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Cytohesin-associated scaffolding protein (CASP) is involved in migration and IFN-γ secretion in Natural Killer cells

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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 highly expressed lymphocyte adaptor protein that forms complexes with vesicles and sorting proteins including SNX27 and Cytohesin-1. In this study we show that by using stably integrated shRNA, CASP has a direct role in the secretion of IFN-γ, and NK cell motility and ability to kill tumor cells. CASP polarizes to the leading edge of migrating NK cells, and to the immunological synapse when engaged with tumor cells. However, CASP is not associated with cytotoxic granule mediated killing. CASP is a multi-faceted protein, which has a very diverse role in NK cell specific immune functions.

1. Introduction

Natural Killer (NK) cells are a subset of lymphocytes that play a central role in innate responses to tumors and viral 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 are highly motile cells that patrol lymphoid and peripheral organs and tissue, ready to react to stimulus by infection. Lymphocyte migration is important for both invasion into tissue from the blood, and infected cell engagement. Migration is a complex polarized event, for 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 [1]. NK cells respond to signals generated by inhibitory receptors on their surfaces to specifically target infected or tumor cells [2]. Upon making contact with a target cell, NK cells release the membrane disrupting protein perforin, and proteolytic serine proteases known as granzymes from secretory granules [3]. 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 [4].

The pathways of cytokine and cytotoxic granules secretion in Natural Killer cells are distinct from one another in later maturation, however may share common origins [5]. 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 [6]. 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) [7]. This specific release contrasts cytokine release, for distinct carriers transport both IFN-γ and TNF to points all over the cell surface for non-polarized release. The separation of these pathways is an important mechanism allowing NK cells to simultaneously kill target cells and to recruit other immune cells [8].

Before NK cells are able to release their toxic payload from cytotoxic granules, they must be able to travel towards stimuli such as infected cells. 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

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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 [9].

Cytohesin associated scaffolding protein (CASP a.k.a CYBR, CYTIP, PSCDBP, B3-1) is a lymphocyte specific adaptor protein 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. 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 [10]. When CASP is knocked down in ccRCC, there is complete inhibition of lung colonization, indicating a role in tumor metastasis. According to Vanharanta et al. [10], the epigenetic expansion in ccRCC of CXCR4 allows for chemotaxis while CASP promotes survivability.

In this study, we show CASP polarizes toward the leading edge of migrating NK cells, associating in the same pseudopod as the chemokine receptor CXCR4. A CASP knockdown causes NK cell migration to be impeded. We also show that CASP has a role in overall NK cell cytotoxicity when challenged against tumor cell line K562. In addition when CASP is knocked down, the secretion of IFN-γ is reduced when stimulated by either tumor cells or degranulation inducing artificial stimuli.

2. Materials and methods

2.1. Cells and antibodies

Human cell line NK92 was a gift from Dr. D. Burshtyn, (University of Alberta). NK92 was grown in RPMI 1640 supplemented with 12.5% FBS, 12.5% horse serum (Gibco), 100 units/mL of IL-2 (PeproTech), and antibiotics. Subcellular marker antibodies used were: anti-CXCR4 1:200 (R&D Systems); rat PDZ-2F9 anti-sera; anti-beta tubulin (Genetech) anti-actin (Sigma).

2.2. Lentivirus

For lentivirus production, HEK 293T cells were plated to 30–40% confluency in 10 cm tissue culture dishes. The next day, cells were transfected with pLKO.1 shCASP (TRCN0000141641) [10] or control lentiviral vectors, along with psPAX2 and pMD2.G packaging vectors. Briefly, 3.3 μg of pLKO.1, 2 μg psPAX2 and 1 μg of pMD2.G were diluted in 500 μl of Optimem media (Life Sciences). In a separate tube, 18 μl of 1 mg/mL, pH 7.0, polyethylenimine (PEI) (Sigma) was diluted with 500 μl of Optimem media. After 5 min, the two tubes were combined and the DNA-PEI mix was left to incubate for another 15 min. Cells were washed twice in PBS and fed 4 mL of serum free, antibiotic free DMEM. Subsequently, the mix was added dropwise to HEK293T cells and the transfection reaction was left to proceed for 6 h. The serum free transfection mix was then replaced with 10 mL fresh DMEM supplemented with 10% FBS. After 2 days the virus containing media was collected, syringe filtered through a 0.45 μm filter, and stored at −80 °C as aliquots.

For infection, 2.5×10^5 cells NK92 cells were incubated in 1 mL of IL-2 media supplemented with 100 mM HEPES buffer, and 8 μg/mL of polybrene. An aliquot (1 mL) of the frozen virus was then added to the cells in a 6 well plate (BD). The plate was then spinoculated at 2000 rpm for 1 h. After infection, the cells were left to recover for 24 h before the media was replaced with 10 mL of IL-2 media. Two days later the cells were subjected to antibiotic selection with 2 μg/mL of puromycin. After 14 days cells grown under puromycin selection were deemed stable transductants. Individual cells were then isolated by dilution and grown out as clonal populations.

2.3. 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, DNA primers qCASP S2 (5′ – AGA TCG GGA AAC CTG CT – 3′) and qCASP AS2 (5′ – GGG GCA ATT AGC TGC ATC ACC – 3′) were used 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, a *t*-test was performed for significance.

2.4. Lysates

Cells were grown ($\sim 1 \times 10^7$ cells/mL) and 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 lysates were then sheared using a 23 gauge syringe and were then sonicated for 2 s on ice. The sample was then centrifuged at 10,000*g* for 10 min and the supernatant was then removed.

2.5. Western blot

NK92 protein lysate (60 μg) were used from the NK92, CASP knockdown, and non-specific cell lines. Loading dye was then added to the samples and then boiled for 5 min. Proteins were resolved on a 15% polyacrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) Hybond-P paper (Amersham Bioscience). Blots were stained in Ponceau to confirm protein transfer to the PVDF paper. The PVDF was then blocked with 5% QuickBlocker Membrane Blocking Agent (Chemicon International) containing 0.2% sodium azide overnight at 4 °C. Blots were rinsed 3 times with tris-buffered saline (TBS) with tween (TBST). The anti-CASP primary antibodies (1:5000 PDZ2F9 rat anti-sera) were then added to the blot for 2 h. Washed blots were then immersed in secondary antibodies (conjugated to horse-radish peroxidase [HRP]) 1 h, washed and then incubated with SuperSignal West femto maximum sensitivity substrate (Thermo Scientific) for various times and exposed films were quantified for protein levels using densitometry with ImageJ software. Significance of knockdown was calculated by *t*-test.

2.6. Immunocytochemistry

NK92 cells (2×10^5 cells) were permitted 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).

2.7. Migration assay

NK92, NK92 Scrambled, and NK92 CASP knockdown (2.5×10^5 cells) were aliquotted in triplicate. Cell were washed in serum-free RMPI 1640 (Life) 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) was 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. Cells were incubated for 6 h and then permeable supports were washed twice with PBS. The supports were emptied of liquid and replaced into the respective wells. Supports were removed and the remaining media containing migrated cells was harvested. The media was centrifuged at 400*g* for 5 min and then resuspended in 100 μl IL-2 supplemented media containing 10% WST-8 Cell proliferation dye (Cayman Chemical) in a 96-well plate. Cells were incubated for 2 h and then readings were taken at 450 nm on a SpectraMax plus 384.

2.8. Cytotoxicity assay

The protocol was adapted from Promega Cytotox 96 Non-Radioactive Cytotoxicity Assay. Tumor cell line K562 was used at target cell line, while Natural Killer cell lines NK92, and NK92 CASP knockdown were used as effector cells. Effector: target cell ratios ranged as follows: 20:1, 10:1, 5:1, 2.5:1, and 1:1. Unstimulated NK cells and tumor cells were used for LDH control and corrected for in LDH release. A *t*-test was performed for significance. Percentage cytotoxicity was calculated as follows:

$$\frac{\text{Experimental} - \text{Effector (NK cell) spontaneous} - \text{Target (K562) Spontaneous}}{\text{Target maximum (NK cell)} - \text{Target (K562) spontaneous}} \times 100 \quad (1)$$

2.9. IFN-γ ELISA

The protocol was adapted from eBioscience Human IFN gamma ELISA Ready-SET-Go! assay kit. Natural Killer cell lines of NK92, NK92 Scrambled, and NK92 CASP knockdown were and stimulated with either tumor 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.

2.10. Conjugation assays

For conjugation assays, 5×10^5 killer cells NK92 were combined with the target cell line (K562) at a 1:1 ratio and centrifuged for 5 min at 250*g* 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 63× oil objective lens (Zeiss).

3. Results

3.1. CASP plays a role in cytotoxicity

CASP knockdown by stably integrated lentiviral shRNA targeted against CASP (TRCN0000141641) showed 70% ($P < 0.05$) reduction in CASP mRNA expression when compared to NK92 and non-specific shRNA control (Fig. 1A). Western blot analysis also confirms also that the CASP protein is knocked-down by 80% ($P < 0.05$) (Fig. 1B).

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

At lower effector to target cell ratios there is little difference seen. The dramatic decrease seen in tumor cell survivability indicates that CASP has some role in the cytotoxic and/or degranulation pathways. NK mediated cytotoxicity against tumors is a

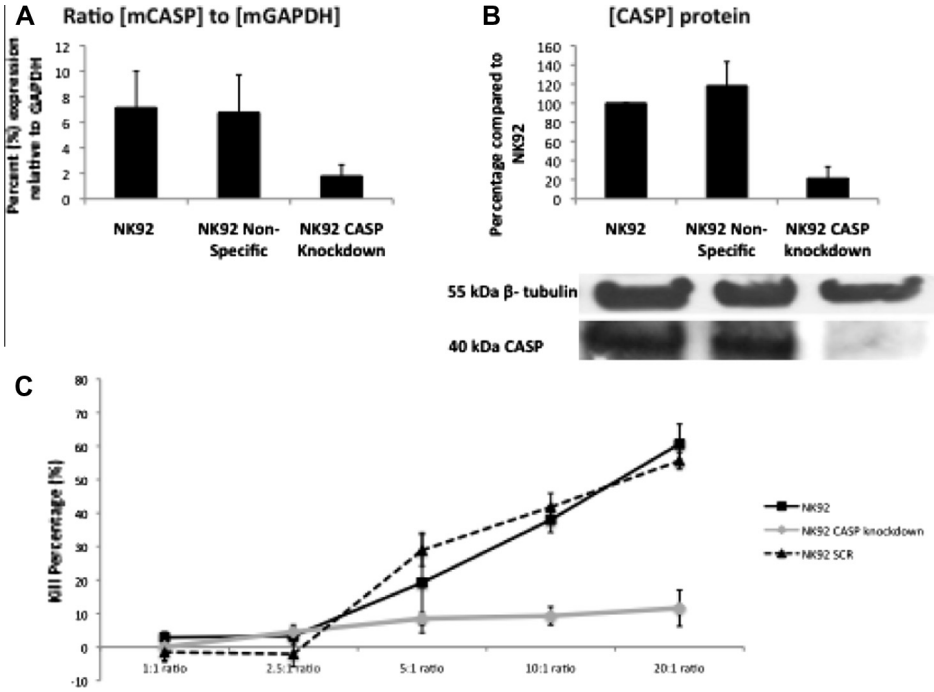

Fig. 1. (A) NK92 CASP knockdown (shRNA clone id TRCN0000141641) has approximately 70% reduction in mRNA expression compared to NK92 cells. This experiment was replicated three times with similar results ($P < 0.05$). (B) CASP protein expression was normalized to tubulin expression. CASP protein expression is knocked down approximately 80% compared to non-transformed NK92 cells ($P < 0.05$). (C) Cytotoxicity was measured through the release of LDH from K562 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 ($P < 0.05$).

complex sequential series of events involving events such as chemotaxis, recognition, activation and secretion, and each step can have profound outcomes on the final result of killing tumors or virally infected cells. We chose to test some of these events to determine the role CASP plays in NK cell mediated cytotoxicity.

3.2. CASP's role in Natural Killer cell migration

For NK cells to effectively kill tumors or virally infected cells they must sense and migrate towards these cells. Chemotacting NK cells can effectively migrate to the chemoattractant SDF-1α. NK cells lacking CASP are significantly impeded in this migration (Fig. 2A). 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.

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

Confocal microscopy shows that CASP polarizes during migration on slides coated with poly-L-lysine when subjected to a SDF-1α (Fig. 2B).

The leading edge of migrating and chemotacting NK cells is denoted by the polarization of the chemokine receptor CXCR4. CASP is present in the area of adhesion of the attached migrating cell, as well as locating in the same area as the pseudopod with CXCR4. When CASP is knocked down there is only an extremely faint signal with the anti-CASP antisera (PDZ 2F9) as expected. Also, when CASP is knocked down, CXCR4 does not appear to polarize uniformly in a single pseudopod in front of the migrating cell, and appears to localize in an extension of the plasma membrane that is distinct from the body of the cell (Fig. 2B).

3.4. The release and degranulation of the Natural Killer cell cytokine IFN-γ involves CASP

Endogenous release of IFN-γ is decreased in CASP knockdown cell lines when either artificially stimulated or challenged with tumor cell line K562. The non-specific shRNA stable cell line showed similar release as the NK92 cell line, approximately 40,000 pg/mL of IFN-γ when artificially stimulated, and 5000 pg/mL when stimulated with tumor cells (Fig. 3) ($P < 0.05$).

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 3 h due to the direct stimulation of the JAK–STAT cytokine release pathway. After measuring extracellular levels of IFN-γ, the residual intercellular levels of IFN-γ after cell lysis were measured. Residual levels are also markedly lower in CASP knock down NK92.

3.5. CASP is not involved in lytic granule polarization

CASP does indeed polarize to the IS in NK cells when conjugated to tumor cells (Fig. 4A). Upon stimulation, the granules polarize along with the microtubule-organizing center and other cytoskeletal machinery [11]. 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. 4B).

4. Discussion

NK cells are sentinels of the immune system and must be able to quickly and independently move in order to interact with self to ensure they are normal cells in a healthy body. When they

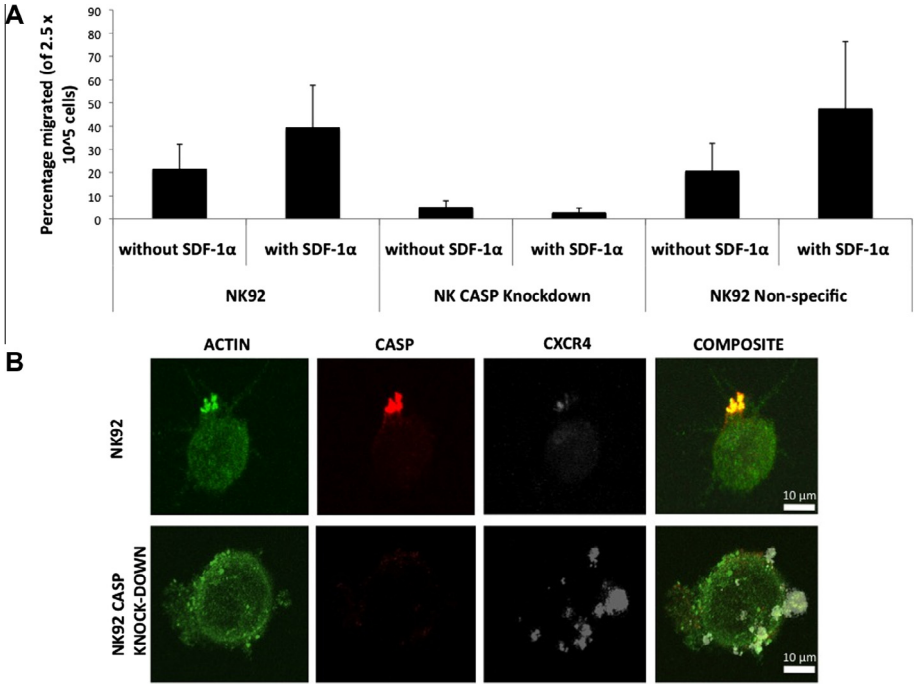

Fig. 2. (A) Migration of NK cell lines through a size selective membrane when stimulated by the chemoattractant SDF-1α. Stable CASP shRNA interference proves to have a significant impairment of migration when SDF-1α is present ($P = 0.026$) compared to the control NK92 cell line. Non-specific shRNA control line also shows similar results to the NK92 cell line. (B) NK cell migration phenotypes viewed under 63× magnification with a Zeiss 510 LSM stacked image of 10 μm. 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. NK cells lacking CASP demonstrate no formation of pseudopods nor a distinct leading edge as denoted by the chemokine receptor CXCR4.

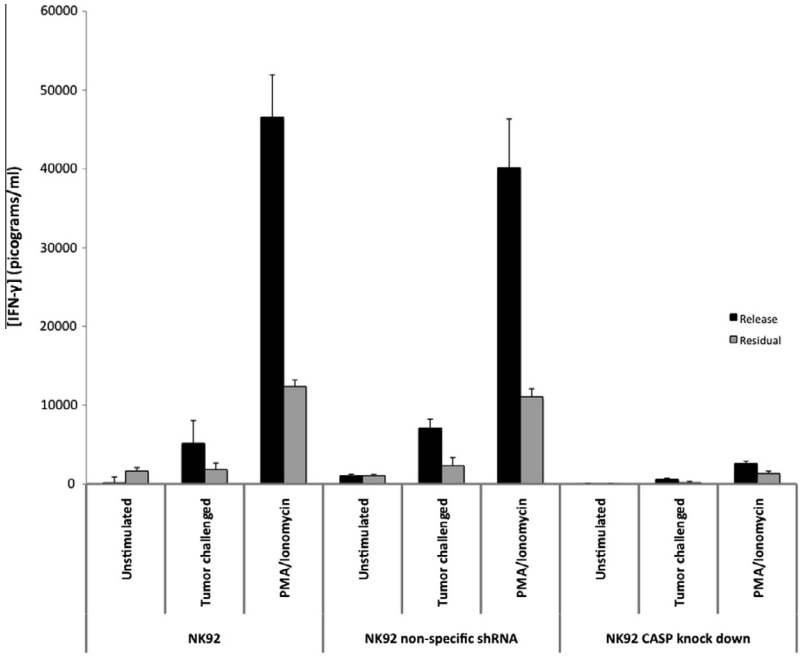

Fig. 3. Degranulation and IFN-γ release comparisons between NK92 cells, stable non-specific shRNA NK92 transduced cells and stable CASP shRNA knockdown NK92 cell line under different stimuli. In both the non-specific shRNA line and the NK92 cell lines, stimulus by ionomycin and PMA caused massive IFN-γ secretion as compared to the natural (K562) stimulus with a 10:1 ratio of effector (NK92): tumor target (k562) cell lines. Residual amounts of IFN-γ were collected by total cell lysis. 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 stimulated with tumor cell line K562 or PMA/ionomycin, respectively.

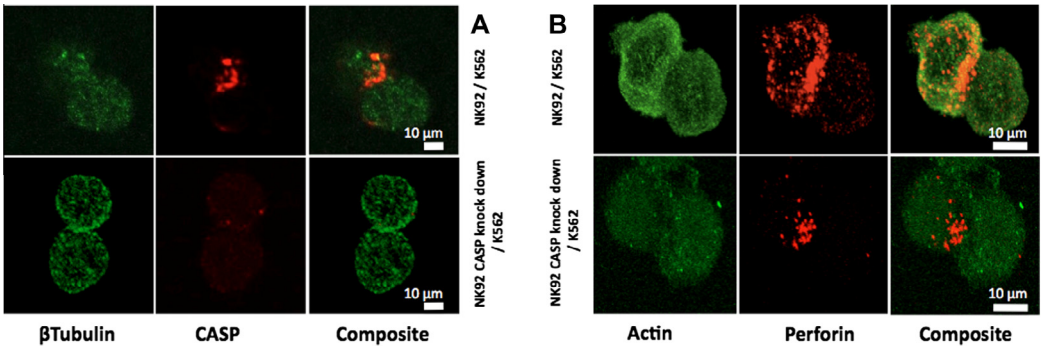

Fig. 4. (A) NK cell conjugation and cytotoxicity phenotypes viewed under 63× magnification with a Zeiss 510 LSM. NK92 cells demonstrate polarization of CASP to the IS. NK cells lacking CASP demonstrate no detection of CASP polarization at the IS. (B) NK cell conjugation and cytotoxicity phenotypes viewed under 63× magnification with a Zeiss 510 LSM. 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.

identify an infected or transformed cell, they must destroy the cell to prevent its spread. It does so by releasing its cytotoxic contents and releasing molecules to recruit other immune cells to the site of infection.

There have been two CASP knockout models produced in mice. Lymphocytes that were harvested from CASP knockout mice were largely unaffected from when tested in the traditional Boyden chamber migration assay like the one performed in this study [12]. NK cells only make up approximately 3–5% of the overall lymphocyte population, and their specific inhibition of migration may not be evident when using total lymphocytes [13]. 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) [14]. Coppola et al. [15] did mention that CASP knockout mice had a small but reproducible decrease in migration to CXCL12, however they looked at T cells and not NK cells, which may be different. Other *in vivo* mouse CASP knockout models show that overall lymphocyte migration to targeted sites such as the spleen, lymph and peritoneal cavity was significantly reduced when challenged with Moloney murine sarcoma virus [16]. The contrasting results for *ex* versus *in vivo* shows that there is significance in the microenvironment in lymphocyte migration when CASP is not present.

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 T-cells, CASP shows a cytoplasmic and vesicular localization

associated with the cell cortex, a cytoplasmic domain directly below the plasma membrane [12]. ARNO, a family member of Cytohesin-1 also has a role in actin remodeling [16]. CASP could have influence in the restructuring of the cell and the formation of the leading edge by its interaction with ARNO.

IFN-γ degranulation is reduced when is CASP knocked-down, indicating an overall effect on NK activation and kill response. In an innate immune response, NK cells secrete IFN-γ, which then recruits macrophages and activates superoxides, promotes Th_1 differentiation, and triggers normal cell expression of MHC Class I and II [17]. Most importantly though, is that NK cells self stimulate with IFN-γ [18]. IFN-γ stimulation of NK cells has also been shown to enhance tumor cell adhesion through the upregulation of ICAM-1 in target antigen presenting cells [19]. Confocal analysis of cellular distribution of perforin shows that it continues to polarize to the IS in CASP knockdowns when conjugated with tumor cells, ruling out that CASP has a role in cytotoxic granule movement.

Although we have not shown CASP is directly involved in cytotoxic granule polarization and delivery to the IS, CASP does have a role in killing. This could be due to 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 [20]. CASPs binding partner SNX27 has been shown to play a role in the recycling of endosomes to the plasma membrane [21]. 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.

This study shows that CASP has a role in both migration and cytokine release ultimately affecting NK cell cytotoxicity. Both affected functions are largely regulated and stimulated by surface receptors on the cell membrane. When CASP is knocked down, the shuttling and recycling of these receptors may be aberrated, causing the phenotypes observed in this study. Further investigations into CASPs role in the mechanics of the leading edge formation through receptor shuttling or cytoskeletal restructuring pathways would prove fruitful, as well as the pathways it may be involved with in cytokine secretion.

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