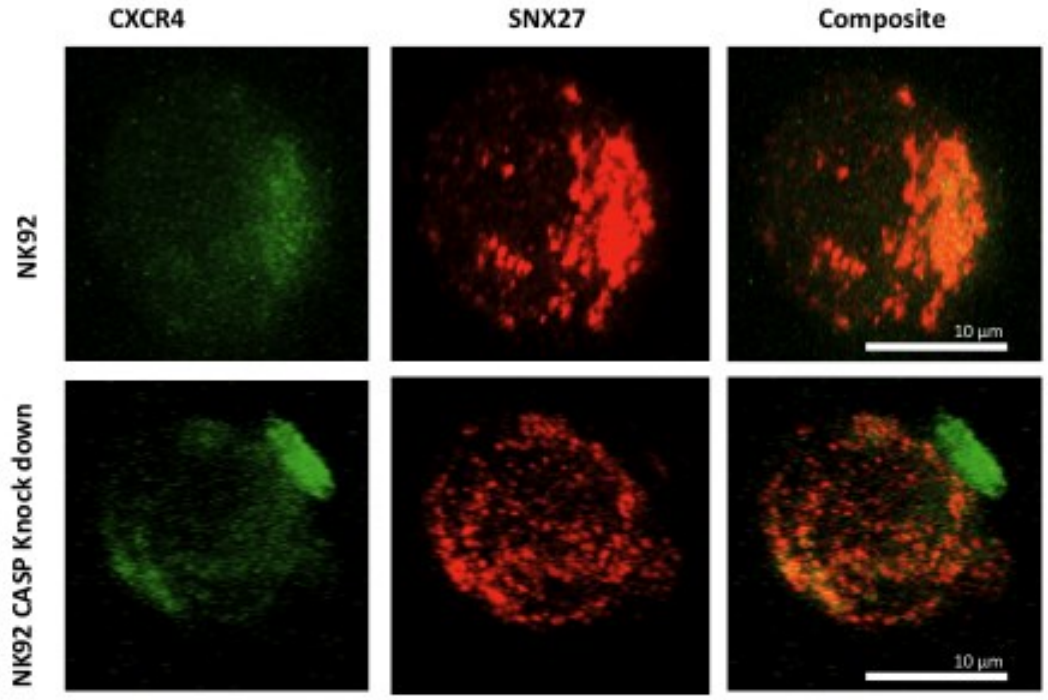

Figure 4.4 Cellular localization of CASPs binding partner SNX27 when CASP is knocked down. CXCR4 clearly localizes toward the leading edge in migrating wild-type NK92 cells, and SNX27 localizes toward the leading edge as well. When CASP is knocked down, CXCR4 fails to localize to a single unified leading edge in NK cells, and SNX27 appears to be dispersed uniformly throughout the cytoplasm of the cell. These figures are representative of 10 different cells (for each line) that were subjected to the same parameters.

4.3.3 Cellular localization of SNX27 during NK cell migration when CASP is knocked down

SNX27 does not polarize to the leading edge of migrating NK-cells *in vivo* when CASP is knocked down. Leading edge marker CXCR4 clearly indicates the leading edge of the migrating NK cell, however SNX27 is uniformly distributed throughout the cytoplasm (Fig 4.4). SNX27 therefore distributes independently of the G-protein coupled chemokine receptor and does not have a role in its sorting to the leading front of the membrane. We have previously shown that CASP and SNX27 co-localize in resting lymphocytes (MacNeil, Mansour, & Pohajdak, 2007), however, it was difficult to see co-localization in migrating NK cells due to potential steric hindrance caused by protein complexes formed with CASP.

4.4 Discussion

Natural killer cells act as the immune systems first line of defense against tumors and virus infection. In contrast to neutrophils and monocytes, natural killer cells may leave tissues through lymph, emigrate from the blood stream into lymphoid and non-lymphoid tissues, and recirculate multiple times during their life history (Le Bouteiller et al., 2011). It is essential for them to be able to migrate into peripheral tissues in order to come into contact with targets. When contact is made they can elicit either a kill-response or secrete cytokines to recruit other immune cells to combat infection or disease. Without the ability to do so, the immune system would be severely compromised.

CASP is shown to have an essential role in NK cell migration *ex vivo*. When CASP is not present, NK cells are no longer capable of migrating through a size-selective

membrane representative of transendothelial migration. CASP is shown to polarize toward the leading edge of migrating cells, however it does not interact or have a role in the polarization of the chemokine receptor CXCR4 on the cell surface. SNX27 does not polarize toward the leading edge when CASP is not present. It appears that CASP has a role in SNX27 sorting and polarizing in migrating cells.

There have been two CASP knockout models produced in mice. Lymphocytes that were harvested from the CASP knockout mice were largely unaffected from wild type when tested in the traditional Boyden chamber migration assay like the one performed in this study (Boehm et al., 2003). It should be noted that NK cells only make up approximately 3-5% of the overall lymphocyte population, and their specific inhibition of migration would not be evident when observing a total lymphocyte count (Berrington et al, 2005). Real-time PCR has shown that normal CASP expression in mice (knockout model organism) is markedly lower than in humans, particularly in the thymus (~7-fold) and lymph node (~20-fold) (Watford et al, 2006). However, *in vivo* mouse CASP knockout models show that overall lymphocyte migration to targeted sites was significantly reduced when challenged with Moloney murine sarcoma virus (Boehm et al., 2003). The contrasting results for *ex* versus *in vivo* shows that there is significance in the microenvironment in lymphocyte migration when CASP is not present.

What is also of note is that CXCR4 appears to have a greater expression at several different localized fronts on the cell, perhaps indicating CASP acts in recycling the membrane protein from the membrane to localize it at one single unified leading edge. CASP's binding partner cytohesin-1 has been shown to have a role in cytoskeletal-rearrangement and endocytic trafficking, and it has previously been shown that in Jurkat

cytotoxic T-cells, CASP shows a cytoplasmic and vesicular localization associated with the cell cortex, a cytoplasmic domain directly below the plasma membrane, that is usually rich in actin filaments (Boehm et al., 2003). ARNO, a family member of Cytohesin-1 has a role in actin remodeling (Frank, Hatfield, & Casanova, 1998), and CASP could have a role in the restructuring of the cell and the formation of the leading edge and pseudopods toward an attractant by its interaction with ARNO.

GRASP, CASP's homologue has also been shown to have an essential role in epithelial cell migration. In a very recent study by Attar and Santy (2013), GRASP has been shown to bind to both Dock180 and cytohesin 2 and bridges the two guanosine exchange factors. What is most interesting is that GRASP interacts with Dock180 independently of its ability to bind cytohesin 2 via the SH3 domain of Dock180 and the proline rich domain of GRASP, located very near to the n-terminal PDZ domain (Attar & Santy, 2013). Knockdown of GRASP inhibits human growth factor-induced migration in MDCK epithelial cells. This is interesting because there is yet no defined binding partner for the PDZ binding domain of CASP, hinting and providing further evidence that CASP is potentially interacting with some other lymphocyte specific protein that acts in migration.

Further studies should be carried out to see if CASP has a role in the sorting of membrane proteins, adhesins or vesicles carrying proteins that help in invasion into tissue.

Chapter 5 The Role of CASP in NK Cell Degranulation and Cytotoxicity

5.1 Introduction

Natural Killer (NK) cells are a subset of lymphocytes that play a central role in innate responses to tumors and infections. They are a key link between the innate and adaptive immune response as they secrete large amounts of cytokines and chemokines that can shape and drive the ensuing adaptive immune response.

NK cells respond to signals generated by activating and inhibitory receptors on their surfaces to specifically target infected or tumor cells. Upon making contact with a target infected cell, NK cells release the membrane disrupting protein perforin, and proteolytic serine proteases known as granzymes from secretory granules (Trapani & Smyth, 2002). A second essential function of NK cells, especially early in viral infections, is to release antiviral cytokines, such as IFN-γ and TNF-α, as immunodefensive agents that activate and recruit other inflammatory cells (Biron et al., 1999).

The pathways of cytokine and cytotoxic granules secretion are distinct from one another in later maturation, however may share common origins (Krzewski & Coligan, 2012). Cytokines in post-Golgi compartments colocalize with markers of the recycling endosome (RE). REs are functionally required for cytokine release because inactivation of RE associated proteins Rab11 and vesicle-associated membrane protein-3 blocked cytokine surface delivery and release (Reefman et al., 2010). In contrast, cytotoxic granules that include granzymes and perforin are located in preformed granules until they come into contact with a target cell. The cytotoxic granules are then released in a

polarized fashion at the Immunological Synapse (IS). This specific release contrasts cytokine release, for distinct carriers transport both IFN-γ and TNF to points all over the cell surface, including within the synapse, for non-polarized release (Reefman et al, 2010). The separation of these pathways is an important mechanism allowing NK cells to simultaneously kill target cells and to recruit other immune cells, with different sets of secreted mediators, and thus to fulfill their role in immunity (Lodoen & Lanier, 2006).

It has been shown that CASP has a dynamic role in cellular polarization during migration and cytotoxicity. It is thought to localize to the endosomal network due to its binding partner SNX27, it has also been shown that ARNO, another of CASP's binding partnersin the cytohesin family, is located at early endosomes ultimately playing a role in apical endocytosis (Hurtado-Lorenzo et al., 2006). CASP may have a role in the sorting of early endosomes and knocking it down could cause the inability to sort early endosomes to mature as either cytotoxic granules or cytokine secretory endosomes, for both cytokine secretion and lysosomal maturation to secretory lysosomes (AKA cytotoxic granules).

In this study we show that CASP has a role in overall NK cell cytotoxicity when challenged against tumor cell line K562. In addition when CASP is lacking, the secretion of IFN-γ is reduced when stimulated by either tumor cells or degranulation inducing artificial stimuli.

5.2 Methods

5.2.1 Immunocytochemistry

Two x 10^5 cells were allowed to adhere to poly-L-lysine coated slides (Lab Scientific) for 15 min at 37 °C. Cells were then fixed with 4% paraformaldehyde. Cells

were permeabilized with 0.2% Triton X-100 in PBS (PBS–TX) for 5 min. Slides were blocked with 1% BSA in PBS–TX and primary antibodies/antisera were incubated at room temperature for 30 min. Slides were then washed before applying Cy3 and Alexa 488 conjugated secondary antibodies (Jackson ImmunoResearch and Molecular Probes). Finally, slides were washed extensively before application of VectaShield mounting medium (Vector Laboratories).

5.2.2 Conjugation assays

For conjugation assays, 5 x 10^5 killer cells NK92 were combined with the target cell line (K562) at a 1:1 ratio and centrifuged for 5 min at very low speed for conjugate formation. Conjugates were incubated for either 15 or 30 min at 37°C prior to incubation on poly-L-lysine coated slides and immunocytochemistry, as described above. All cells were viewed and imaged using an LSM 510 laser scanning confocal microscope with a 63x oil objective lens (Zeiss).

5.2.3 IFN-γ ELISA

The following protocol was adapted from eBioscience Human IFN gamma ELISA Ready-SET-Go! assay kit. First, a 96 well plate was coated with 100 μl/well of capture antibody in Coating Buffer (eBioscience) overnight at 4 °C. The next day, 10^5 cells /well of Natural killer cell lines of NK92, NK92 Scrambled, and NK92 CASP knockdown were and stimulated with either tumour cells (K562) or artificial stimulus (phorbol-12-myristate 13-acetate [PMA] and ionomycin) to cause degranulation. For tumor cell stimulation, an effector to target ratio of 10:1 was used. Cells were centrifuged at 400g for 5 minutes to allow for contact and incubated for 3 hours. Cells stimulated artificially were incubated for 3 hours in 0.5 μg/ml of Ionomycin and 2.5 μg/ml of PMA in IL-2

supplemented media. Control cells were simply incubated for 3 hours without stimulation. The IFN-γ ELISA 96 well plate was washed 5 times with wash buffer, (0.5% Tween-20 in PBS) then blocked with Assay diluent for 1 hour. After incubation the cells were centrifuged; the supernatant of each sample was removed and added to the coated wells in the 96 well plate, and the remaining pellet was resuspended in 0.1% TritonX-100 in PBS, centrifuged at 16 000 rpm for 2 minutes and the supernatant was added to the coated 96 well plate. The plate was then incubated overnight at 4 °C.

The following day, the supernatant were removed and the plate was washed 5X with wash buffer. 100 µl of detection antibody was then added to the wells (diluted 1:250 in assay buffer) for 1 hour at room temperature. The wells were then washed 5X and 100 u of avidin-horse radish peroxidase (HRP) (diluted 1:250) were added to the wells for a half hour at room temperature. The avidin-HRP was removed and the wells were washed again 7X in wash buffer. 100ul of substrate solution was then added to the wells and allowed to incubate at room temperature for 15 minutes. After incubation, 50 µl of stop solution (1 M acetic acid) was added to the wells and the plate was read on a SpectraMax plus 384 at 450 nm.

5.2.4 LDH cytotoxicity assay

The following protocol was adapted from Promega Cytotox 96 Non-Radioactive Cytotoxicity Assay. The tumor cell line K562 (10^4 cells per well) was seeded into a Costar V-bottom 96 well plate in Optimem media (Gibco, Life sciences) supplemented with 5% FBS. Natural killer cell lines NK92, NK92 Scrambled, and NK92 CASP knockdown were added to wells containing the tumor cells ranging from effector: target cell ratios as follows: 20:1, 10:1, 5:1, 2.5:1, and 1:1. Controls of just the NK cell lines at

the different ratios (Effector spontaneous release), just the media, and just K562 (Target spontaneous release and maximum release) were also added to the plate. The plate was centrifuged at 1000 RPM for 4 minutes for the NK cells to come into contact with the K562 cells. The plate was then incubated at 37° C 5% CO_2 for 5 hours. 45 minutes prior to the end of the incubation time, 10 µl of lysis buffer (0.1% Triton-x 100 in PBS) were added to just K562 for 100% lysis. After incubation, the plate was again centrifuged at 1000 RPM for 4 minutes. 50 µl aliquots from all wells were then transferred to a 96-well flat bottom Costar enzymatic assay plate. 50 ul of Substrate Mix (Promega Cytotox kit) was then added to each well. The plate was then incubated for a half hour in the dark at room temperature. 50 ul of Stop solution (1 M acetic acid) was then added to each well. Absorbance was then read at 490 nm on SpectraMax plus 384.

Percentage cytotoxicity was calculated as follows:

$$\frac{\text{Experimental- Effector Spontaneous- Target Spontaneous}}{\text{Target Maximum- Target spontaneous}} \times 100$$

5.3 Results

5.3.1 CASP is essential for NK cell cytotoxicity

NK cell cytotoxicity was quantified by measuring lactate dehydrogenase (LDH) release from tumor cells undergoing apoptosis. NK cells (wild-type NK92, and CASP knockdown NK92) and tumor (K562) cells were conjugated for a period of 5 hours. From the experiment, NK cell cytotoxicity is reduced from 60% to 10% at an effector: target cell (E:T) ratio of 20:1 (Fig 5.1) when CASP is knocked down.

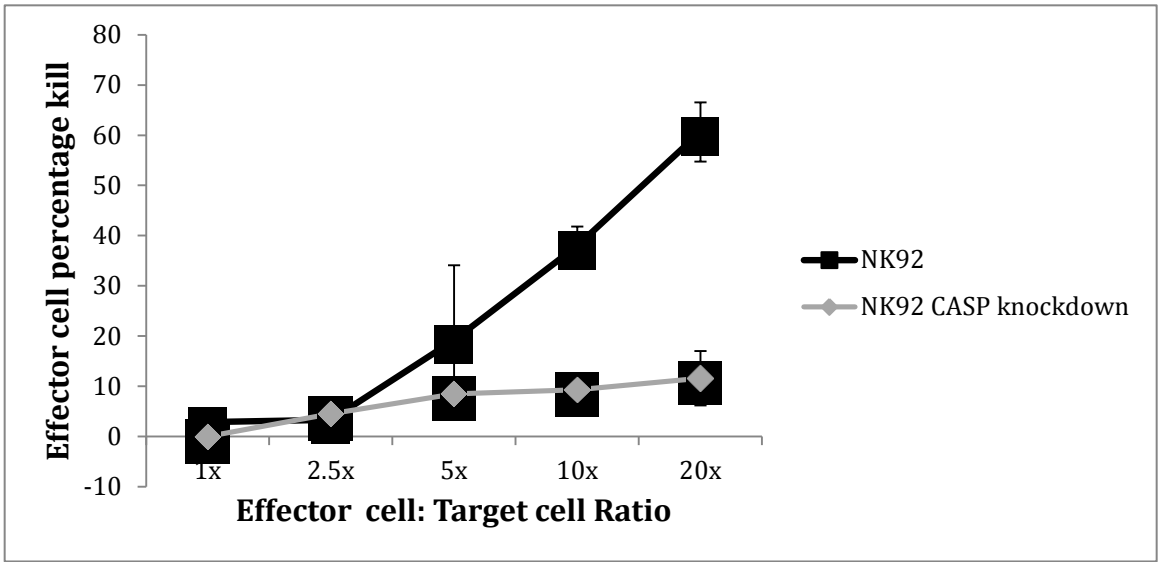

Figure 5.1 Kill curve comparing wild-type NK92 cells and a CASP shRNA stable knockdown line. Kill efficiency is measured through the release of LDH into the environment from tumor cells undergoing apoptosis. Cells were tested at various effector (NK92) to target tumor cell (K562) ratios, ranging from 1:1 to 20:1. Overall kill efficiency is reduced significantly when CASP is knocked down in NK cells that are artificially conjugated and come into contact with tumor cells by means of centrifugation. This figure is representative of three separate experiments.

At lower effector to target cell ratios there is little difference seen. The drastic change seen in tumor cell survivability indicates that CASP has some role in the cytotoxic and degranulation pathways. This result provided reason for further study into CASPs specific molecular role in cytolysis.

5.3.2 CASP does not mediate the release of cytotoxic granules at the Immunological synapse

CASP does indeed polarize to the IS in NK cells when conjugated to tumor cells (Fig 5.2). We have previously shown that SNX27 also polarizes to the IS, however it does so independently of cytotoxic granules (MacNeil & Pohajdak, 2007). When CASP is knocked down, there is nearly no detectable signal in NK-tumor cell conjugates.

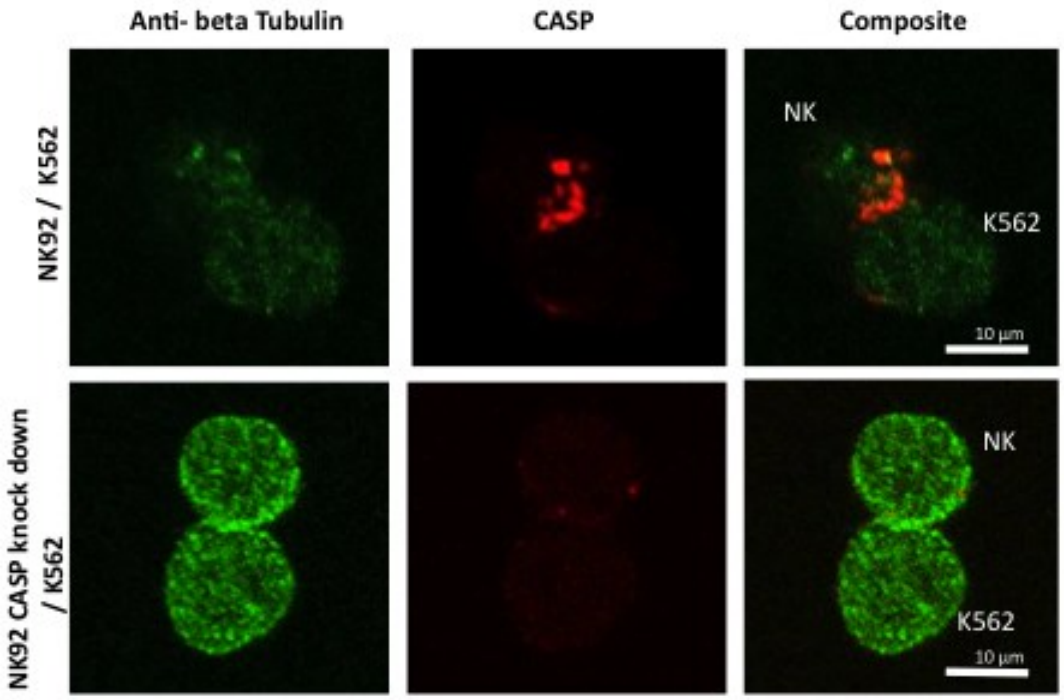

Figure 5.2 Cellular localization of CASP during tumor cell engagement between wild-type NK cells and CASP knockdowns. NK cell conjugation and cytotoxicity phenotypes viewed under 63x magnification with a Zeiss 510 laser-scanning microscope. Wild-type NK92 cells demonstrate polarization of CASP to the immunological synapse (IS). PDZ 2F9 is antisera against CASP. NK cells lacking CASP demonstrate no detection of CASP polarization at the IS. These figures are representative of 25 cell conjugates viewed in both wild-type NK92 and CASP knockdown NK92, tumor cell (K562) conjugates. Conjugation was performed three times for each cell line.

It is known that in natural killer cells cytotoxic granules are synthesized before cell activation, and are stored and constrained into pools located within the cytoplasm. Upon stimulation, the granules polarize along with the microtubule-organizing center and other cytoskeletal machinery (Stinchcombe, Majorovits, Bossi, Fuller, & Griffiths, 2006). In NK cells where CASP is knocked down, cytotoxic granules containing perforin still polarize to the IS, indicating the CASP does not have a direct role in cytotoxic granule movement (Fig 5.3).

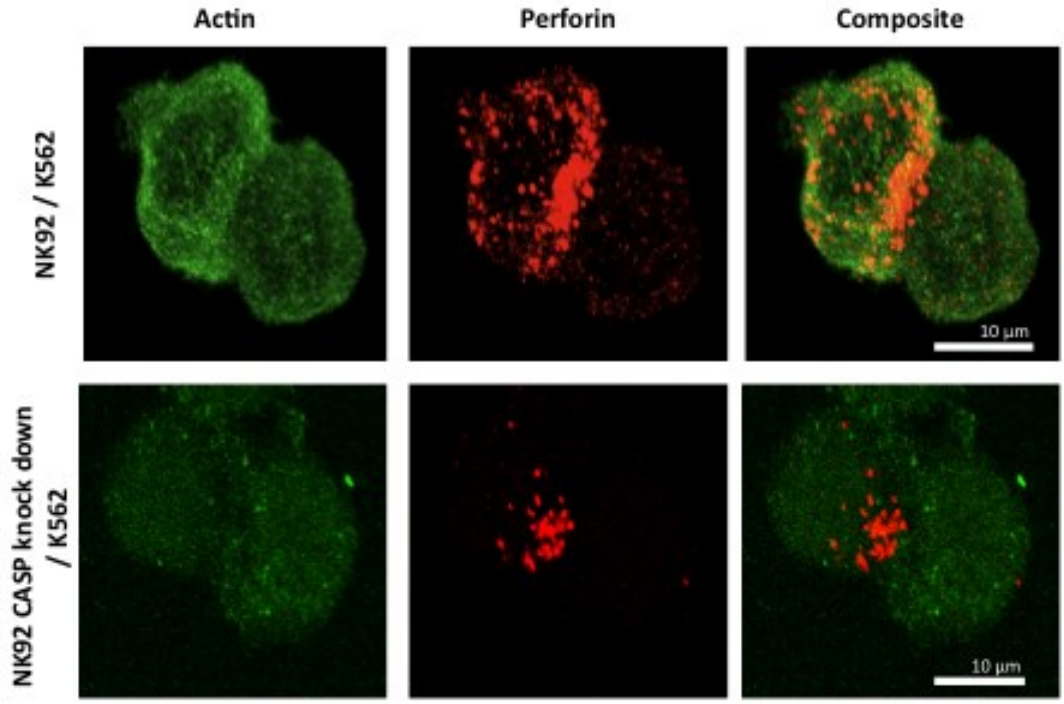

Figure 5.3 Cellular localization of cytotoxic granules containing perforin between wild-type and CASP knock down cell lines. NK cell conjugation and cytotoxicity phenotypes viewed under 63x magnification with a Zeiss 510 laser scanning microscope. Wild-type NK92 cells demonstrate polarization of cytoxic granules containing perforin to the immunological synapse (IS). NK cells lacking CASP demonstrate polarization of perforin containing granules to the IS, but a reduction in perforin signal. Again, conjugation was performed three times for each cell line.

5.3.3 The release and degranulation of the natural killer cell cytokine IFN-γ is mediated by CASP

Endogenous release of IFN-γ is decreased in CASP knockdown cell lines when either artificially stimulated or challenged with tumour cell line K562. The non-specific shRNA stable cell line showed similar release as the wild-type NK92 cell line, approximately 40 000 pg/ml of IFN-γ when artificially stimulated, and 5000 pg/ ml when stimulated with tumor cells (Fig 5.4).

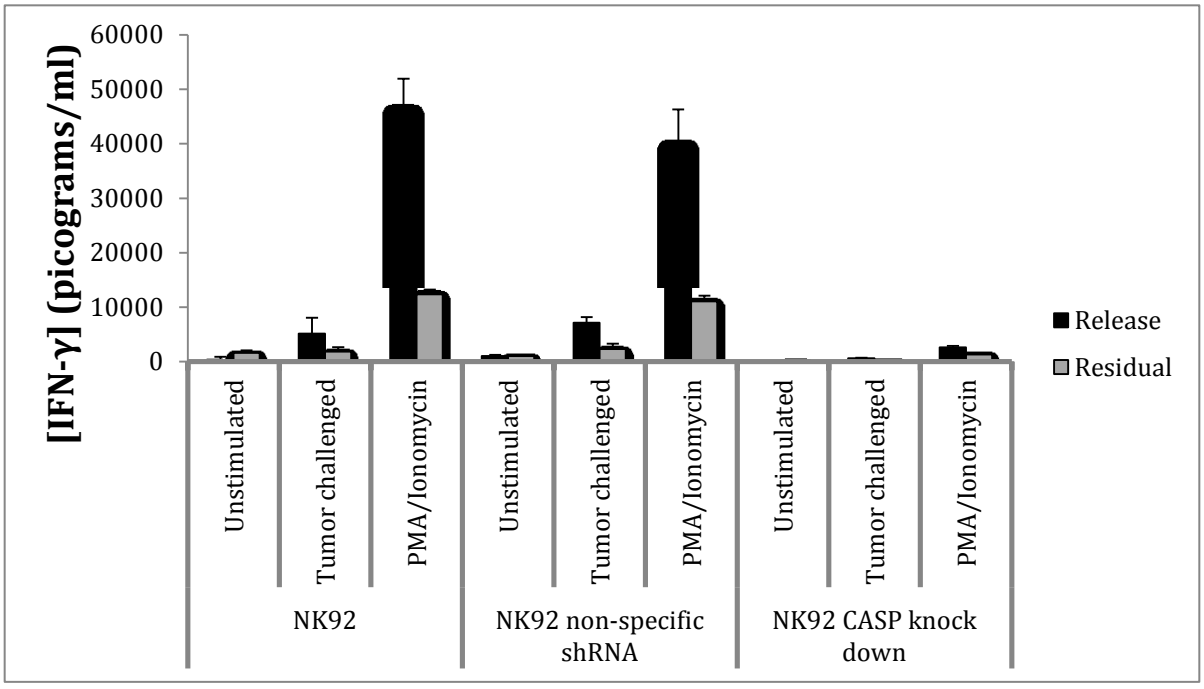

Figure 5.4 Degranulation and IFN-γ release comparisons between wild-type NK92 cells, stable non-specific shRNA NK92 tranduced cells and stable CASP shRNA knockdown NK92 cell line under different stimuli. In both the non-specific shRNA line and the wild-type NK92 cell lines, stimulus by ionomycin and PMA caused massive degranulation as compared to stimulus with 10:1 ratio of effector (NK92): tumor target (k562) cell lines. Cell lines were stimulated for 3 hours with either the artificial stimulus or tumor cell contact. Residual amounts of IFN-γ were collected by total cell lysis with 0.5% Tritonx-100 in PBS. Overall degranulation in stable CASP shRNA knockdown cell line is significantly impaired compared to wild-type and non-specific lines. This figure is representative of three separate experiements. Comparison of the non-specific NK92 transformed line to NK92 did not show significant difference. CASP knockdown showed p values of 0.026 and 0.0001 as compared to NK92 when stiumulated with tumor cell line K562 or PMA/ionomycin, respectively.

Release of IFN-γ by artificial stimulation is also impaired when CASP is knocked down, to near undetectable levels. NK cells artificially stimulated with PMA and Ionomycin have higher production and release of IFN-γ as compared to stimulation with tumor cells for the same incubation time of three hours due to the direct stimulation of the JAK-STAT cytokine release pathway. In the literature, systemic levels of IFN-γ release by NK cells typically peaks around 6 hours in mouse models after endotoxin stimulus, so the three hour incubation with tumor cells is representative of max release on a cellular level (Varma et al, 2002). After measuring extracellular levels of IFN-γ, the residual

intercellular levels of IFN-γ after cell lysis were measured. Residual levels are markedly lower in CASP knock down NK92.

5.4 Discussion

CASP has a role in killing tumor cells as well as the release of IFN-γ. Confocal analysis of cellular distribution of perforin shows that it continues to polarize to the IS when conjugated with tumor cells, ruling out that CASP has a role in cytotoxic granule movement.

Since IFN-γ degranulation is reduced in CASP knockdowns, this could have an overall effect on NK activation and kill response. In an innate immune response, NK cells are the predominant lymphocyte that secrete IFN-γ. IFN-γ release then recruites macrophages and activates superoxides, promotes Th_1 differentiation, and triggers normal cell expression of MHC Class I and II (Schoenborn & Wilson, 2007). Most importantly though, is that NK cells self stimulate with IFN-γ (Wang et al, 2012). When CASP is knocked down, the inability to secrete IFN impairs a more robust NK activation, and could be the reason why the NK cells are not more effective in killing when challenged with the K562 tumor cell line. IFN-γ stimulation of NK cells has been shown to enhance tumor cell adhesion through the upregulation of ICAM-1 in target antigen presenting cells (Wang, Jaw, Stutzman, Zou, & Sun, 2012). This links the two processes of NK cell degranulation of cytokines and its effect on NK cell cytotoxicity in people who could be suffering from lymphocyte cancers like lymphomas or leukemia.

What is also interesting is that several studies show CASP plays a role in mediating detachment of cell–cell interactions between dendritic cells (DC) and cytotoxic T cells (Heib et al, 2012). CASPs binding partner Cytohesin-1 supposedly binds to the β-

2 chain of lymphocyte-function associated antigen-1 (LFA-1), enhancing the binding activity of ICAM-1, a receptor essential for DC binding. Inhibition of CASP expression in Jurkat cytotoxic T cells showed T cell-DC adhesion increase in time it took for the cells to break contact (Hofer et al., 2006). This shows CASP may have a significant role in the attachment of cytotoxic cells to target antigen-presenting cells that share lymphoid lineage. CASP mediates the release of cytokines that upregulate adhesion molecules in target cells, and then controls the detachment at the IS in order to move on to the next cell, to continue killing, resulting in a strong immune response.

Although I have not shown CASP is directly involved in cytotoxic granule polarization and delivery to the IS, CASP does have a role on overall kill efficiency. This could be due to CASP having a role in the recycling of cytotoxic effector molecules. Cytotoxic granule delivery is a semi conservative event. Complete release of granule contents does not always occur, which may promote the efficient recycling of lytic components into the natural killer cell, conserving the ability to target additional cells (Liu et al, 2011). CASPs binding partner SNX27 has been shown to play a role in the recycling of endosomes to the plasma membrane (Temkin et al., 2011). Knocking down CASP could be interrupting this recycling machinery. The reduced amount of perforin seen in the CASP knockdowns could be due to a complete release event, depleting internal stocks of preformed granules. With CASP knocked down, recycling of cytotoxic molecules could be preventing internalization to pools that would be delivered upon the next contact with tumor cells.

Additional avenues to explore for a more complete insight into the role of CASP in cytotoxicity would be to look for the degranulation marker lysosomal associated

membrane protein-1 (LAMP-1, CD107a) (Winchester, 2001). LAMP-1 is a highly glycosylated membrane protein that represents approximately 50% of the proteins in the lysosomal membrane (Fukuda, 1991; Kannan et al., 1996). Members of the LAMP family have short cytosolic tails, which are thought to interact with trans Golgi mediators that are involved in sorting and targeting proteins to the lysosomal pathway, the precursor of cytotoxic granule maturation. An increase in overall surface expressed of CD107a after stimulation in CASP knockdown NK cells compared to wild-type would indicate CASP's role in this mechanism, by possibly recycling effector molecules and re-releasing them. Also, the quantification of granzyme B release in a similar fashion to the IFN-γ, would provide a more robust involvement of CASP in cytotoxic granule recycling. If more GrB is released extra-cellularly compared to wild-type, that would indicate that CASP knockdown cells can not recycle GrB. Also, immunocytochemistry could be used to explore the cellular localization of these processes.

Cytotoxic granules largely have the same membrane proteins and proteases found in generic lysosomes, however, they are distinct due to the fact that recycling endosomes serve to separate the terminal stages of secretory pathways to regulate and segregate cell killing and cytokine release (de Saint Basile et al, 2010). CASP is known to be involved in both recycling endosome trafficking and sorting through SNX27, as well as early endosome trafficking through Cytohesin-1 and ARNO. Knocking down CASP results in the aberrant maturation of endosomes that carry cytokines that are to be secreted (IFN-γ) (Fig 5.5). Although CASP is not directly involved in delivering the cytotoxic payload to tumor cells, the failure of releasing cytokines necessary for activation of NK kill response has a drastic effect on NK cells ability to clear infection.

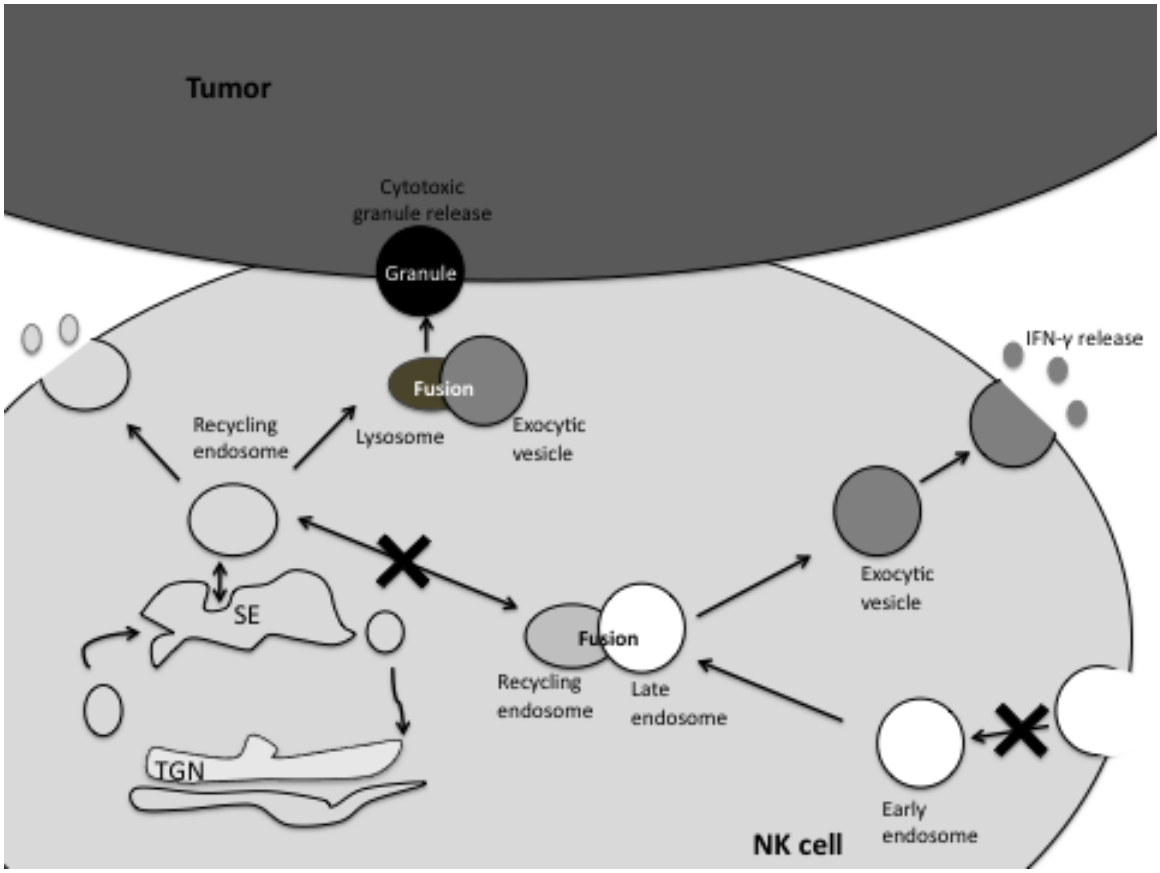

Figure 5.5 Model depicting the involvement of CASP in cytotoxic granule formation and cytokine vesicle release. CASP is involved in the trafficking of early endosomes through its interaction with the Cytohesin/ARNO family of proteins, as well as recycling endosomes through its interaction with SNX27. The lack of CASP in NK cells may potentially affect the formation of cytotoxic granules, and the release of cytokines. Lack of CASP may prevent necessary early endosome trafficking required for the maturation and release of exocytic vesicles containing cytokines involved in immune cell recruitment and activation, such as IFN-γ. This impairment of proper vesicle sorting and shuttling significantly disables NK cell function in immune responses. TGN= Trans Golgi Network, SE= Sorting Endosome

Chapter 6 Conclusion

The work presented here is the first to report the direct role and effects of CASP in cytotoxic cell function. These changes in phenotype when CASP is knocked down lead to new perspectives and pathways of investigation, and shed light on the potential role of CASP in NK cell migration and cytotoxicity. The discovery of CASPs ubiquitination and targeting to the proteasome for degradation and also it's cleavage by granzyme B is intriguing because it shows alternative modification of the protein after translation, adding to an already complex portrait of described molecular interactions and regulation.

The specific granzyme B cleavage of CASP would disrupt CASP interaction with SNX27 and effectively disrupt endosomal trafficking in NK cells, also preventing interaction with cytohesin/ARNO (MacNeil et al, 2007; Mansour et al, 2002), and potentially the fusion, trafficking, sorting and maturation of cytokine vesicles and cytotoxic granules. Granzyme B is located within granules, while CASP is cytoplasmic or associated with the exterior of endosomes. This cleavage would only occur if the granzyme was released within cytotoxic cells through the disruption of granule formation, or by recycling from the immunological synapse. Granzyme B can also become cytosolic if delivered to another lymphocyte that was infected such as a dendritic cell. Usually, targets of granzymes and the caspase cascade generally disrupt critical functions for the cell, such as tubulin structure (Adrain et al, 2006; Bovenschen et al., 2013; Goping et al, 2006).This targeting of CASP by granzyme B suggests that disruption of CASP interaction with SNX27 may have a role in cell death and apoptosis, perhaps interrupting vesicle shuttling of necessary cellular functions.

The ubiquitination of CASP is an indication that CASP is targeted by regular cellular processes, and targeted to the proteasome for degradation recycling. This degradation by the proteasome is most likely the largest player in the regulation of CASP expression, giving reason why it has such a generally low basal expression. Limiting endogenous levels of CASP by ubiquitination could limit the release of cytokine and cytotoxic granules before proper stimulation and target cell interaction has occurred. The effects of CASP regulation through ubiquitination could be tested by inhibiting the proteasome with inhibitors such as bortezomib, and quanitifying if cytokine release is increased without prior stimulation.

Recently, it has been shown that in patients with frequently recurring Herpes, NK cell degranulation is reduced (Murugin et al., 2011). It has also been shown that in infected dendritic cells, herpes simplex virus type 1 induces the rapid degradation of CASP (Theodoridis et al., 2011). This degradation of CASP has been linked to the impairment of dendritic cell migration and adhesion. Herpes virus could also be targeting CASP specifically to suppress one of the key players in innate immune defense in order to establish itself for infection by rendering NK cells incapable of migrating for detection, or incapable of degranulation to recruit other immune cells.

What is interesting is that there is no NK cell function of migration or cytotoxicity recovery due to GRASP, the neuron-specific homologue of CASP. The shRNA target that works in my CASP knockdown does not target mature GRASP mRNA (not shown). We have shown that GRASP is expressed in extremely low levels in lymphocytes (unpublished data), and this drastic loss of NK cell function after knockdown in cytotoxicity and migration indicates that GRASP does not rescue CASP function.

Further studies should be carried out to determine how exactly CASP is playing a role in migration of NK cells. The loss of the ability may be due to the inability to restructure overall cellular morphology through improper shuttling of necessary receptors and proteins to the leading edge. This identification of CASP involvement in migration opens up a new field of research.

There have been several limiting factors in working with CASP that have restricted its research. Being a small adaptor protein that is often embedded in complexes, steric hindrance plays a major role in its detection with antibodies *in situ*. It also has very poor antigenic properties, explaining why I have tested both homemade and nearly every commercially available polyclonal and monoclonal antibody on the market for detection by Western blot and immunofluorescence.

CASP has been a particularly difficult protein to assign a specific role in the cell. Current research has shown that CASP has been studied in many different experimental approaches, and many different cell lineages. CASP was first identified as a cytotoxic lymphocyte specific protein, and the work I have presented in this thesis has provided new insight into the role of CASP in characteristic NK cell function. My work has demonstrated an unreported direct involvement of CASP in NK cell migration and cytotoxicity, something that will pave the way for further study on the role of this protein in trafficking and signaling processes specific to cytotoxic lymphocytes. I am confident that my contributions to this field will produce new future investigations and provide useful information to the scientific community in whole.

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