LYTIC GRANULE CONVERGENCE TO THE MICROTUBULE ORGANIZING CENTER IN NATURAL KILLER CELLS

Ashley Mentlik James

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2012

Jordan S. Orange MD, PhD, Professor of Pediatrics, Pathology and Immunology, Supervisor of Dissertation

---

Daniel S. Kessler, PhD, Associate Professor of Cell and Developmental Biology, Graduate Group Chairperson

Dissertation Committee

Janis K. Burkhardt, PhD, Associate Professor of Pathology and Laboratory Medicine

Erika L.F. Holzbaur, PhD, Professor of Physiology

Michael S. Marks, PhD, Professor of Pathology and Laboratory Medicine

Phong Tran, PhD, Associate Professor of Cell and Developmental Biology

LYTIC GRANULE CONVERGENCE TO THE MICROTUBULE ORGANIZING CENTER IN NATURAL KILLER CELLS

DEDICATION

I would like to dedicate my thesis to Christopher Calvin Garsky who was taken from us before he could complete his graduate studies. We supported each other when things got difficult and now here I am and I wish so badly that you were still here. Even though you are not here, I know you are watching over us, always ready with a snide comment. I miss you so much.

And Peanut, I did this for you. I hope this encourages you to be the absolute best version of yourself that you can be.

ACKNOWLEDGMENTS

I would like to thank first and foremost the two people without whom I would never have gotten this far: my husband Kyle James and my advisor Jordan Orange. Kyle, you deserve an honorary PhD for the amount of emotional and intellectual support you have given me. It truly was a dissertation worth of support. I love you so much and I am so lucky to have you in my life. Jordan, you kept me going, making you proud and not letting you down were huge motivating factors in my graduate career. You always believed in me, even when so many others did not, even when I did not believe in myself. I will always remember and appreciate your enthusiasm for science and discovery, and the respect you showed me.

I would also like to thank my committee members, Drs. Jan Burkhardt, Erika Holzbaur, Mickey Marks, and Phong Tran. I could not have asked for a more constructive, candid and informed group of scientists to help me navigate this process. Your time, advice, resources, and knowledge were invaluable to me.

To the members of the Orange lab, you made it not feel like work. You made scientific discussion stimulating but when we needed to take a break and be silly, you let me lead the silly parade. You were not just colleagues; you will always be friends. Specifically, I would like to thank Drs. Emily Mace, Keri Sanborn, and Greg Rak, mostly because your intelligence always made me strive to be better, but also because you are the most humble and impressive group of people I have ever been fortunate enough to know.

I cannot thank my family and friends enough for their love and support. Mom and Dad, you made me feel stronger when I could not, would not go any further. Thanks for always reminding me “If it was easy, everyone would do it” and for loving me and being proud of me no matter what. I know my whole family is beside me, thanks for raising me with so much love. Thanks to Sean, Mia, Dan, Derek, Nick and Julie, for being true friends, and shining points in my life. I cannot say enough about how much I appreciate you all.

This work was supported in part by NIAID research supplement to NIH Grant AI067946. Additional gratitude must be paid to those who recognize that what we do is so important.

ABSTRACT

LYTIC GRANULE CONVERGENCE TO THE MICROTUBULE ORGANIZING CENTER IN NATURAL KILLER CELLS

Ashley Mentlik James

Jordan S. Orange, MD, PhD

Natural killer (NK) cells are lymphocytes of the innate immune system that participate in host defense by secreting the contents of specialized secretory organelles termed lytic granules onto virally infected or tumorigenic cells in order to terminate them. Lysis of these target cells is a highly regulated, stepwise process beginning with actin rearrangement into an immunological synapse (IS) at the contact site between NK cell and target cell, followed by microtubule organizing center (MTOC) polarization towards the target cell, and culminating in the release of lytic granule contents through the actin network at the IS. The NK cell is capable of directing lytic granules along microtubules to the IS as lytic granule contents are secreted only onto susceptible target cells while bystanding cells are protected. Through quantitative fluorescence microscopy of NK cell lines and primary NK cells, we, in fact, find that lytic granules organize around the MTOC prior to MTOC polarization to the IS. This process is dynein-dependent but independent of F-actin and microtubule reorganization. Interestingly, lytic granule convergence is a default preparatory step, found to occur even after adhesion to a non-susceptible cell. We further find that the process of lytic granule convergence is triggered both by activating receptors and soluble cytokine. In both scenarios, through the use of specific inhibitors, we demonstrate that the trigger for lytic granule convergence is Src kinase-dependent. Thus, lytic granule convergence represents a novel preparatory step in NK cell cytotoxicity, prerequisite to directed secretion. This dynein-dependent mechanism is induced very early by Src kinases after receptor and cytokine stimulation, the purpose of which may be to ensure precision in the delivery of deadly lytic granule contents.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

^{51}Cr	Chromium Radioisotype
Ab	Antibody
ADAP	Adhesion- and Degranulation-Promoting Adaptor Protein
ADCC	Antibody-Dependent Cellular Cytotoxicity
Arp2/3	Actin-Related Protein 2/3
AWD	Area-Weighted Distance
Ca^{++}	Calcium
CC1	First Coiled-Coil Domain of $p150^{Glued}$
Cdc42	Cell Division Control Protein 42
CIP4	Cdc42-interacting protein 4
CLF	Crude Lysosomal Fraction
cSMAC	Central Supramolecular Activation Cluster
CTL	Cytotoxic T Lymphocyte
DAG	Diacylglycerol
DHC	Dynein Heavy Chain
Dia	Diaphanous
DIC	Differential Interference Contrast
DIC	Dynein Intermediate Chain
DMSO	Dimethyl Sulfoxide
EBV	Epstein-Barr Virus
eNK	*ex vivo* NK Cell
Erk	Extracellular Signal-Related Kinase
F-actin	Filamentous Actin
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
GFP	Green Fluorescent Protein
Grb2	Growth Factor Receptor-Bound Protein 2
GzmB	Granzyme B
HLA	Human Leukocyte Antigen
ICAM-1	Intracellular Adhesion Molecule 1
IFNγ	Interferon Gamma
Ig	Immunoglobulin
IL-2/-10	Interleukin 2/10
IS	Immunological Synapse
IP_3	Inositol Triphosphate
JAK	Janus-Activated Kinase
JNK	c-Jun N-Terminal Kinase
KIR	Killer Immunoglobulin-Like Receptor
LAD-1	Leukocyte Adhesion Deficiency 1
LAMP-1	Lysosomal-Associated Membrane Protein 1
LAT	Linker for Activation of T Cells
Lck	Lymphocyte-Specific Protein Tyrosine Kinase
LFA-1	Leukocyte Function-Associated Antigen 1
mAb	Monoclonal Antibody
MEK	Mitogen-Activated Protein Kinase Kinase
MGD	MTOC to Granule Distance
MHC	Major Histocompatibility Complex
MICA/B	MHC Class I chain-related protein A/B
MIP-1	Macrophage Inflammatory Protein 1
MTOC	Microtubule Organizing Center

NCR	Natural Cytotoxicity Receptor
NF-κB	Nuclear Factor-κB
NK	Natural Killer Cell
PAK1	P21 Protein-Activated Kinase 1
PALM	Photoactivation Localization Microscopy
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-Kinase
PLCγ	Phospholipase C Gamma
PLG	Purified Lytic Granules
PNL	Postnuclear Lysate
pSMAC	Peripheral Supramolecular Activation Cluster
Pyk2	Proline-Rich Tyrosine Kinase 2
Rac1	Ras-Related C3 Botulinum Toxin 1
Ras	Rat Sarcoma (Small GTPase)
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SH	Src Homology
SHC	Src Homology 2 Domain-Containing Transforming Protein C
SHP-1	Src Homology Phosphatase 1
SLP-76	Src Homology 2 Domain-Containing Leukocyte Protein of 76 Kilodaltons
Src	Sarcoma (Protein Tyrosine Kinase)
STAT	Signal Transducer and Activator of Transcription
STED	Stimulated Emission Depletion
STORM	Stochasitic Optical Reconstruction Microscopy
Syk	Spleen Tyrosine Kinase
TCR	T Cell Receptor
TIRF	Total Internal Reflection Fluorescence
TL	Transmitted Light
TNF	Tumor Necrosis Factor
ULBP	UL16-Binding Partner
WASp	Wiskott Aldrich Syndrome Protein
WCL	Whole Cell Lysate
ZAP-70	Zeta Chain-Associated Protein of 70 Kilodaltons

LIST OF ELECTRONIC FILES

File	Format	Size
Video 1A: MTOC polarization in a YTS GFP-tubulin cell conjugated to a susceptible target cell	QuickTime Movie	4.8 MB
Video 1B: MTOC polarization in an NK92 GFP-tubulin cell conjugated to a susceptible target cell	QuickTime Movie	2.2 MB
Video 1C: MTOC positioning in a YTS GFP-tubulin cell conjugated to a non-susceptible target cell	QuickTime Movie	1.7 MB
Video 2: Lytic granule movement relative to the MTOC in YTS cells exposed to immobilized antibodies	QuickTime Movie	1.6 MB
Video 3A: Lytic granule dynamics in a resting YTS cell	QuickTime Movie	2.0 MB
Video 3B: Lytic granule dynamics in a resting NK92 cell	QuickTime Movie	2.0 MB
Video 3C: Lytic granule dynamics in an activated YTS cell	QuickTime Movie	8.4 MB
Video 3D: Lytic granule dynamics in an activated NK92 cell	QuickTime Movie	2.5 MB
Video 4: MTOC and lytic granule dynamics in an ex vivo NK cell conjugating with a susceptible target cell	QuickTime Movie	2.5 MB
Video 5A: Effect of Taxol treatment on MTOC dynamics and lytic granule convergence	QuickTime Movie	948 KB
Video 5B: Effect of Cytochalasin D treatment on MTOC dynamics and lytic granule convergence	QuickTime Movie	396 KB
Video 6: Effect of p50 dynamitin, or $p150^{Glued}$ dynactin first coiled coil domain (CC1) overexpression on lytic granule dynamics relative to a granule centroid	QuickTime Movie	1.1 MB
Video 7A: Lytic granule behavior in control DMSO-treated NK cells after CD28 or CD11a triggering	QuickTime Movie	504 KB
Video 7B: Lytic granule behavior in PP2-treated NK cells after CD28 or CD11a triggering	QuickTime Movie	572 KB
Video 8A: Lytic granule behavior in LY294002-treated NK cells after CD28 or CD11a triggering	QuickTime Movie	2.6 MB
Video 8B: Lytic granule behavior in Wortmannin-treated NK cells after CD28 or CD11a triggering	QuickTime Movie	2.5 MB
Video 8C: Lytic granule behavior in PD98059-treated NK cells after CD28 or CD11a triggering	QuickTime Movie	3.5 MB
Video 8D: Lytic granule behavior in U73122-treated NK cells after CD28 or CD11a triggering	QuickTime Movie	924 KB
Video 9A: Lytic granule dynamics relative to a granule centroid in a YTS GFP-KIR2DL1 cell conjugated to a non-cognate KIR ligand-expressing target cell	QuickTime Movie	168 KB
Video 9B: Lytic granule dynamics relative to a granule centroid in a YTS GFP-KIR2DL1 cell conjugated to a cognate KIR ligand-expressing target cell	QuickTime Movie	92 KB
Video 10A: NK cell lytic granule movement after IL-2 addition	QuickTime Movie	4.2 MB
Video 10B: NK cell lytic granule movement after IL-10 addition	QuickTime Movie	1.8 MB
Video 10C: Lytic granule movement in a ZM449829-treated NK cell after IL-2 addition	QuickTime Movie	3.4 MB
Video 10D: Lytic granule movement in a PP2-treated NK cell after IL-2 addition	QuickTime Movie	2.4 MB

CHAPTER 1: INTRODUCTION

SUMMARY

Natural killer (NK) cell cytotoxicity is crucial to the clearance of infection and tumors in the body. The progression of NK cell cytotoxicity must be tightly regulated so that only infected or stressed cells are targeted for lysis. NK cells pass through a number of checkpoints beginning with recognition of target cells by specific receptor-ligand pairings. Activation signaling triggers formation of an F-actin-rich immunological synapse (IS) between the NK cell and target cell. I have discovered that lytic granules converge to the microtubule organizing center (MTOC) as soon as adhesion occurs, but only after further propagation of triggering signaling does the MTOC then polarize with the lytic granules to the IS, where the lytic granules pass through the F-actin cortex to fuse with the membrane and secrete the contents onto the target cell. This thesis focuses on lytic granule convergence to the MTOC, the signaling contributing to its progress, and its potential importance in precision in NK cell cytotoxicity.

INTRODUCTION: NK CELL-MEDIATED CYTOTOXICITY

NK cells are key lymphocytes of the innate immune system, a first line of defense against pathogens. They are extremely important for ridding the system of virally infected, stressed, or cancerous target cells (reviewed in (Orange and Ballas, 2006)). NK cells function by secreting the contents of preformed specialized secretory lysosomes termed lytic granules onto stressed or infected target cells. This task is accomplished by forming an immunological synapse (IS) with the susceptible target cell, which is rich in filamentous actin (F-actin) and effectively confines the lethal contents of the lytic granules to the target cell while bystanding cells are protected. This process is termed directed secretion and mature NK cells are inherently able to facilitate this effector function without further priming, making them rapid first responders to danger (Wulfing et al., 2003). Because mature NK cells are always capable of killing, cytotoxicity is also strictly

regulated. If not carefully controlled, inappropriate NK cell activation is linked to inflammatory diseases and autoimmunity (Perricone et al., 2008).

NK cells also participate in inflammation by secreting chemokines such as macrophage inflammatory protein (MIP) -1α and MIP-1β and cytokines such as interferon γ (IFNγ) and tumor necrosis factor α (TNFα) to regulate and enhance the immune response (Fauriat et al., 2011). The purpose of chemokine secretion is to alert the immune system of danger, which will hasten recruitment of more immune cells to the site of infection. Cytokine secretion, which requires stronger NK cell activation signals, has a concomitantly stronger ability to direct an effective immune response by decreasing viral load (Orange et al., 1995) and increasing tumor cell susceptibility to NK cell lysis (Berthou et al., 2000). Loss of NK cell function has severe implications for increased susceptibility to infection and cancer, among others (reviewed in (Wood et al., 2011)).

While NK cells are traditionally considered members of the innate immune system – meaning that they do not create immunological memory like memory $CD4^+$ T cells – several groups have discovered that NK cells may be capable of developing memory specific to certain antigens (Sun et al., 2009) (and reviewed in (Paust and von Andrian, 2011)) or at the least, memory of prior activation (Cooper et al., 2009) (and reviewed in (Cooper and Yokoyama, 2010)). Even as innate immune system members, NK cells are capable of affecting adaptive immune responses, either through cytokine secretion or direct interaction with adaptive immune cells (Lunemann et al., 2009). NK cells have clearly been shown to be powerful immune regulators in many ways. Together, the value of the NK cell in the immune system is that NK cells are able to respond rapidly to many diverse forms of immunological challenge.

The most recognizable characteristic of NK cells is their ability to mobilize lytic granules in order to secrete their contents onto target cells. Once lytic granules reach and fuse with the NK cell plasma membrane, their contents are spilled out into the space between the NK cell and the target cell, to be taken up by the target cell. Lytic granules contain pore-forming perforin and serine proteases called granzymes that are capable of inducing apoptosis in a target cell

(Zychlinsky et al., 1990). Originally, it was believed that perforin created pores in the target cell membrane through which granzymes could pass (Criado et al., 1985; Gershenfeld et al., 1988). However, it has since been discovered that the pores formed by perforin are not large enough for granzymes. Rather, the pores formed by perforin cause membrane repair mechanisms in the target cell and subsequent endocytosis of the lytic granule contents (Thiery et al., 2010). The granzymes are then capable of activating the caspase cascade in the target cell, leading to apoptosis (reviewed in (Smyth et al., 2005)).

Upon target cell recognition, the NK cell begins immediate cytoskeletal rearrangement. F-actin reorganizes to create the signaling platform that will become the mature IS and the NK cell membrane deforms to cup the target cell (Carpen et al., 1983). Once the IS is mature, the MTOC and lytic granules polarize towards it. In cytotoxic T lymphocytes (CTLs), it has been shown that microtubules anchor in the actin network at the IS and the MTOC is pulled through the cytoplasm, possibly reeled in by tension placed on the microtubules by motor proteins in the IS (Combs et al., 2006; Kuhn and Poenie, 2002). As the MTOC and lytic granules approach the IS, actin is cleared from discreet locations in the interface to make conduits through which lytic granules can pass (Brown et al., 2011; Rak et al., 2011), myosin IIA is required to assist lytic granule passage through the dense actin network (Sanborn et al., 2009) and lytic granules fuse with the plasma membrane to exocytose perforin and granzymes onto the target cell. Later in the introduction, these topics will be expounded upon more thoroughly.

Once an NK cell encounters a susceptible target cell, complex signaling networks initiate the cytotoxic response. Signaling begins with engagement of NK cell surface receptors and several distinct signaling pathways coordinately accomplish the lytic event. These will be addressed more specifically below. The Arp2/3 signaling pathway is responsible for reorganizing F-actin at the interface between the NK cell and the target cell into the IS (Riteau et al., 2003). The Erk1/2 pathway is responsible for MTOC polarization to the IS (Chen et al., 2006). The calcium mobilization pathway is required for release of lytic granule contents onto the target cell (Upshaw et al., 2005). NK cells are also capable of calcium-independent lysis through

engagement of the Fas ligand, a TNF family member. Stressed cells that express Fas (CD95) can be recognized by NK cells solely by Fas ligand, which most NK cells express constitutively. Fas engagement causes caspase activation and subsequent apoptosis in the target cell without any release of cytotoxic material by the NK cell (Eischen and Leibson, 1997).

There is significant evidence to suggest that NK cell cytotoxicity is a sequential process as MTOC polarization does not occur if actin is not present at the IS, and degranulation does not occur if the MTOC and lytic granules are not present at the IS (Graham et al., 2006; Orange et al., 2003; Wulfing et al., 2003). From precise control to protection of healthy cells, the more we learn about NK cell killing, the more elegant the process proves to be.

STAGES OF NK CELL CYTOTOXICITY: FORMATION OF THE LYTIC IS

The stepwise progression of the formation of the lytic NK cell immunological synapse (IS) can be broken into stages (reviewed in (Orange, 2008)). The NK cell must proceed sequentially through these checkpoints so as not to harm surrounding cells or lyse a cell that is not susceptible. Directed secretion is comprised of initiation, effector, and termination stages.

Initiation Stage

During initiation, an NK cell recognizes and binds to a susceptible target cell and activation signaling allows for adhesion (Figure 1.1A). Integrins such as leukocyte function-associated antigen 1 (LFA-1, CD11a and CD18) and macrophage receptor 1 (MAC-1, CD11b and CD18) are important for firm adhesion (Davis et al., 1999; Vyas et al., 2001). As integrins recognize intracellular adhesion molecules (ICAM) on target cells, they cluster at the cell-cell interface and initiate signaling that will soon begin rearranging actin into a firm bridge (Riteau et al., 2003). One report contends that LFA-1 is capable of inducing cytotoxicity in some NK cells (Barber et al., 2004).

Some triggering receptor signaling may begin now if ligands are present on the target cell but inhibitory signaling will also occur now if the NK cell recognizes major histocompatibility

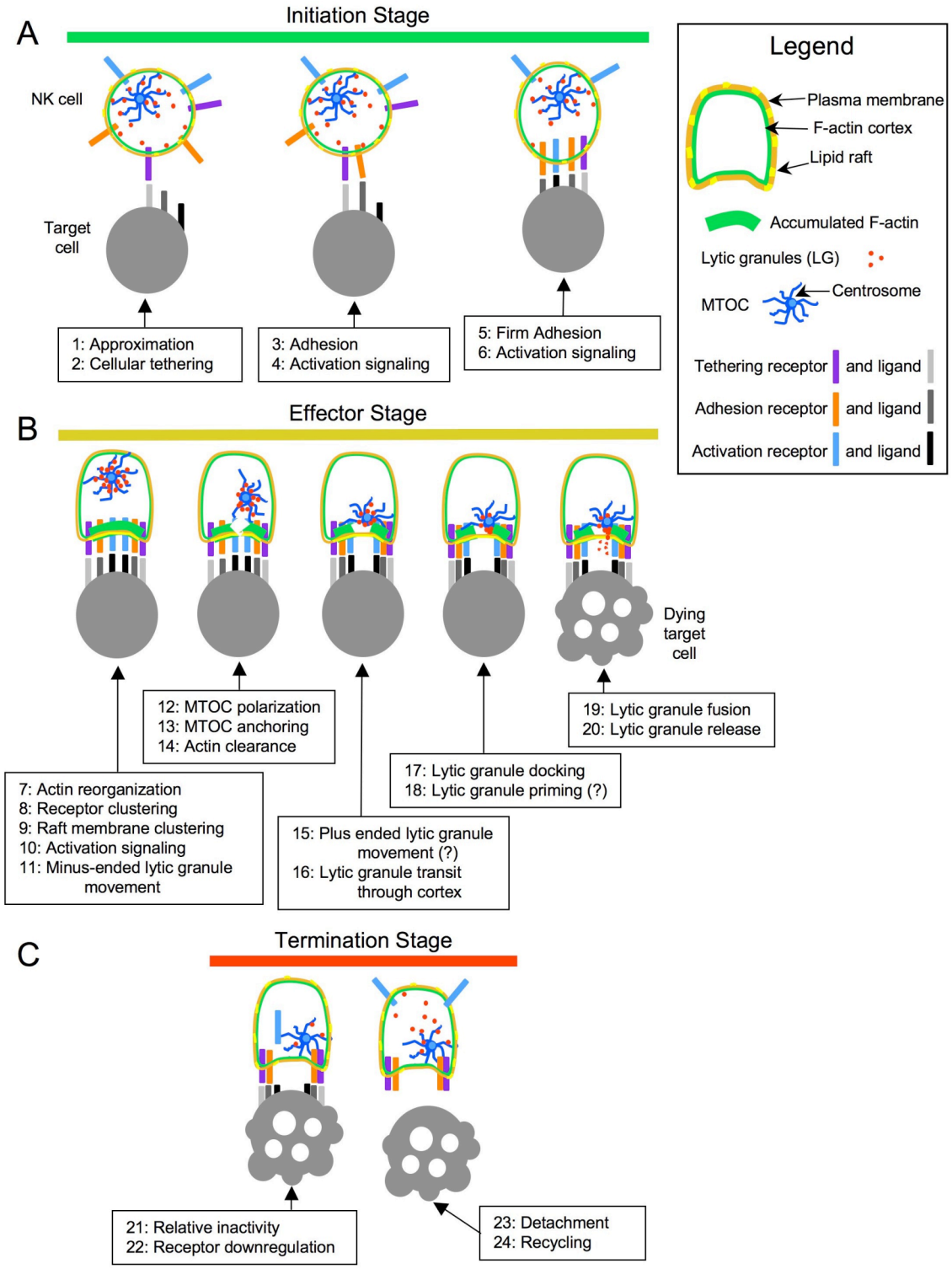

Figure 1.1. Stages of NK cell cytotoxicity.
The three stages of NK cell cytotoxicity. In the initiation phase (A), an NK cell encounters a target cell and adhesion is established. Activation signaling begins and receptors cluster at the cell-cell contact zone. In the effector stage (B), F-actin reorganizes at the contact zone to form the IS and Lytic granules traffic in a

complex (MHC) class I molecules that classify the target cell as 'self' and not a threat (reviewed in (Ljunggren and Karre, 1990). If this happens, inhibitory signaling can inhibit actin reorganization and cease activation signaling (Endt et al., 2007; Masilamani et al., 2006). If inhibitory signaling does not predominate, actin continues to build up at the IS to prepare for the approach of the MTOC and lytic granules.

Effector Stage

Next, during the effector stage, cytoskeletal reorganization causes formation of an IS and polarization of the MTOC with lytic granules to the IS (Figure 1.1B). After polarization, clearance of actin at the IS then allows lytic granules to fuse with the membrane and lytic granule contents to be secreted. Assuming triggering receptor engagement predominates during the cell-cell interaction, the first step in the effector stage is formation of an actin-rich IS (reviewed in (Orange, 2008)). Clustering of integrins brought on by engagement of ligand on the target cell causes a conformational change in the integrin into a high-affinity state that promotes strong ligand binding (inside-out signaling). Increased ligand binding propagates signaling from the cell surface into the NK cell cytoplasm that will lead to actin polymerization (outside-in signaling (Lub et al., 1997)). Initiated by the Rho family guanine-nucleotide exchange factor Vav1 after integrin clustering and signaling, actin reorganization at the IS is necessary for all following steps of the effector stage (Orange et al., 2003; Riteau et al., 2003).

Occurring independently but perhaps simultaneously is minus-end directed lytic granule traffic towards the MTOC (Mentlik et al., 2010). Because NK cells are important members of the innate immune system but do not possess immunological memory, regulation of NK cell

---

minus-end directed manner along microtubules towards the MTOC. When triggering signaling predominates, the MTOC then polarizes with the lytic granules towards the IS. Actin clears from the IS to make conduits for lytic granules to pass through and lytic granules dock and fuse with the NK cell plasma membrane and granule contents are secreted onto the target cell. In the termination stage (C), the target cell has died and the NK cell remains attached while downregulating activation receptors. Finally the NK cell detaches and is able to recycle cytotoxic ability to be ready for the next target cell encounter.

Figure adapted from Orange JS, "Formation and function of the lytic NK-cell immunological synapse." Nature Reviews Immunology. September 2008. Image copyright Orange JS, provided with permission for reproduction.

cytotoxicity may occur at multiple steps. Lytic granule convergence to the MTOC may ensure that the granules do not fuse with the NK cell membrane anywhere other than at the IS, and only after MTOC polarization. This process is distinct from that in CTLs in which the MTOC is seen to polarize first and then lytic granules traffic along microtubules to the docked MTOC to be delivered to the IS (reviewed in (Stinchcombe and Griffiths, 2007)). The minus-end directed motor cytoplasmic dynein is necessary for lytic granule transport to the MTOC, but how it is activated to preferentially transport lytic granules to the MTOC and hold them there is unknown (Mentlik et al., 2010). Interestingly, this step is a checkpoint independent of cytolytic commitment, as lytic granules still converge to the MTOC even in non-cytolytic interactions. Additionally, cytotoxicity is severely impaired in NK cells that do not undergo convergence of lytic granules, marking this step as a possible prerequisite to further progression of cytotoxicity (AMJ - manuscript submitted).

Continued triggering signaling by means of receptor clustering is necessary for a strong NK cell response. In the T cell IS, receptors are arranged into microclusters termed supramolecular activation clusters (SMAC), presumably to organize and concentrate signaling to discrete locations and so that once a receptor has transmitted a signal, it may be internalized from discrete locations back into the T cell for recycling and downregulation (Monks et al., 1998). T cell receptors (TCR) cluster into the center of the IS, termed the central or cSMAC and integrin molecules migrate and cluster into a ring around the periphery of the cSMAC; the pSMAC (van der Merwe et al., 2000). While existence of distinct cSMAC and pSMAC have not been proven in NK cells, there is evidence that important triggering receptors and signaling molecules do congregate in the center of the IS, again, presumably to concentrate signaling strength (Giurisato et al., 2007; Vyas et al., 2001).

Next, for delivery of lytic granules, the MTOC must polarize towards the IS. MTOC polarization requires mitogen activated protein kinase (MAPK) signaling as well as continued Vav1 signaling, the formin Diaphanous (an actin nucleator distinct from Arp2/3, hDia1), and the focal adhesion kinase family member proline-rich tyrosine kinase 2 (Pyk2) (Butler and Cooper, 2009; Chen et al., 2006; Graham et al., 2006; Li et al., 2008; Sancho et al., 2000). Physically, the

MTOC may be propelled through the cytoplasm towards the IS by virtue of localized diacylglycerol (DAG) and tension placed on anchored microtubules in the IS (Combs et al., 2006; Kuhn and Poenie, 2002; Quann et al., 2009). This phenomenon has been studied in CTL killing but has not been confirmed in NK cells. It was originally theorized in CTLs that lytic granules had to travel along microtubules in a plus-ended, kinesin-dependent manner to get to the IS from the MTOC and subsequently, lytic granule movement was observed in a kinesin-dependent manner along microtubules *in vitro* (Burkhardt et al., 1993). However, this finding has been contradicted by studies showing that the CTL MTOC approximates to the IS closely enough so as not to need plus-ended microtubule traffic (Stinchcombe et al., 2006). Here again, neither scenario has been confirmed or denied in NK cells. Lastly, there is some evidence to support MTOC anchoring in the IS by Cdc42-interacting protein 4 (CIP4) in NK cells (Banerjee et al., 2007). CIP4 contains actin- and microtubule-binding domains and so may facilitate a stable anchor for the MTOC in the actin at the IS. Studies in CTLs have shown a necessity for Src family kinase member Lck in MTOC docking at the IS (Tsun et al., 2011). Lytic granule delivery in CTLs also varies depending on signal strength, but delivery in NK cells is probably all-or-nothing (Beal et al., 2009). Once again, without an intact F-actin-rich IS, the MTOC will not polarize, highlighting the likelihood and utility of an anchoring mechanism.

As the MTOC approaches and perhaps anchors in the IS, actin is cleared from distinct regions of the IS to make conduits for lytic granules to pass through (Brown et al., 2011; Rak et al., 2011). It is not known how actin is disassembled from specific areas of the IS, but targets for study now may be actin disassembling proteins such as coronin 1A and cofilin (AMJ – unpublished). Interestingly, even though actin is cleared from parts of the IS, lytic granules cannot pass through the remaining actin network unaided. The actin-dependent motor protein myosin IIA has been shown to be necessary for transit of lytic granules through the actin network at the IS (Andzelm et al., 2007; Sanborn et al., 2009). Myosin IIA has been found localized to lytic granules and facilitates interaction of lytic granules with actin. Finally, once the lytic granules have passed through the IS, the granule membrane can fuse with the plasma membrane and the contents are

secreted into the cleft between NK cell and target cell (Rak et al., 2011). The target cell takes up the lytic granule contents and begins to undergo apoptosis.

Termination Stage

Finally, the termination stage sees downregulation of activity and detachment of the NK cell from the lysed target cell, after which the NK cell can move on to the next susceptible target cell (Figure 1.1C). Although not perfectly understood, it is known that after a target cell has been lysed, the NK cell remains attached but inactive while accumulated activating receptors are degraded (McCann et al., 2007; Sandusky et al., 2006). In CTLs, T cell receptor microclusters migrate towards the center of the synapse and become ubiquitinated, internalized and degraded. This way, new signaling molecules can replenish the old and sustain signaling (Lee et al., 2003; Wiedemann et al., 2005). Prolonged attachment may also serve to continue protecting bystanding cells from effector molecules (Orange, 2008).

After detachment, the NK cell is able to recycle its cytotoxic potential. The NK cell is able to internalize granule membrane proteins, such as lysosomal-associated membrane protein 1 (LAMP-1), in order to generate new lytic granules (Liu et al., 2009) and re-express triggering receptors. NK cells are serial killers, and can continue generating new lytic granules without needing prior challenge, unlike CTLs (Wulfing et al., 2003). Therefore, while NK cells may require tighter regulation so as not to harm healthy cells, they are always ready to dispose of the next susceptible target.

NK CELL SIGNALING

Unlike in the major effectors of the adaptive immune system, $CD8^+$ T cells, NK cell receptors do not undergo recombination in order to create antigen-specific activation receptors. Instead, NK cells utilize germline-encoded receptors that recognize common ligands present on infected and cancerous cells. Because of this, NK cell activity is tightly regulated via signaling checkpoints. It is also important that NK cells respond to their environment. NK cell are also capable of responding to circulating inflammatory cytokines.

Receptor-mediated signaling

Physical interactions between NK cells and potential target cells are negotiated through receptor-ligand pairing (Figure 1.2). First, integrins, such as LFA-1, associate with ICAM-1 on target cells. Intracellular interaction with Talin and other proteins facilitates association of integrins with actin as it is being polymerized and reorganized (Mace et al., 2009). Actin accumulation then allows for aggregation of more integrins on the cell surface so that firm adhesion can be established. Meanwhile, Pyk2 and Vav1 are phosphorylated, and Vav1 initiates actin reorganization (Gismondi et al., 2000; Riteau et al., 2003). The importance of integrin signaling, specifically through β2 integrin CD18, is illustrated by a severe immunodeficiency known as leukocyte adhesion deficiency (LAD-I). Characterized by a lack of CD18, patient NK cells cannot effectively bind target cells and therefore, patients suffer from recurrent infections (Kishimoto et al., 1987).

NK cells have several types of triggering receptors. These include the natural cytotoxicity receptor (NCR) family, which recognize viral hemaglutinin, tumor cell ligands and other as yet undiscovered ligands (Arnon et al., 2006; Brandt et al., 2009), and NKG2D, which recognizes UL16-binding partners and MHC class I chain-related proteins A and B, among others (Bauer et al., 1999; Cosman et al., 2001). These receptors transmit signal through intracellular binding partners such as DAP10/12 or CD3ζ that contain tyrosine residues in the intracellular regions (reviewed in (Watzl and Long, 2010)). These tyrosines can then be phosphorylated by Src family kinases in order to transduce signaling (Figure 1.2). Src family kinases are very early mediators of NK cell signaling. Many family members have been found to be necessary for NK cell cytotoxic function and seem to serve redundant roles (Augugliaro et al., 2003; Mason et al., 2006). After Src kinase-mediated phosphorylation, these receptor phospho-tyrosine residues become a platform for all the signaling that follows.

Among the phospho-tyrosine binding partners that are well studied in NK cells, phosphatidylinositol-3 kinase (PI3K) signaling can trigger MTOC and granule polarization by activating the Erk1/2 pathway (Chen et al., 2006). Another common binding complex consists of

growth factor receptor-bound protein 2 (Grb2), Vav1 and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) (reviewed in (Lanier, 2008)). SLP-76 activates phospholipase Cγ (PLCγ), which has also been linked to MTOC polarization through c-Jun N-terminal kinase (JNK) (Chen et al., 2007; Li et al., 2008). SLP-76 activation also leads to calcium mobilization that will eventually make degranulation possible. Activation of PLCγ leads to depletion of intracellular calcium stores. Subsequent store-operated calcium entry is necessary for granule content secretion (Upshaw et al., 2005). Syk tyrosine kinase and zeta chain-associated protein kinase 70 (ZAP-70) have also been implicated in PI3K and SLP-76 activation (reviewed in (Tassi et al., 2006)) but Syk and ZAP-70 are both dispensable in NK cell cytotoxicity; they represent a redundant activation pathway, but not one that we will discuss further (Billadeau et al., 2003).

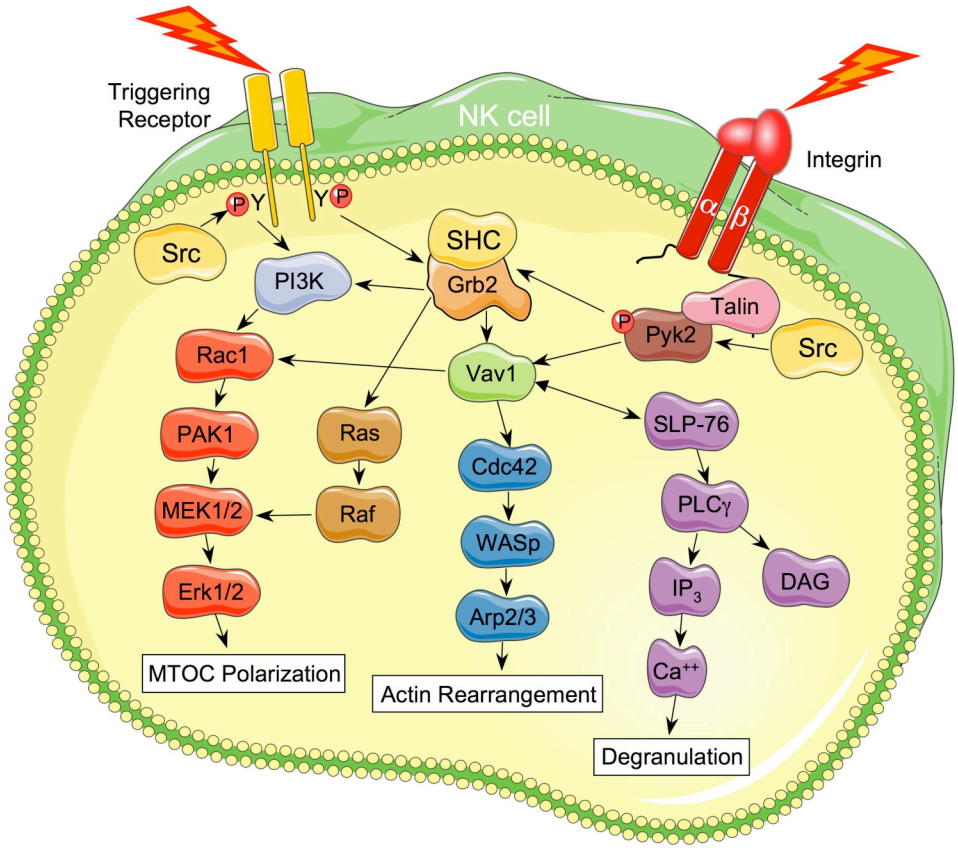

Figure 1.2. Model of receptor-mediated signaling in NK cells.
Diagram outlining some of the major NK cell signaling pathways that contribute to NK cell-mediated cytotoxicity. Integrin signaling proceeds through Talin and other signaling partners to phosphorylate Pyk2 and Vav1, resulting in actin remodeling. Many NK cell triggering receptors associate with or signal through intracellular tyrosine reside-containing adaptors, which are phosphorylated by Src kinases. Downstream signaling occurs through PI3K, Grb2, and Vav1, resulting in MTOC polarization and degranulation.

NK cells also have inhibitory receptors to regulate responses to healthy cells. Killer immunoglobulin-like (KIR) receptors recognize major histocompatibility complex (MHC) class I molecules on healthy target cells utilizing what is widely agreed to be recognition of self (Ljunggren and Karre, 1990). If KIR engagement predominates triggering receptor engagement, actin reorganization and any other triggering signaling is inhibited via specialized phosphatases such as Src homology phosphatase 1 (SHP-1) (Cuevas et al., 1999; Peterson and Long, 2008; Stebbins et al., 2003; Watzl and Long, 2003). KIR receptor intracellular domains also have tyrosine residues that are phosphorylated by Src family kinases; in this case, phospho-tyrosine residues are necessary for SHP-1 recruitment (reviewed in (Vivier et al., 2004)). If the cell that the NK cell encounters is healthy, then this SHP-1-mediated dephosphorylation leads to disruption of actin and detachment of the NK cell from the healthy cell (Burshtyn et al., 2000).

Cytokine-mediated signaling

NK cells are also capable of responding rapidly to circulating inflammatory cytokines. This is important *in vivo* because NK cells must migrate towards areas of infection (Beider et al., 2003; Wald et al., 2006). The cytokine interleukin 2 (IL-2), which is usually secreted by $CD4^+$ memory T cells, is one of the most potent activators of NK cells, heightening the activity state of the NK cell to better prepare it for large-scale infection or tumors (reviewed in (Malek and Bayer, 2004)). Once the IL-2 receptor is engaged, it contributes to the activation of several signaling pathways that increase NK cell responsiveness and contribute to NK cell cytotoxicity (Figure 1.3).

In NK cells, canonical signaling triggered by IL-2 is responsible for transcription of target genes. IL-2 stimulation increases expression of activating receptors, production of IFNγ, perforin and granzymes, and decreases the threshold for activation of the NK cell (Farag and Caligiuri, 2004). Most canonical IL-2 signaling occurs through Janus activated kinase/signal transducers and activators of transcription (JAK/STAT) pathways. JAK molecules are associated with the γ subunit of the IL-2 receptor and become phosphorylated when IL-2 is present (Johnston et al., 1994). IL-2 receptor intracellular domains are also phosphorylated to allow binding of downstream

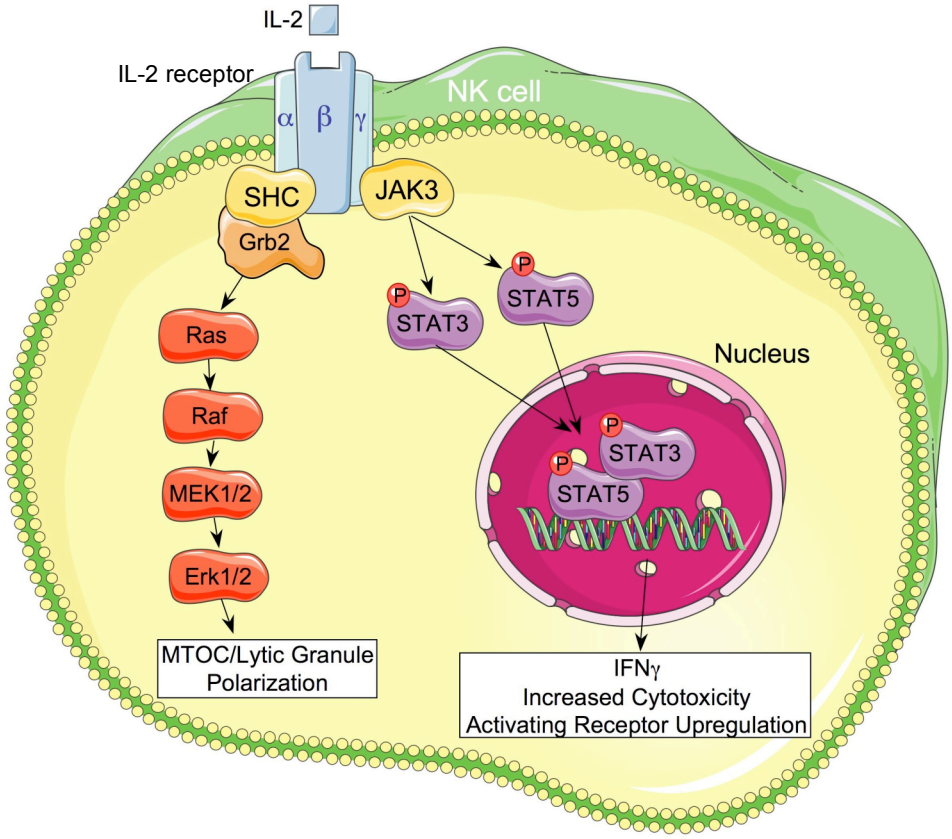

Figure 1.3. Model of cytokine-mediated signaling in NK cells.
Diagram outlining the major canonical and non-canonical signaling pathways that result from IL-2 stimulation. Canonical: after IL-2 is detected by the IL-2 receptor, the kinase JAK3 phosphorylates the IL-2 receptor as well as STAT molecules. The STAT molecules dimerize and translocate to the nucleus to initiate transcription. Non-canonical: the adaptor molecule SHC binds to phosphorylated residues on the IL-2 receptor and activates Grb2. Grb2 activates Ras and the Erk1/2 pathway to enhance MTOC and granule polarization.

effectors. JAKs are also capable of autophosphorylation and IL-2 receptor phosphorylation (Kurzer et al., 2004). Downstream of JAKs, STAT molecules are phosphorylated and dimerize (Johnston et al., 1995). The dimers translocate to the nucleus where they begin transcription of target genes.

Non-canonical IL-2 signaling is equally important for enhancing cytotoxicity. For the IL-2 receptor (β subunit), non-canonical signaling is facilitated through the adaptor Src homology 2 domain-containing transforming protein C (SHC). SHC signaling continues through Grb2 to activate the small GTPase Ras (Ravichandran and Burakoff, 1994) and PI3K (Merida et al., 1991). Ras and PI3K signaling culminates in Erk1/2 activation, and so, IL-2 signaling aids in

MTOC and granule polarization should the NK cell encounter a target cell after IL-2 stimulation. Because it contributes to NK cell cytotoxicity physically, non-canonical IL-2 signaling may be subject to the same regulation as in receptor-mediated cytotoxicity checkpoints. As a common signaling mediator between receptor- and cytokine-mediated signaling, Grb2 may have a role in regulating early events common to both modes of NK cell activation.

THESIS OBJECTIVES

The objective of this thesis is to examine the movement of lytic granules in NK cells and the signaling that affects lytic granule movement. After initial observations, I hypothesized that lytic granules converge to the MTOC in order to ensure precise delivery of their contents to the IS. In Chapter 2, lytic granule convergence is characterized in detail and we established the requirements for lytic granule convergence to the MTOC in NK cells. Lytic granules converge to the MTOC even without a commitment to cytotoxicity, which marks convergence as a preparatory step that the NK cell undergoes any time it encounters a potential target cell. We also found that dynein is present on lytic granules and is indispensable for minus-ended lytic granule convergence to the MTOC. In Chapter 3, I characterize NK cell signaling as it pertains to lytic granule convergence. We further establish that lytic granule convergence is a very early step in NK cell activation as only Src kinase activity is upstream of compaction. Additionally, soluble cytokine stimulation is sufficient to trigger lytic granule convergence. This highlights the importance of convergence further because it exemplifies a heightened state of preparedness of the NK cell, while simultaneously ensuring that granule contents are not prematurely released. Finally, in Chapter 4, I discuss the implications of these studies and future directions for this research.

CHAPTER 2: RAPID LYTIC GRANULE CONVERGENCE TO THE MTOC IN NATURAL KILLER CELLS IS DEPENDENT UPON DYNEIN BUT NOT CYTOLYTIC COMMITMENT[1]

SUMMARY

Natural killer cells are lymphocytes specialized to participate in host defense through their innate ability to mediate cytotoxicity by secreting the contents of preformed secretory lysosomes (lytic granules) directly onto a target cell. This form of directed secretion requires the formation of an immunological synapse (IS) and occurs stepwise with actin reorganization at the IS, preceding microtubule organizing center (MTOC) polarization to the synapse. Since MTOC polarization to the synapse is required for polarization of lytic granules, we attempted to define their inter-relationship. We found that when compared to the time required for MTOC polarization, lytic granules converged to the MTOC rapidly. The MTOC-directed movement of lytic granules was independent of actin and microtubule reorganization, dependent upon dynein motor function, occurred prior to MTOC polarization, and did not require a commitment to cytotoxicity. This defines a novel paradigm for rapid MTOC-directed transport as a prerequisite for directed secretion; one that may prepare, but not commit cells for precision secretory function.

INTRODUCTION

Natural killer (NK) cells are lymphocytes of the innate immune system capable of killing stressed or infected cells by secreting lytic effector molecules through an immunological synapse (IS) at the interface with the target cell. The NK cell IS forms and functions in a stepwise progression (Orange, 2008; Orange et al., 2003; Wulfing et al., 2003). A major objective of this

[1] A version of this chapter has been published as:

Mentlik AN, Sanborn KB, Holzbaur, EL, Orange JS.
Rapid lytic granule convergence to the MTOC in natural killer cells is dependent upon dynein but not cytolytic commitment.
Molecular Biology of the Cell. July 2010; Vol. 21, pp 2241-2256.

process is the polarization of lytic granules, a form of secretory lysosome containing the pore-forming molecule perforin, towards the target cell so that their contents can be released. Cytoskeletal reorganization is necessary for maturation of the NK cell IS as F-actin accumulates at the IS and is required for reorientation of the MTOC (Orange et al., 2003; Wulfing et al., 2003). The microtubule network is ultimately responsible for delivery of lytic granules to the IS and the MTOC is required for their approximating the cytotoxic cell membrane at the IS (Banerjee et al., 2007; Katz et al., 1982; Stinchcombe et al., 2006). In NK cells, precision in directed secretion of lytic granules enables destruction of specific target cells without harming neighboring cells within a complex environment.

Several receptor systems have been characterized for their ability to participate in IS formation and granule polarization. The LFA-1 integrin is required for target cell adhesion and directed secretion through early signaling that initiates actin rearrangements and enables granule focusing to the IS (Barber et al., 2004; Liu et al., 2009). Engagement of more specialized NK cell triggering receptors such as NKp30, or CD28 on some NK cell lines and subsets, is critical for full NK cell activation and cytotoxicity (Chen et al., 2006; Pende et al., 1999). The activity of triggering receptors includes induction of MTOC polarization to the signaling platform, which facilitates lytic granules joining the synaptic membrane (Bryceson et al., 2005; Chen et al., 2006; Vyas et al., 2001). Several direct activation-induced requirements for MTOC polarization in cytotoxic lymphocytes have been identified (Banerjee et al., 2007; Gomez et al., 2007; Stinchcombe et al., 2006). Thus far, all microtubule-directed events required for lytic granule polarization to the IS, however, are dependent upon some prerequisite actin-dependent function.

Once lytic granules polarize to the IS, the actin motor myosin IIA facilitates lytic granule transit through the F-actin-rich NK cell IS (Andzelm et al., 2007; Sanborn et al., 2009). Although plus-ends of microtubules insert into the F-actin network at the IS, there has not been any demonstrated requirement for plus-ended lytic granule movement in approximating lytic granules to the synaptic membrane. Instead, lytic granules in association with the MTOC have been defined as the only prerequisite (Jenkins et al., 2009; Stinchcombe et al., 2006). Although a plus-

ended motor for lytic granule movement along microtubules in cytotoxic cells has been identified (Burkhardt et al., 1993), presumably the approximation of lytic granules to the MTOC requires minus-end-directed traffic at some point. A specific motor required for, or detailed kinetics of MTOC-directed traffic of lytic granules in cytotoxic cells has not been defined. Analogies in other cell systems in which minus-ended organelle traffic along microtubules occurs similarly to enable directed secretion have also not been identified.

Given that NK cells function in innate immunity, having multiple defined points of regulation is useful in controlling the lethal function of directed secretion for cytotoxicity. Thus, we evaluated the kinetic relationship between the lytic granules, the MTOC, and the IS in NK cells. We found that lytic granule traffic to the MTOC was rapid, occurring long before MTOC polarization to the IS. We have defined this movement as convergence relative to the MTOC, which is meant to indicate that lytic granules amass in close proximity to the MTOC. This rapid minus-end-directed lytic granule movement and convergence was independent of a commitment to cytotoxicity and occurred independently of microtubule and F-actin reorganization. We also defined the minus-end-directed motor used by lytic granules as dynein and found that traffic of lytic granules to the MTOC was required for IS maturation. We propose that rapid lytic granule convergence to the MTOC defines a new checkpoint in cytotoxicity whereby an NK cell prepares for cytotoxicity upon adhesion to a target cell but requires additional activation signaling to polarize this now fully armed lytic machinery. It also defines a new paradigm in organelle traffic for directed secretion; one in which cargoes are compacted to the MTOC to enable efficiency and precision in delivery.

RESULTS

MTOC polarization occurs slowly and only in cytolytic conjugates

NK cell cytotoxicity is a stepwise process relying upon checkpoints that govern sub-cellular reorganization culminating in directed secretion to facilitate destruction of a target cell (Wulfing et al., 2003). One critical checkpoint in the cytolytic process is the polarization of the

MTOC and lytic granules to the IS so that the lytic granule contents can be secreted. Because NK cells are cytotoxic lymphocytes of the innate immune system with important roles in routine immune surveillance, we hypothesized that the process of MTOC polarization would be gradual, providing ample opportunity for regulation. Thus, we initially evaluated the kinetics of MTOC polarization towards the NK cell IS in real time using immortalized NK cell lines, YTS and NK92, stably expressing GFP-tubulin. Since MTOC polarization is also required for lytic granule reorientation to the IS, NK cells were loaded with LysoTracker Red dye to enable their visualization during the process of MTOC movement.

GFP-tubulin, LysoTracker-loaded YTS or NK92 cells were added to live cell imaging chambers to which target cells were immobilized using specific antibodies. The 721.221 B lymphoblastoid cell line and K562 erythroleukemic cell line were used as target cells for YTS and NK92 cells, respectively, in order to enable formation of cytolytic conjugates which can give rise to cytotoxicity (Figure 2.1). K562 and YTS cells were also evaluated as examples of non-cytolytic

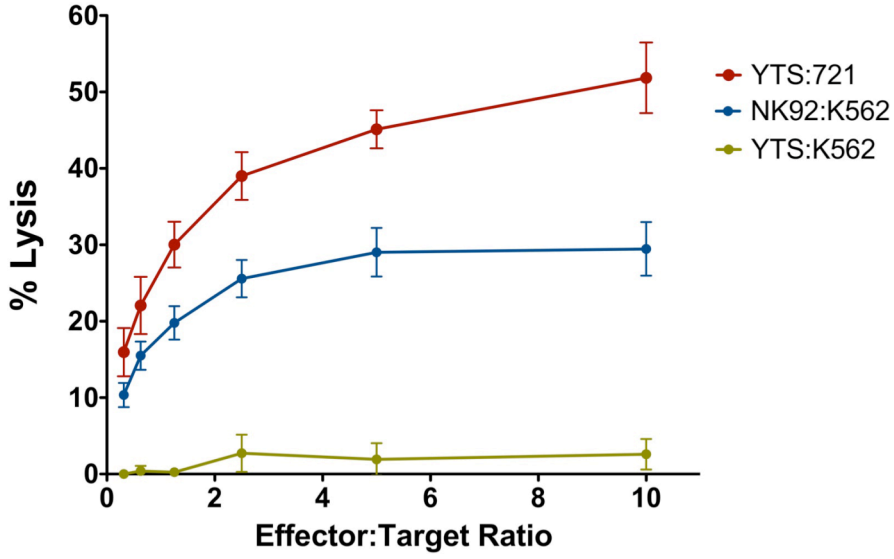

Figure 2.1. Chromium-release assays evaluating models of cytolytic and non-cytolytic conjugation.
Cytolytic activity of NK cells against ^{51}Cr-labeled target cells measured in a 4hr chromium release assay. The different effector and target cell combinations represent those used comparatively in Figures 1 and 2 and include: YTS cells and susceptible 721.221 target cells (red line), NK92 cells and susceptible K562 target cells (blue line), as well as YTS NK cells and non-susceptible K562 target cells (green line). Each line represents the mean from three independent experiments with the error bars demonstrating ± SD of the independent experiments.

conjugates, which do not give rise to cytotoxicity, since YTS cells do not kill K562 cells (Figure 2.1). NK cells contacting target cells were identified and imaged every 15 s within a single *z*-axis plane containing the MTOC for a minimum of 30 min. The MTOC was identified as a solitary intense cluster of GFP-tubulin, which corresponded to the MTOC as defined by pericentrin localization (Figure 2.2A). In cytolytic conjugates between either YTS and 721.221 cells (Figure 2.2B; and Video 1A), or NK92 and K562 cells (Figure 2.2C; and Video 1B), the MTOC gradually tracked towards the IS. In contrast, in non-cytolytic conjugates between YTS and K562 cells, this gradual directed movement was not observed (Figure 2.2D; and Video 1C). To quantify the kinetics of MTOC movement, the distance between the MTOC and the center of the IS (defined as the center of the interface between NK and target cell) was measured at each time point. The mean MTOC polarization time to the IS was 35.45 ± 5.73 min in YTS cells and 36.19 ± 2.38 min in NK92 cells (Figure 2.2E,F). In contrast, MTOC polarization did not occur in non-cytolytic conjugates and the MTOC to IS distance remained constant (Figure 2.2G). Thus, MTOC polarization was specific to the cytolytic IS and occurred gradually.

Lytic granules converge to the MTOC rapidly and prior to polarization

Microtubules and the MTOC are required for lytic granule delivery to the IS (Orange et al., 2003), with the MTOC directing the final steps in lytic granules reaching the cell membrane (Stinchcombe et al., 2006). The relationship of the lytic granules to the MTOC prior to its final polarization to the IS, however, is unclear. Although the MTOC was found to gradually track to the IS, the lytic granules were noted to be gathered around the MTOC in newly formed cytolytic conjugates. In these initial experiments, imaging began 1-4 min after cytolytic conjugate formation and lytic granules were found to be in close proximity to the MTOC either at the outset of imaging or soon afterwards (Figure 2.3A,B). In time lapse images, once lytic granules converged to the MTOC, they appeared sustained in this conformation as the MTOC polarized to the IS. The coalescing of lytic granules was independent of cytolytic activation, or MTOC polarization, as it was also found in non-cytolytic conjugates soon after their formation (Figure 2.3C). Thus, we hypothesized that lytic granules first cluster to the MTOC in order to be delivered with it to the IS.

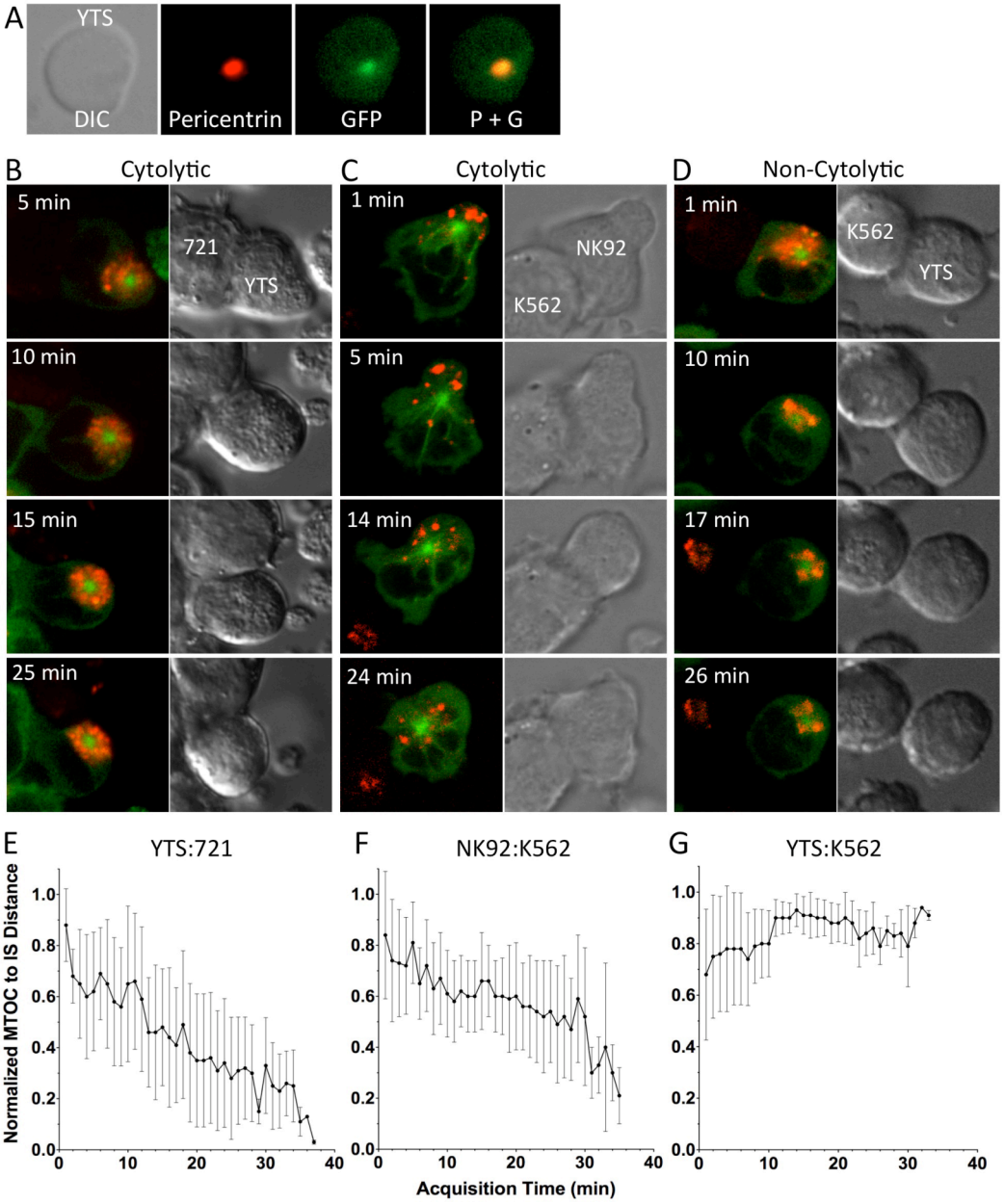

Figure 2.2. Dynamics of MTOC movement relative to the NK cell IS.
(A) DIC (left) and fluorescence images of a YTS GFP-tubulin cell fixed and stained with anti-pericentrin polyclonal antibody. Green fluorescence represents GFP-tubulin and red the pericentrin antibody as identified using an AlexaFluor-conjugated anti-rabbit mAb. An overlay of fluorescent images is shown (right). Time-lapse images of MTOC movement in: (B) a YTS GFP-tubulin cell conjugated with a susceptible 721.221 target cell (from Video 1A), (C) an NK92 GFP-tubulin cell conjugated with a susceptible K562 target cell (from Video 1B), and (D) a YTS GFP-tubulin cell conjugated with a non-susceptible K562 target cell (from Video 1C). Each image pair shows confocal immunofluorescence in the plane of the MTOC on the left and DIC on the right. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. Mean distance ± SD between the MTOC and the IS, normalized to the largest distance of the MTOC from the IS in that cell, as a function of conjugation time in: (E) YTS with 721.221 cells (n = 10), (F) NK92 with K562 cells (n = 9), and (G) YTS with K562 cells (n = 10).

To objectively define lytic granule movement relative to the MTOC, equations were developed to define the MTOC to granule distance. Specifically, the *x*,*y* coordinates of the

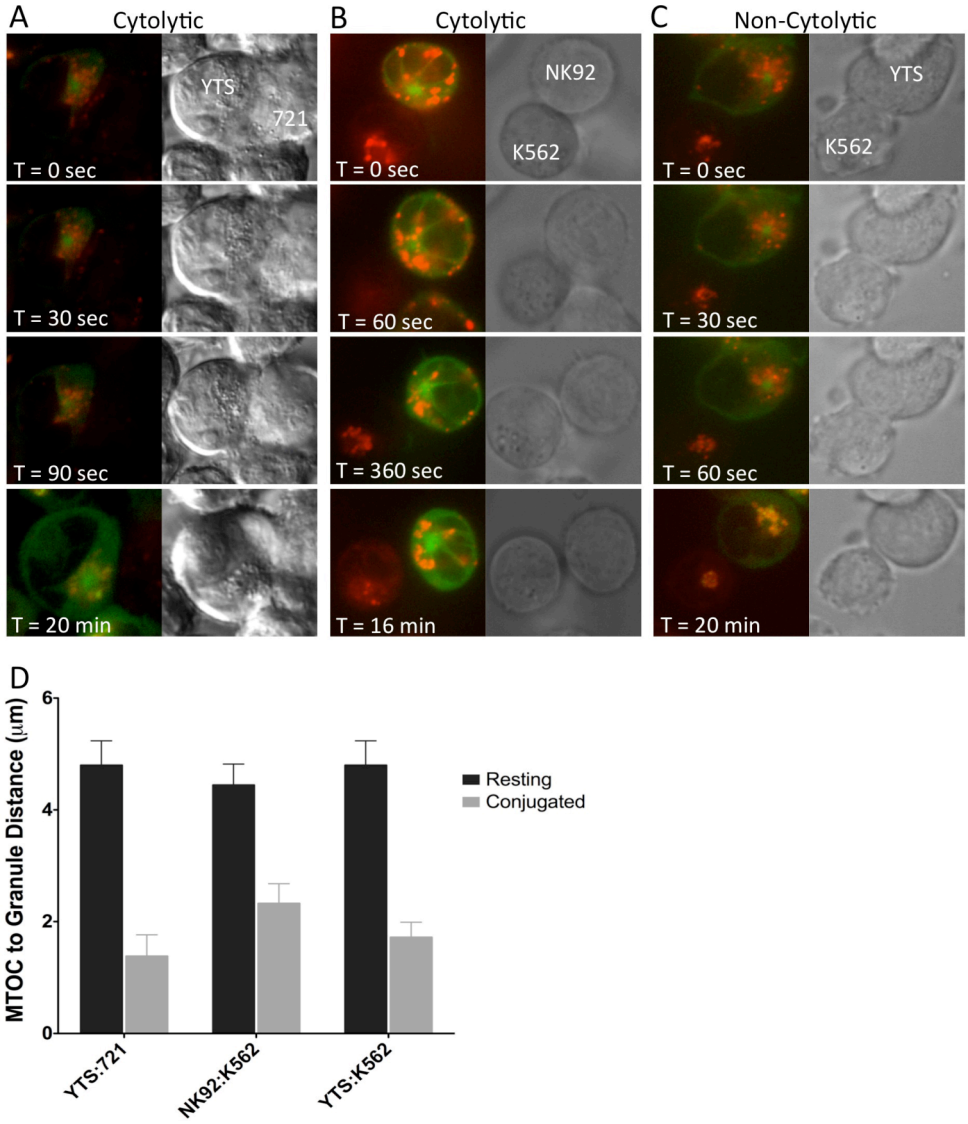

Figure 2.3. Dynamics of lytic granule movement relative to the MTOC in unconjugated or target cell-conjugated NK cells.

Time-lapse images of lytic granule movement in conjugates between: (A) a YTS GFP-tubulin and a susceptible 721.221 target cell, (B) an NK92 GFP-tubulin and a susceptible K562 target cell, and (C) a YTS GFP-tubulin and a non-susceptible K562 target cell. In each pair of images, confocal immunofluorescence in the plane of the MTOC is shown on the left and DIC on the right. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. T = 0 refers to the time image acquisition began which was between 1-5 min after NK cells were added to the imaging chamber. (D) Quantitative analysis of lytic granule movement relative to the MTOC as measured by mean MTOC to granule distance in individual time points averaged over all measured time points ± SD in conjugates between: YTS and 721.221 target cells (n = 10), NK92 and K562 target cells (n = 9), and YTS and K562 target cells (n = 10). All mean distances of lytic granules from the MTOC were significantly different in conjugates compared to unconjugated NK cells ($p < 0.05$).

centroid of each lytic granule was related to that of the MTOC. The mean value was determined for all lytic granules within a single NK cell at each individual time point. Compared to distances in unconjugated YTS and NK92 cells, the average distance of granules from the MTOC over all time points was reduced at least 2-fold (Figure 2.3D). Even in the absence of MTOC polarization, in YTS non-cytolytic conjugates, there was a reduction in the distance of the lytic granules from the MTOC over all time points similar to that in cytolytic conjugates. In either cytolytic or non-cytolytic conjugates, differences in the mean distance of lytic granules from the MTOC were not identified

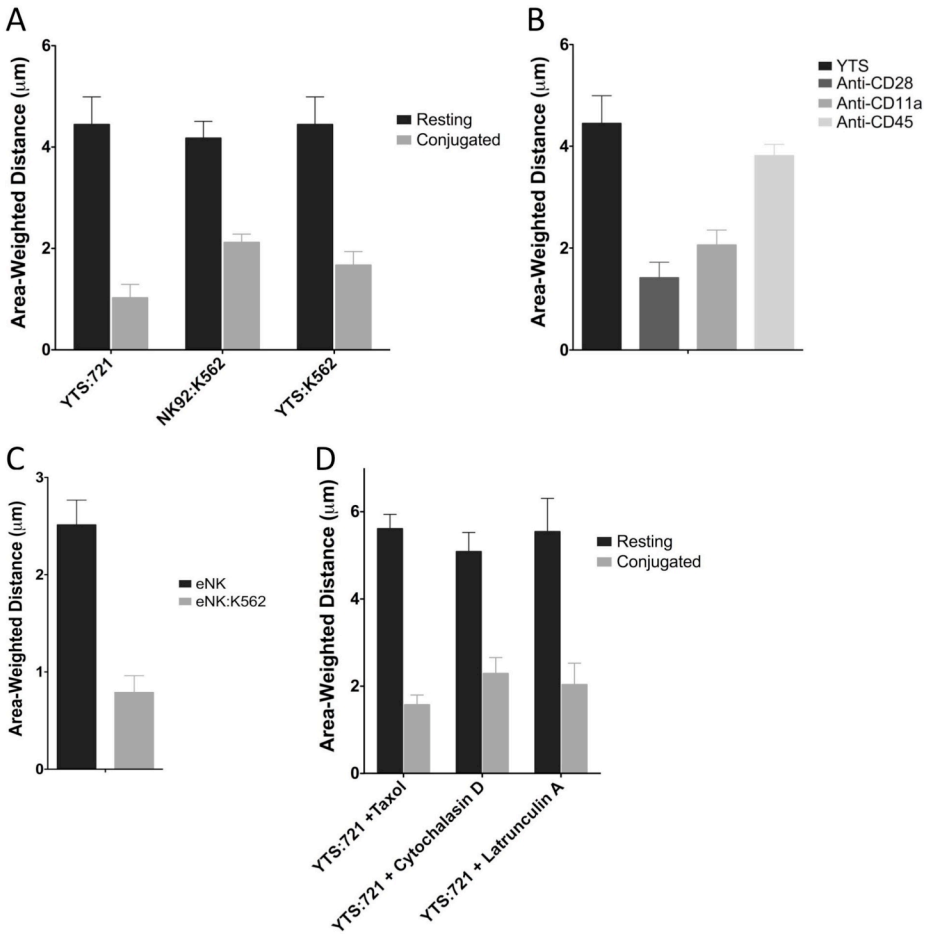

Figure 2.4. Effect of conjugation, activation stimuli, Taxol treatment, Cytochalasin D treatment, or Latrunculin A treatment on lytic granule area-weighted distance from the MTOC.
Quantitative analyses of lytic granule movement relative to the MTOC as measured by mean area-weighted distance ± SD across all time points in: (A) GFP-tubulin expressing NK cells in isolation (black), or conjugated with target cells (gray) as specified on the x-axis, (B) YTS GFP-tubulin cells in control or antibody-coated imaging chambers as defined in the inset legend, (C) eNK cells in isolation (black) or conjugated to susceptible K562 target cells (gray), and (D) Taxol-, Cytochalasin D-, or Latrunculin A-treated YTS GFP-tubulin cells in isolation (black) or conjugated to susceptible 721.221 target cells (gray). Each data point represents the mean over all time points recorded in 8-10 cells.

once they had converged to the MTOC. Thus, lytic granules remained converged around the MTOC throughout conjugation whether the MTOC was polarized or not.

A simple measure of lytic granule to MTOC distance, however, did not account for groups of granules that could not be resolved individually using confocal microscopy. Thus, an equation was developed to give additional weight to lytic granule groupings (area-weighted distance). Using this equation over all time points, the same trends of lytic granules being clustered to the MTOC after NK cell conjugation were identified (Figure 2.4A). Thus, lytic granules were

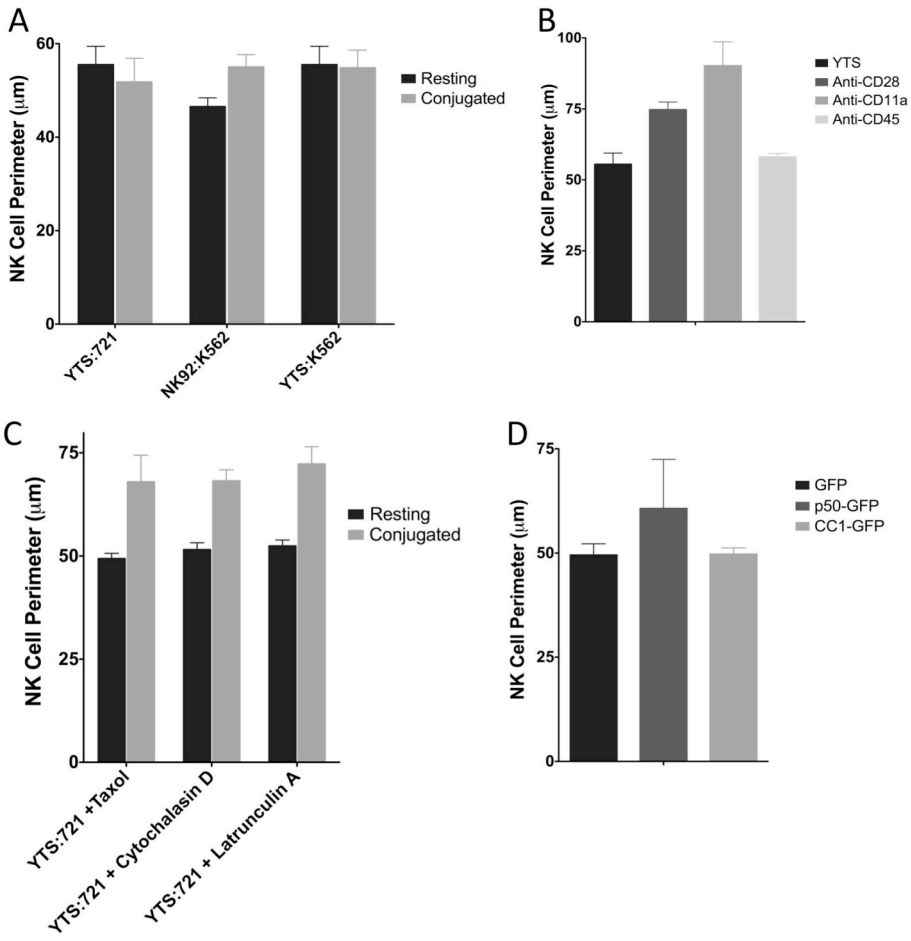

Figure 2.5. Effect of conjugation, activation stimuli, Taxol treatment, Cytochalasin D treatment, Latrunculin A treatment, or dynein disruption on NK cell perimeter.
Quantitative analyses of mean NK cell perimeter ± SD across all time points in: (A) GFP-tubulin expressing NK cells in isolation (black), or conjugated with target cells (gray) as specified on the x-axis, (B) YTS GFP-tubulin cells in control or antibody-coated imaging chambers as defined in the inset legend, (C) Taxol-, Cytochalasin D-, or Latrunculin A-treated YTS GFP-tubulin cells in isolation (black) or conjugated to susceptible 721.221 target cells (gray), and (D) GFP-, p50-GFP-, or CC1-GFP-nucleofected YTS cells conjugated to susceptible 721.221 target cells as defined in the inset legend. Each data point represents the mean over all time points recorded in 5-10 cells.

accumulated around the MTOC, were sustained in that position and were independent of cytotoxicity triggering signals.

The measurements thus far also did not take into account any cell spreading that might occur during the course of the experiments, which could bias measurements by pulling granules in the direction of the cell footprint. When specifically measured, however, the mean cell perimeter at individual time points did not substantively change over all time points (Figure 2.5A). Thus, it is unlikely that granule movements were a feature of global cell expansion or contraction.

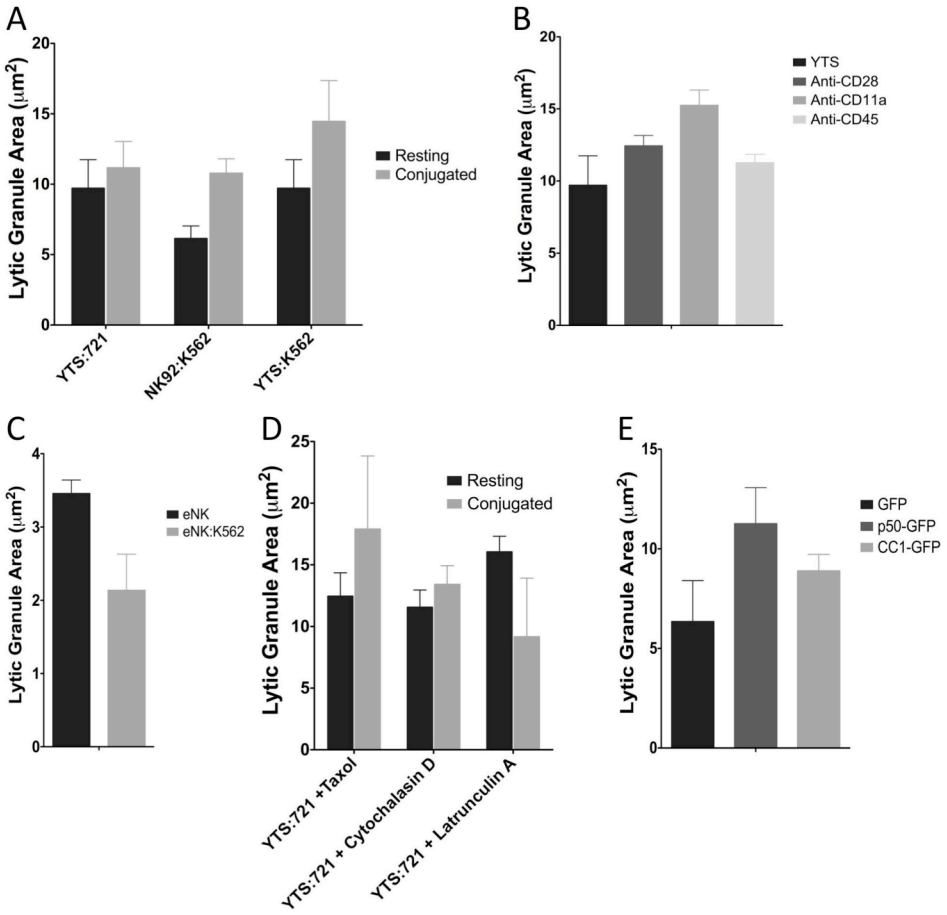

Figure 2.6. Effect of conjugation, activation stimuli, Taxol treatment, Cytochalasin D treatment, Latrunculin A treatment, or dynein disruption on lytic granule area.
Quantitative analyses of mean total lytic granule area ± SD across all time points in: (A) GFP-tubulin expressing NK cells in isolation (black), or conjugated with target cells (gray) as specified on the x-axis, (B) YTS GFP-tubulin cells in control or antibody-coated imaging chambers as defined in the inset legend, (C) eNK cells in isolation (black) or conjugated to susceptible K562 target cells (gray), (D) Taxol-, Cytochalasin D-, or Latrunculin A-treated YTS GFP-tubulin cells in isolation (black) or conjugated to susceptible 721.221 target cells (gray), and (E) GFP-, p50-GFP-, or CC1-GFP-nucleofected YTS cells conjugated to susceptible 721.221 target cells as defined in the inset legend. Each data point represents the mean over all time points measured in 5-10 cells.

The measurements could also be affected by changes in the total lytic granule area in an *x,y* plane. Thus, total lytic granule area was measured in the *x,y* plane of the MTOC in all time points and the mean value did not vary consistently over time (Figure 2.6A). These consistencies were also observed for the other NK cells measured throughout this work (Figures 2.5B-D; 2.6B-E).

Finally, since the lytic granules in live cells were visualized using LysoTracker-loaded NK cells, an additional method of visualizing lytic granules was pursued to allow for confirmation of measurements of convergence. Here, YTS cells were either left unconjugated, or conjugated to 721.221 target cells, followed by fixation and staining for perforin and tubulin to parallel the live cell experiments and enable detection of the lytic granules and MTOC, respectively. In unconjugated cells the perforin-defined lytic granules were diffusely localized relative to the MTOC, whereas in cells that had been conjugated to a target cell for 5 or 30 min, the lytic granules were converged to the MTOC (Figure 2.7A). This was confirmed in repeated experiments using the measurements for mean distance, or area-weighted distance of lytic granule regions from MTOC described above, even though the total area of perforin-defined lytic granules was slightly less than that identified by LysoTracker labeling of live cells (Figure 2.7B-D).

NK cell activation receptors trigger rapid lytic granule convergence to the MTOC

The convergence of lytic granules to the MTOC in non-cytolytic conjugates suggested that adhesion events alone could trigger granule motility. To evaluate the activity of isolated receptors, live cell chamber slides were coated with monoclonal antibodies specific for the CD28 lytic receptor, CD11a, or CD45 as a non-activating receptor, and cells imaged every 10 s after they contacted the glass surface. When slides were not coated with antibody, the lytic granules remained diffusely localized relative to the MTOC (Figure 2.8A). After contacting a surface coated with antibody against CD28 or CD11a, however, rapid lytic granule convergence to the MTOC was observed (Figure 2.8B,C; and Video 2, left and center). YTS cells deposited on surfaces coated with antibody against CD45 adhered, but lytic granules did not converge to the MTOC and remained diffusely localized (Figure 2.8D; and Video 2, right).

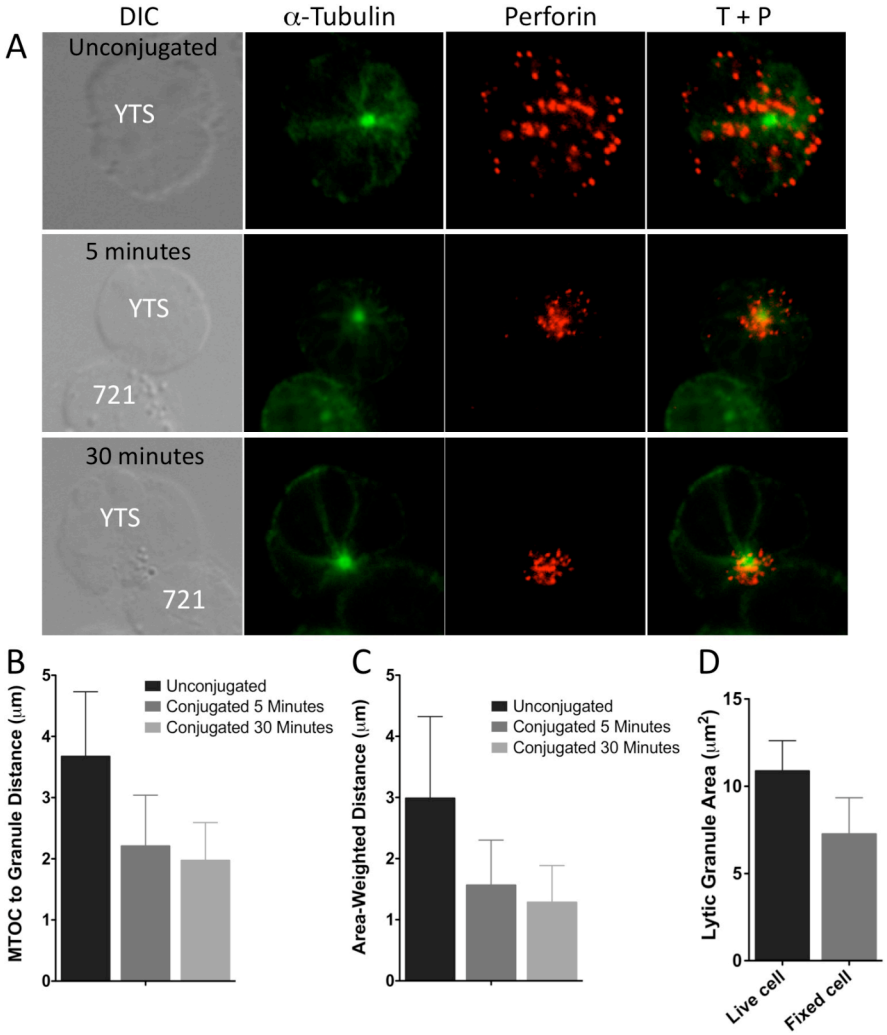

Figure 2.7. Quantitative analysis of lytic granule convergence to the MTOC in fixed cell conjugates.

Microscopy of YTS cells that were (A) unconjugated, or conjugated to 721.221 cells for 5 min, or 30 min prior to fixation. Images show DIC (left) and fluorescence (right) of the fixed cells in which staining was performed with anti-α−tubulin (green) and anti-perforin (red). The rightmost images demonstrate an overlay of tubulin and perforin fluorescence. The biotinylated anti-tubulin mAb was detected with Pacific Blue-streptavidin (pseudo-colored green); the anti-perforin antibody was directly FITC-conjugated (pseudocolored red). This particular pseudo-coloring was performed to enable comparison to similarly colored structures in images in other figures. Lytic granule proximity to the MTOC in unconjugated or conjugated NK cells as measured by mean MTOC to granule distance ± SD (B) and by mean area-weighted distance ± SD (C). Data are representative of 3 independent experiments in which 50 cells were analyzed per condition. Lytic granule distance from the MTOC in fixed conjugated NK cells is significantly less than in fixed unconjugated NK cells ($p < 0.001$). (D) Lytic granule area in the plane of the MTOC as measured by perforin staining in fixed cells compared to the area of the region defined LysoTracker Red staining in live cells used to generate Figure 2.3.

The distances of the lytic granules to the MTOC in these settings were measured directly (Figure 2.8E) and with weighting for lytic granule region area (Figure 2.4B). The mean distance of granules from the MTOC over all time points was decreased greater than two-fold in cells on anti-

CD28- or anti-CD11a-coated surfaces when compared to YTS cells in uncoated chambers. The mean distance of granules from the MTOC over time in YTS cells on the anti-CD45-coated surfaces, however, was not different from that in YTS cells in uncoated chambers (Figure 2.8E). Thus, activating receptors triggered in isolation can specifically induce lytic granule convergence to the MTOC, even if the receptor is not capable of inducing cytotoxicity on its own.

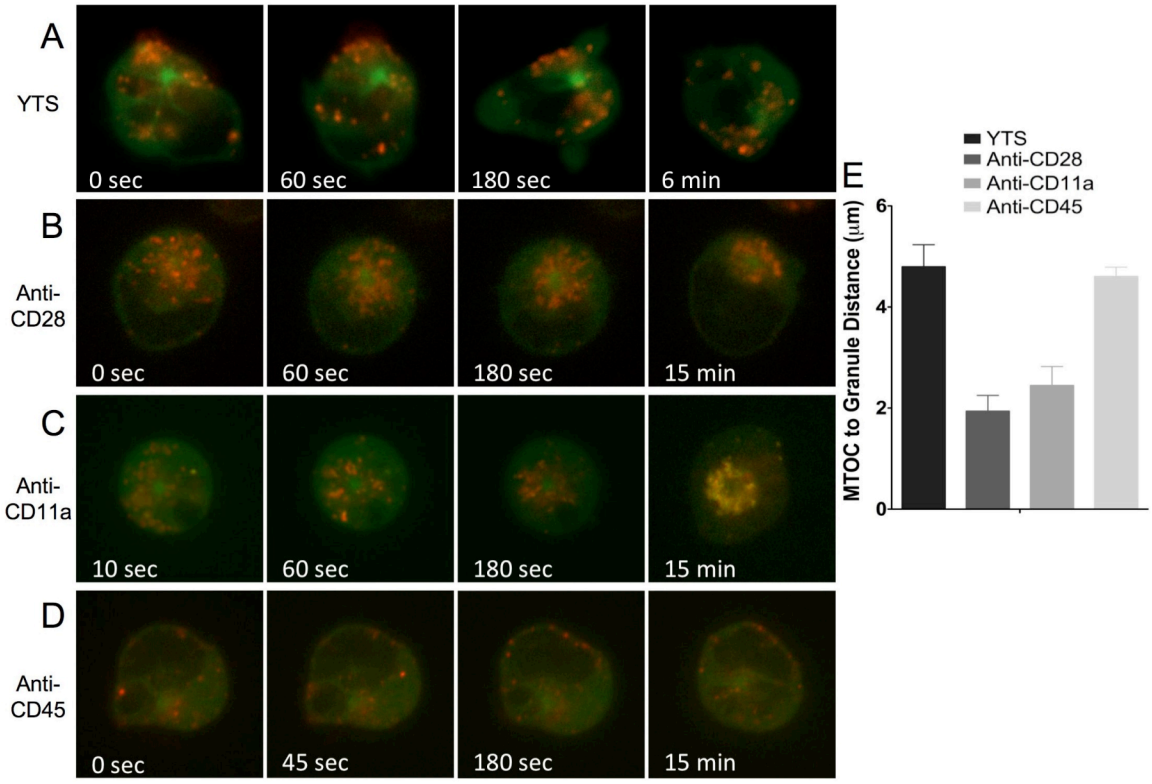

Figure 2.8. Dynamics of lytic granule movement to the MTOC in NK cells induced by immobilized antibodies against cell-surface receptors.

Time-lapse frames from Video 4 of lytic granule movement in a: (A) YTS GFP-tubulin cell on an uncoated surface, (B) YTS GFP-tubulin cell on an anti-CD28-coated surface, (C) YTS GFP-tubulin cell on an anti-CD11a-coated surface, and (D) YTS GFP-tubulin cell on an anti-CD45-coated surface. In each image, confocal immunofluorescence in the plane of the MTOC is shown. Green represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. Zero seconds represents the time at which the NK cell appears to contact the glass surface. (E) Quantitative analyses of lytic granule movement relative to the MTOC measured by mean MTOC to granule distance in individual time points averaged over all measured time points ± SD in 9-10 YTS cells per condition. Mean distances of lytic granules from the MTOC in NK cells on anti-CD28- and anti-CD11a- but not anti-CD45-coated surfaces were significantly different from unconjugated NK cells ($p < 0.05$).

Individual lytic granules rapidly track towards the MTOC only after activation

Although lytic granule movement to the MTOC was implied by the observed clustering around the MTOC at early time points after activation, the individual movements and kinetics of

moving granules were not defined. Thus, in order to resolve lytic granule traffic and in an effort to see individual lytic granules moving, rapid fluorescent image acquisition in resting and activated NK cells was performed. In resting YTS or NK92 cells, lytic granules did not move towards the MTOC (images in Figure 2.9A,B; and Videos 3A and 3B) and remained stationary. When YTS or NK92 cells were deposited onto chamber slides coated with anti-CD28 or anti-NKp30, respectively, individual granules moved rapidly in short bursts towards the MTOC (images in Figure 2.9C,D; and Videos 3C and 3D).

To quantify the lytic granule movements, the distance of each lytic granule from the MTOC was measured at each time point by comparing the *x,y* coordinates of both objects. In resting YTS and NK92 cells, there was negligible change in lytic granule position relative to the MTOC (graphs in Figure 2.9A,B). In activated cells, however, lytic granules demonstrated MTOC-directed movement (graphs in Figure 2.9C,D). The same paradigm was identified for lytic granules in YTS and NK92 cells conjugated to target cells (Figure 2.10A). The movements of lytic granules in activated NK cells were asynchronous, often abrupt and typically short-lived (Figure 2.9C,D). To better gauge directedness of the individual lytic granule tracks, the net velocity of each over its entirety relative to the MTOC was determined. A positive value denoted a net movement towards the MTOC and was found for the mean of all lytic granule tracks in activated cells. These values were significantly different from those in resting NK cells (Figure 2.9E). The lytic granule velocity over the total run length of individual tracks irrespective of their directionality was also measured and was still greater in activated NK cells (Figure 2.10B,C). Although the total run length was significantly greater in activated as compared to resting YTS cells, it was not in NK92 cells and thus the velocity differences cannot be explained by run length alone (Figure 2.10D,E).

To better quantify abrupt transitions in lytic granule position relative to the MTOC, the instantaneous velocity was determined for only those parts of lytic granule tracks that traveled at

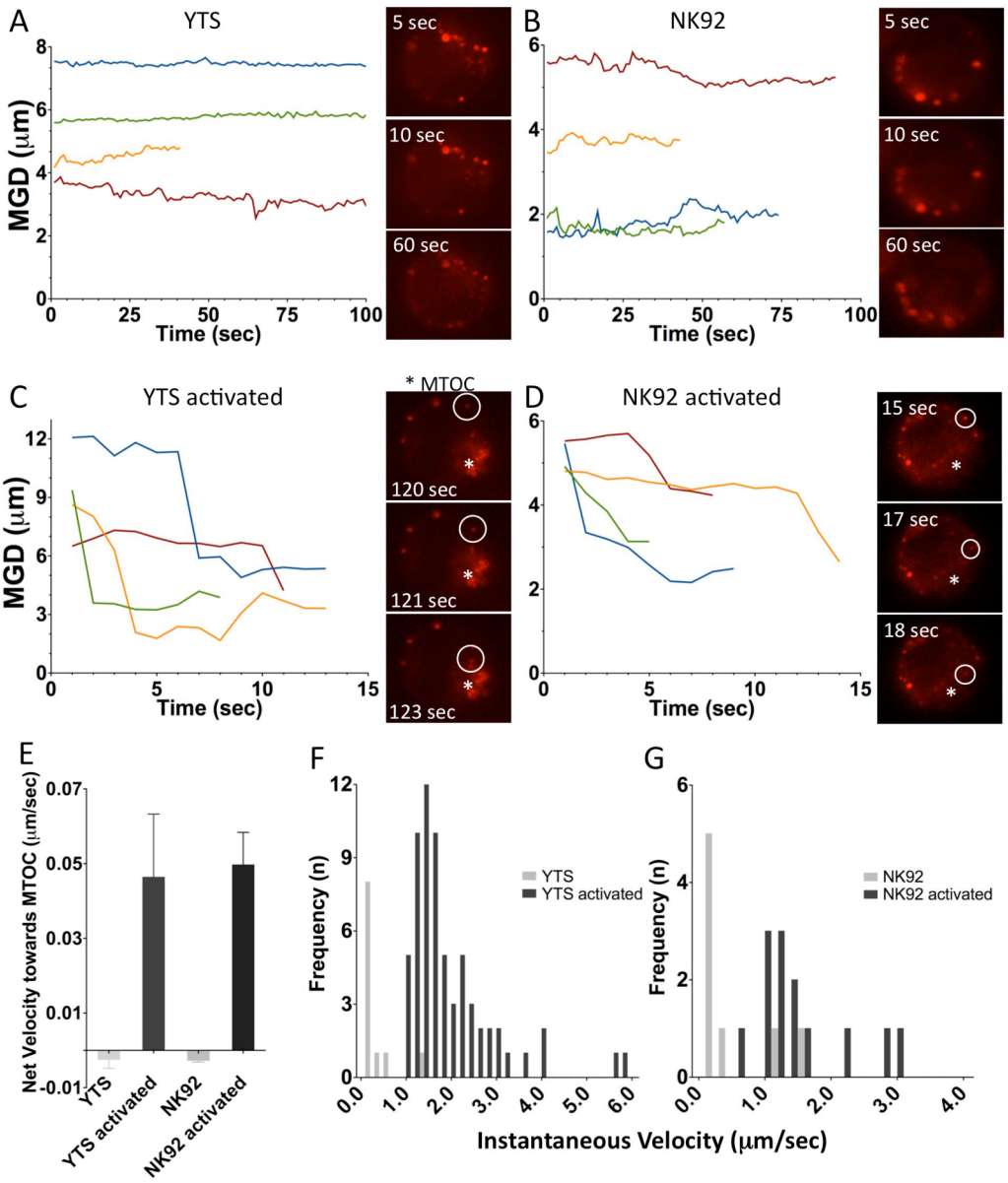

Figure 2.9. Quantitative analyses of rapid lytic granule movement relative to the MTOC in NK cells.

Distance of individual LysoTracker Red-loaded lytic granules from the MTOC as a function of time in a single cell and as visualized in a streaming video sequences in: resting YTS GFP-tubulin (A), or NK92 GFP-tubulin (B) cells, and YTS GFP-tubulin cells on an anti-CD28-coated surface (C), or NK92 GFP-tubulin cells on an anti-NKp30-coated surface (D). Times listed in images, represent seconds elapsed after the NK cell contacted the glass surface. The MTOC as defined by GFP fluorescent acquisition (not shown), is marked (*) and a white circle tracks an individual moving lytic granule. The line graphs shown each demonstrate 4 representative single lytic granule tracks with T=0 representing the first identification of that granule. In activated NK cells these had a total displacement of >1 μm as did >50% of all granules identified. (E) Mean net velocity ± SD of all measured lytic granules over their entire tracks. Each bar represents three cells accounting for 58-141 measured lytic granule tracks; means in activated YTS and NK92 cells were significantly different from resting cells (p=0.045 and p=0.0036, respectively). Histogram of instantaneous velocities of all MTOC-directed lytic granules in YTS GFP-tubulin (F) and NK92 GFP-tubulin cells (G), as they moved towards the MTOC with a displacement of ≥1 μm. Instantaneous velocities in activated YTS and NK92 cells were significantly different from those in resting cells (p<0.0001 and p=0.0035, respectively).

least 1 μm in a minus-end-directed manner. Although few lytic granules in resting cells attained this degree of minus-end-directed movement, the instantaneous velocity in activated cells was significantly greater (Figure 2.9F,G). The mean instantaneous velocity of lytic granules increased from 0.2 ± 0.4 μm/sec in resting to 1.8 ± 1.0 μm/sec in activated YTS cells and from 0.4 ± 0.6 μm/sec in resting to 1.4 ± 0.7 μm/sec in activated NK92 cells. This further demonstrates the directedness of lytic granules to the MTOC after NK cell activation, which occurs soon after the signal is received.

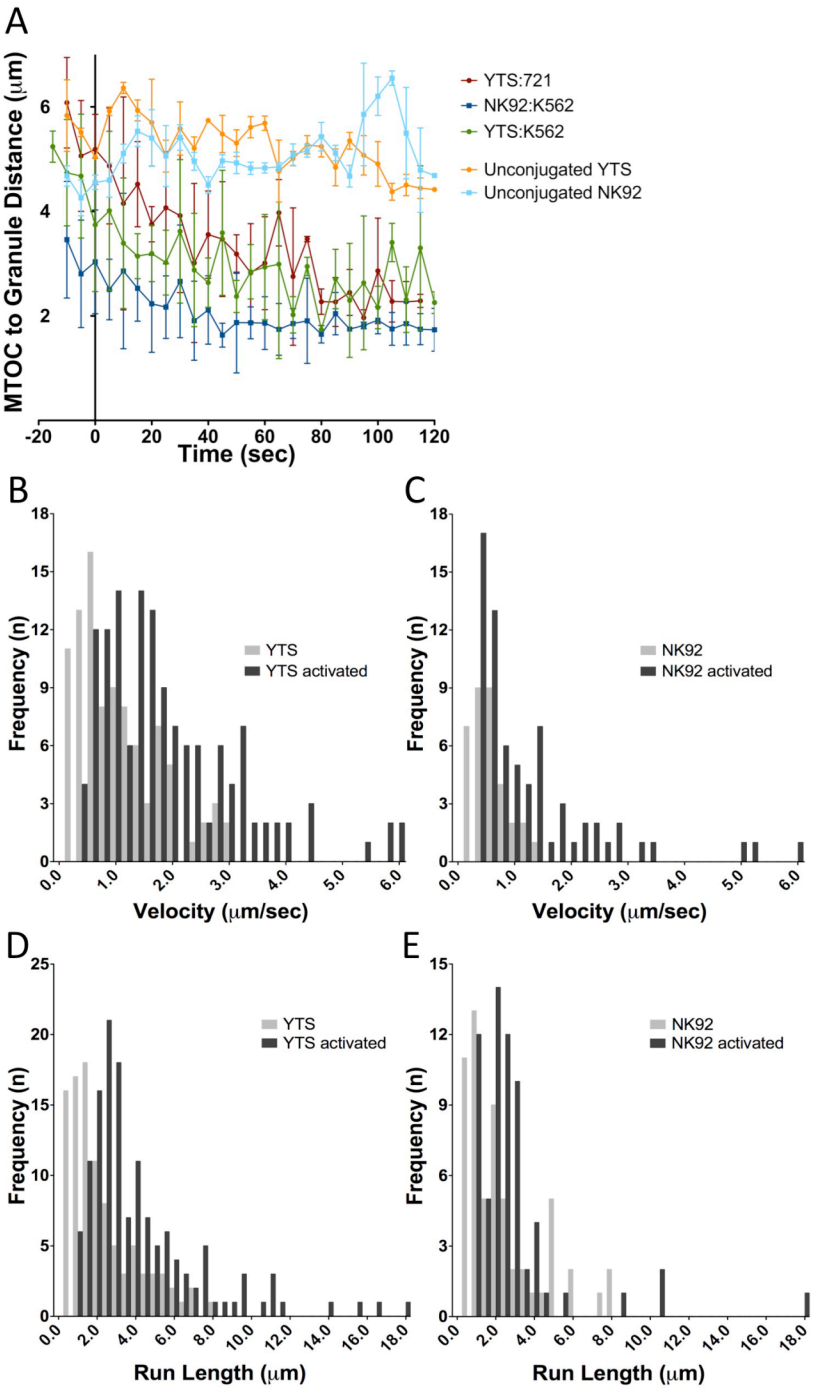

Figure 2.10. Quantification of MTOC-directed lytic granule movement in resting, activated and conjugated NK cells. (A) Mean MTOC to lytic granule distance measured in streaming video of YTS GFP-tubulin or NK92 GFP-tubulin cells unconjugated, or conjugated to susceptible target cells. YTS GFP-tubulin cells were also measured in nonclytolytic conjugates with K562 target cells. One image per second for 15 s prior to and 2 min after conjugation were analyzed for conjugated cells. In unconjugated cells a similar number of images were acquired and measured. Each point represents mean data of lytic granule to MTOC distances at a single time point from 3-6 cells. (B-E) Additional measurements of lytic granule tracks identified in Figure 2.9. Total lytic granule velocity (B and C), or total run length (D and E) over the entirety of individual lytic granule tracks identified in YTS GFP-tubulin

Lytic granule and MTOC dynamics in ex vivo NK cells recapitulate that in NK cell lines

Although our findings thus far were established in two distinct NK cell lines, they might not necessarily translate to ex vivo NK (eNK) cells. Thus, to define the observed dynamics of lytic granules and the MTOC as a general characteristic of NK cells, highly enriched eNK cells were prepared from human blood and an α-tubulin-GFP construct introduced immediately after their isolation. Prior to their conjugation with K562 target cells, eNK cells were incubated with LysoTracker Red dye in order to enable visualization of the lytic granules. In unconjugated eNK cells, a concentration of GFP tubulin signifying the MTOC could be visualized and lytic granules were diffusely arranged (Figure 2.11A). In time-lapse images, the lytic granules remained diffuse. Similar lytic granule patterns were observed in eNK cells prior to conjugation, but soon thereafter, they converged to the MTOC (Figure 2.11B; and Video 4) at which point it began to polarize to the IS.

In repeated assays using eNK cells from different donors, the positioning of the MTOC relative to the IS and the lytic granules relative to the MTOC were quantified. The distance of the MTOC from the IS gradually decreased as a feature of conjugation time (Figure 2.11C). The time required to achieve minimum distance between the MTOC and the IS was also measured in each individual cell and was 22.67 $\pm$ 0.29 min. The distance of the lytic granules to the MTOC, however, decreased rapidly when compared to that in unconjugated eNK cells (Figure 2.11D). At the first time point after target cell contact (6.4 min after acquisition began), the distance of the lytic granules to MTOC had already decreased by 43%. This was substantively before the MTOC was recognized as moving towards the IS, as the MTOC to IS distance at these time points was not significantly different from that at T=0. When the lytic granule regions were weighted according to their area, the rapid convergence to the MTOC was similarly evident (Figure 2.4C),

---

cells (B and D), and NK92 GFP-tubulin cells (C and E) from streaming video sequences recorded in control (gray) or activating antibody-coated (black) imaging chambers. Each bar represents the number of lytic granule tracks having a particular mean velocity or run length as obtained from 3 cells. The velocities of lytic granules in activated YTS and NK92 cells were significantly different from that in resting YTS and NK92 cells ($p < 0.0001$, $p = 0.0007$, respectively). The difference in lytic granule run lengths between activated and resting YTS cells was significant ($p < 0.0001$) while the difference in lytic granule run lengths between activated and resting NK92 cells was not ($p = 0.2$).

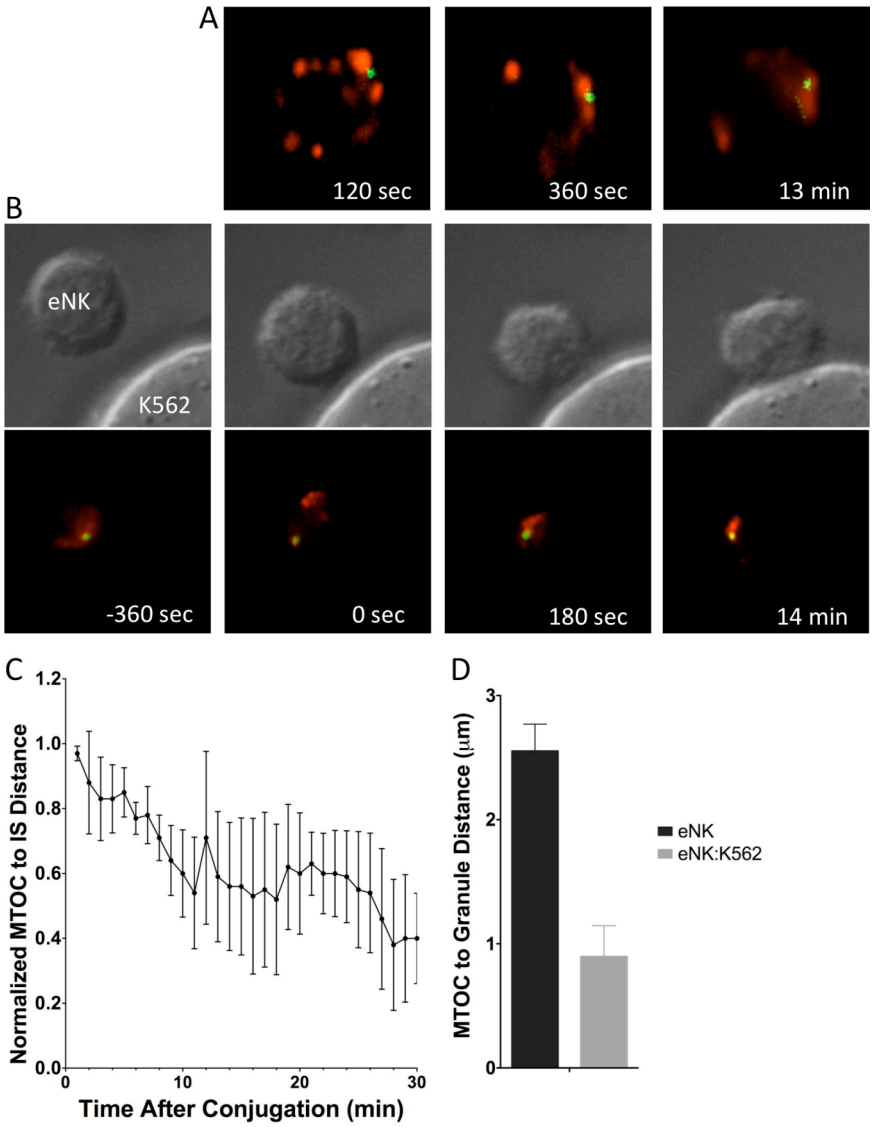

Figure 2.11. Dynamics of MTOC movement relative to the IS and lytic granule movement relative to the MTOC in unconjugated or target cell-conjugated eNK cells.
Time-lapse images of individual eNK cells nucleofected with GFP-tubulin deposited onto an uncoated surface (A), or in conjugation with a susceptible K562 target cell (B; Video 4). Confocal immunofluorescence in the plane of the MTOC is shown (green: GFP-tubulin and red: LysoTracker-loaded acidified lysosomes) and DIC (top). Zero seconds represents the time at which the NK cell appears to contact either the imaging surface or the target cell. Quantitative analyses of mean distance ± SD between the MTOC and the IS normalized to the largest distance of the MTOC from the IS as a function of time (C). Lytic granule movement relative to the MTOC as a function of time measured by mean MTOC to granule distance in individual time points averaged over all measured time points ± SD in 9 cells (D). Mean distance of lytic granules from the MTOC in conjugated eNK cells was significantly different from that in unconjugated eNK cells ($p < 0.05$).

and therefore was not a feature of the relatively small size of eNK cells compared to the cell lines.

Thus, the dynamics defined in the NK cell lines reproduces those observed in eNK cells. This

likely represents the physiological process by which lytic granules and the MTOC approach the IS, with lytic granules first approximating the MTOC and then the MTOC polarizing to the IS.

Lytic granule convergence to the MTOC in activated NK cells occurs independently of microtubule and actin reorganization

Since lytic granule convergence to the MTOC occurred rapidly after IS formation, we next evaluated the dependence of this MTOC-directed traffic upon other known cytoskeletal requirements for IS formation and function. Because microtubule function is required for lytic granule polarization to the IS (Orange et al., 2003), we first asked whether microtubule dynamics and/or MTOC movement was required for lytic granule convergence to the MTOC. Thus, YTS GFP-tubulin cells were treated with Taxol to stabilize microtubules, incubated with LysoTracker Red dye, and conjugated with target cells (Figure 2.12A; and Video 5A). Taxol-treated cells still conjugated with target cells, but as would be expected the MTOC failed to polarize to the IS (Figure 2.12B). While the lytic granules did not polarize to the IS in Taxol-treated cells, they did continue to rapidly converge to the MTOC after target cell conjugation. This was reflected quantitatively by rapidly decreased MTOC to granule distance (Figure 2.12C) as well as area-weighted distance (Figure 2.4D) when compared to Taxol-treated unconjugated YTS cells. The magnitude and kinetics of lytic granule convergence in the Taxol-treated conjugated cells was not different from that in untreated conjugated cells shown in Figure 2.3 ($P = 0.17$, and data not shown). Thus, MTOC-directed lytic granule movement occurs independently of microtubule dynamics and can utilize stabilized microtubules to move towards a fixed MTOC.

To define the dependency of lytic granule convergence to the MTOC upon microtubules, YTS GFP-tubulin cells were treated with Nocodazole prior to conjugation with target cells. Similar to Taxol treatment, the NK cells still conjugated with target cells but due to Nocodazole-induced destabilization, the microtubule network and the MTOC were not visualized. In these cells, although there was no MTOC, clustering of lytic granules within the cell was not found (Figure 2.13). Thus, lytic granule convergence in NK cells after activation requires intact microtubules but does not require microtubule dynamics. This suggests specific motor-driven traffic of lytic

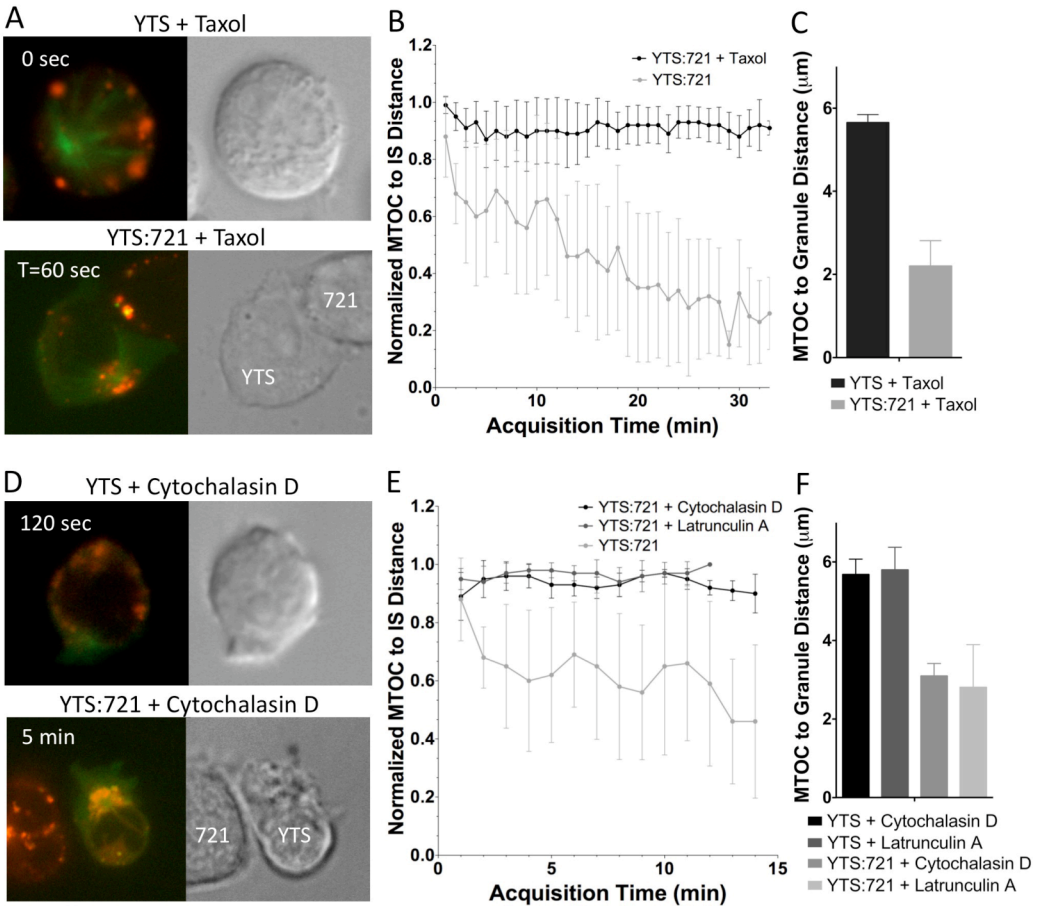

Figure 2.12. Taxol, Cytochalasin D or Latrunculin A treatment does not prevent lytic granule movement to the MTOC in NK cells after target cell conjugation.
Images of YTS GFP-tubulin cells treated with: (A) Taxol (Video 5A) or (D) Cytochalasin D (Video 5B) unconjugated or conjugated with a susceptible 721.221 target cell. In each, confocal immunofluorescence in the plane of the MTOC is shown (left; green: GFP-tubulin, and red: LysoTracker-loaded acidified lysosomes) and DIC (right). Zero seconds defines the time at which the NK cell appears to contact either the imaging surface or the target cell. Quantitative analyses of mean distance ± SD between the MTOC and the IS normalized to the farthest distance of the MTOC from the IS as a function of time in: (B) Taxol- or (E) Cytochalasin D, or Latrunculin A-treated YTS GFP-tubulin cells conjugated with susceptible 721.221 target cells (n = 10, n = 8, respectively). Mean MTOC to lytic granule distance in individual time points average over all measured time points ± SD in: (C) Taxol- or (F) Cytochalasin D, or Latrunculin A -treated YTS GFP-tubulin cells conjugated with susceptible 721.221 target cells. Lytic granule distance from the MTOC in drug-treated conjugated NK cells was significantly different from unconjugated drug-treated NK cells ($p < 0.05$).

granules along microtubules to the MTOC rapidly after target cell recognition.

One of the earliest and most rapid events in formation and function of the NK cell IS is the reorganization of F-actin at the IS. It occurs within minutes and is required for activation receptor clustering at and lytic granule polarization to the NK cell IS (Orange et al., 2003). Thus, we next determined if the large-scale actin reorganization required for cytotoxicity was also a requirement for lytic granule convergence to the MTOC. YTS GFP-tubulin cells were treated with

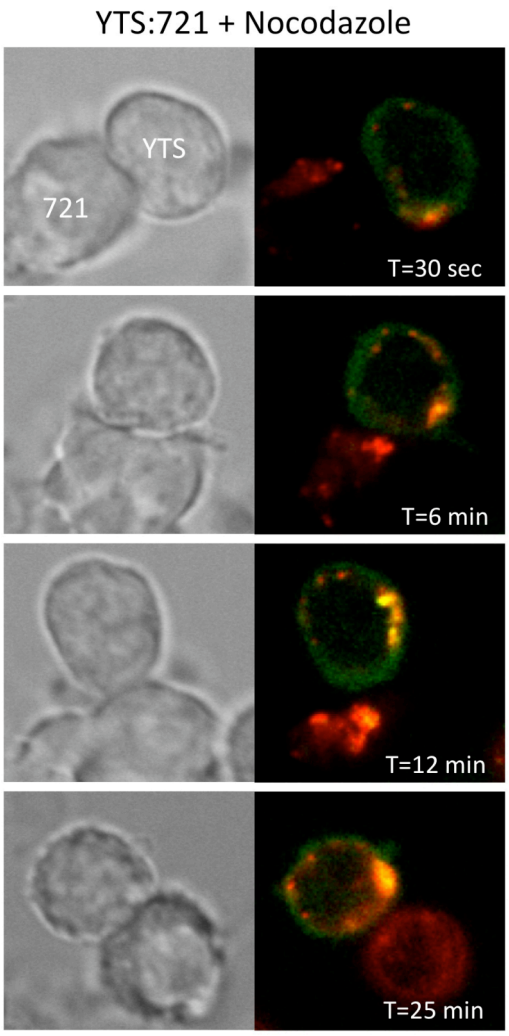

Figure 2.13. Effect of Nocodazole on lytic granule convergence.
Differential interference contrast (left) and fluorescence (right) time-lapse images of a YTS GFP-tubulin cell pre-treated with Nocodazole in conjugation with a susceptible 721.221 cell. Time shown represents the time after acquisition began, which was approximately 1-4 minutes after YTS cells were added to the imaging chamber. Nocodozole was also present in the imaging chamber media throughout the experiment. While GFP-tubulin signal was present, an intensity corresponding to the MTOC was not found throughout the volume of the cell and thus the images represent a single x,y plane intersecting the IS. Convergence of lytic granules was not identified in this or other time-lapse sequences of Nocadazole-treated cells.

Cytochalasin D, or Latrunculin A prior to their conjugation with target cells to prevent F-actin reorganization. In Cytochalasin D-treated cells, conjugates tended to be short-lived and YTS cells were frequently misshapen (Figure 2.12D; and Video 5B). As previously demonstrated, with either inhibitor the MTOC did not polarize to the IS (Figure 2.12E). In contrast, lytic granules converged rapidly to the MTOC after conjugation when compared to Cytochalasin D- or Latrunculin A-treated unconjugated cells. This was true when measuring either the MTOC to granule distance or area-weighted distance (Figures 2.12F and 2.4D). Thus, despite in some cases forming short-lived contacts and with an inhibited ability to reorganize F-actin, lytic granule convergence still occurred. Lytic granule convergence to the MTOC, therefore, is rapid and independent of previously identified cytoskeletal requirements.

Lytic granule convergence to the MTOC during synapse formation requires LFA-1

Since lytic granule convergence to the MTOC during IS formation is rapid and appears to be independent of many previously identified checkpoints in the process of lytic synapse

maturation, we considered the initial adhesion event as one promoting convergence. While a variety of adhesion mechanisms are utilized by human NK cells, those attributable to LFA-1 are

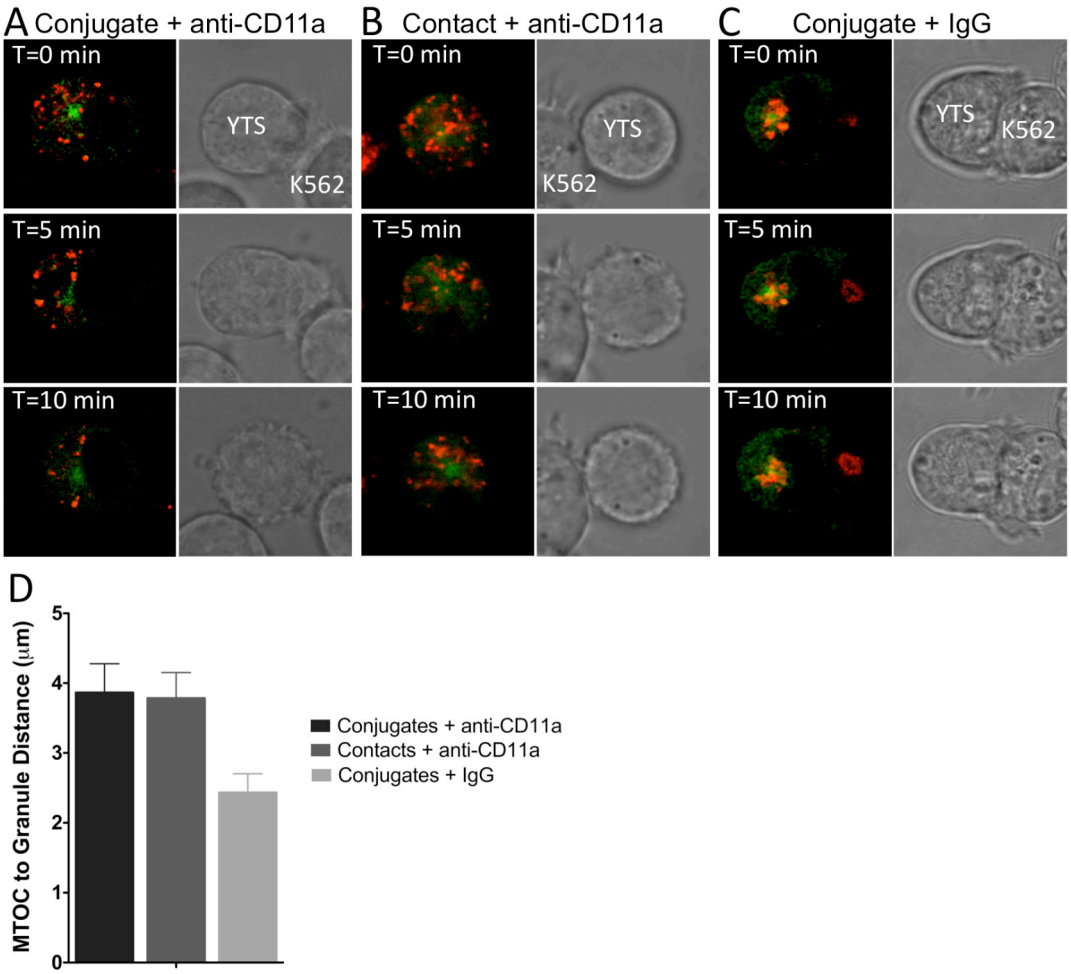

Figure 2.14. LFA-1 blockade prevents lytic granule movement to the MTOC in NK cells after target cell conjugation.

Time-lapse images of lytic granule movement in (A) a YTS GFP-tubulin cell pre-incubated with anti-CD11a and conjugated to a non-susceptible K562 target cell, (B) a YTS GFP-tubulin cell pre-incubated with anti-CD11a and contacting a non-susceptible K562 target cell, and (C) a YTS GFP-tubulin pre-incubated with control IgG and conjugated to a non-susceptible K562 target cell. In each pair of images, confocal immunofluorescence in the plane of the MTOC is shown on the left and DIC on the right. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. T = 0 refers to the time image acquisition began which was between 1-4 min after NK cells were added to the imaging chamber. YTS cells pre-incubated with anti-CD11a conjugated to non-susceptible K562 cells in 50% of cell interactions observed, while in the other 50% the YTS did not demonstrate membrane deformation and are thus described separately as a contact. YTS cells pre-incubated with IgG routinely conjugated to K562 cells. (D) Quantitative analysis of lytic granule movement relative to the MTOC as measured by mean MTOC to granule distance in individual time points averaged over all measured time points ± SD in: conjugates between anti-CD11a-coated YTS and K562 target cells (n = 10), contacts between anti-CD11a-coated YTS and K562 target cells (n = 10), and conjugates between IgG-coated YTS and K562 target cells (n = 5). Mean distances of lytic granules from the MTOC in anti-CD11a-coated YTS cells were significantly greater than in IgG-coated YTS cells ($p < 0.001$).

required (Kohl et al., 1984). Furthermore, ligation of LFA-1 has been shown to enable the focusing of lytic granules to the IS under certain circumstances (Bryceson et al., 2005), and signals downstream of LFA-1 in NK cells are independent of F-actin reorganization (Riteau et al., 2003). Thus, we evaluated the role of LFA-1 in lytic granule convergence by tracking lytic granules after the addition of blocking LFA-1 antibody (anti-CD11a) or control IgG. Non-cytolytic conjugates between YTS and K562 cells were evaluated in these experiments to allow focus upon the signal for convergence without the signal required for cytotoxicity (as would be provided by the 721.221 target cell). In the presence of anti-CD11a antibody only approximately half of conjugates demonstrated an obviously deformed NK cell surface at the IS (Figure 2.14A). The other half made a sustained contact, but failed to demonstrate obvious surface deformation and are referred to as contacts instead of conjugates (Figure 2.14B). In either case, the lytic granules failed to converge to the MTOC when compared to IgG control (Figure 2.14C). The mean MTOC to lytic granule distance was measured across all timepoints and was not found to be different in conjugates or contacts with anti-CD11a blocking antibody, but both were greater than that measured with control IgG (Figure 2.14D). Thus, LFA-1 is required for lytic granule convergence to the MTOC in NK cells.

Dynein/dynactin activity traffics lytic granules to the MTOC

The ability of lytic granules to move towards the MTOC when microtubules were intact, even when they were stabilized, implies a role for a motor protein in their transport. In order to identify the motor used by lytic granules, mass spectrometry of purified NK cell lytic granules was performed. Among a list of 644 proteins identified, the only known minus-end-directed motor component was dynein heavy chain (Figure 2.15A). To confirm this association of dynein with lytic granules and to demonstrate the presence of the dynein/dynactin complex, lytic granules were purified from YTS cells and evaluated by Western blot analysis along with the post nuclear cell lysate and crude lysosomal cell fractions used for lytic granule preparation. The purified lytic granules demonstrated an abundance of granzyme B, as would be expected, and also

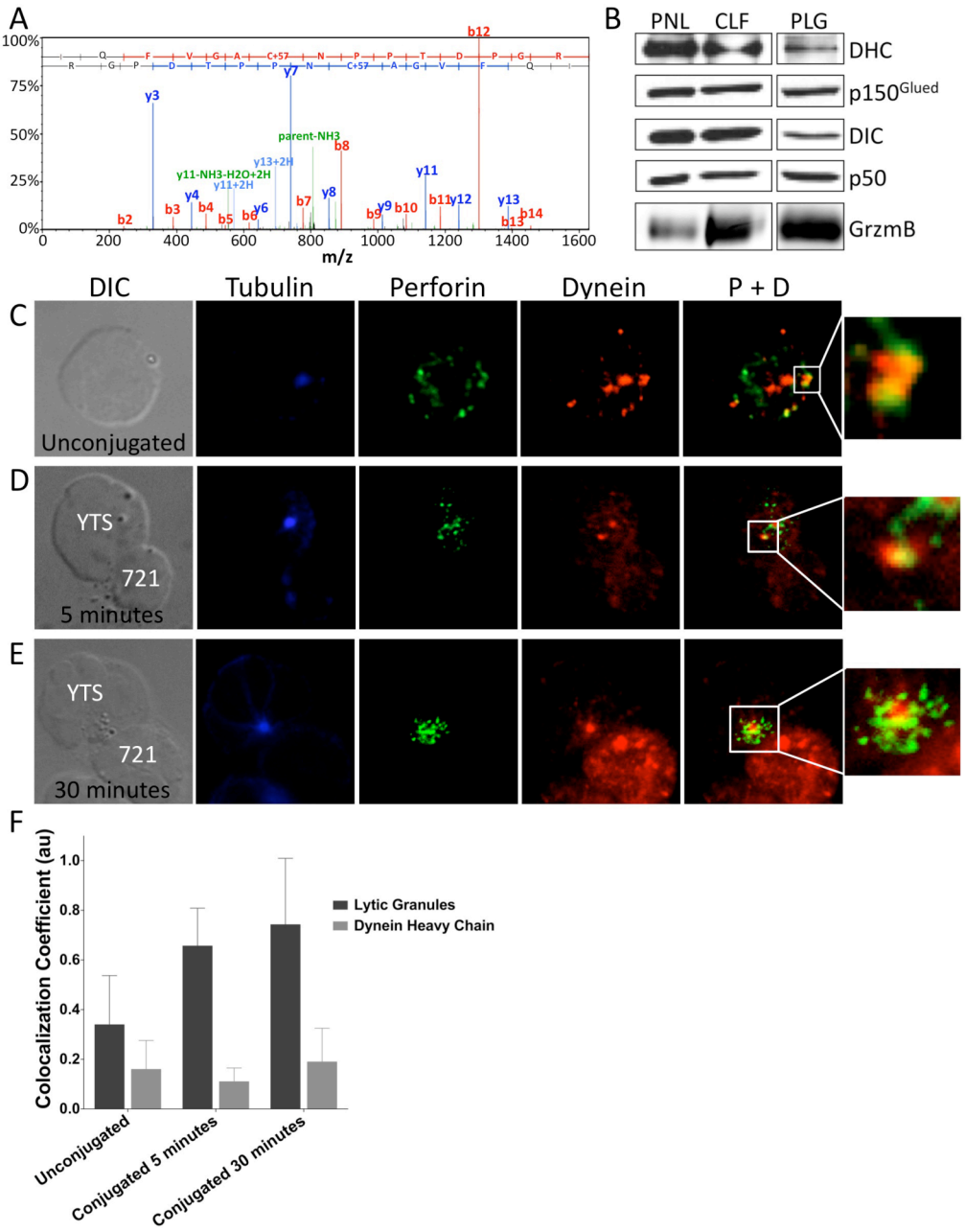

Figure 2.15. Dynein colocalization with perforin-containing lytic granules.
(A) Mass spectrometric analysis of one of 32 unique peptides identified as dynein heavy chain in a tryptic digest of a single protein band from a gel electrophoresis of isolated lytic granules. (B) Dynein heavy chain (DHC), p150^{Glued}, dynein intermediate chain (DIC), p50 dynamitin, and granzyme B (GrzmB) Western blot analyses of the postnuclear lysate (PNL), crude lysosomal fraction (CLF), and purified lytic granules (PLG) from density gradient separation of lytic granules from YTS cells. (C-E) Microscopy of fixed cells showing DIC (left) and fluorescence (right) images of YTS cells unconjugated (C) or conjugated to 721.221 cells for 5 (D) or 30 (E) minutes prior to fixation and staining with anti-α–tubulin (blue), anti-perforin (green), and anti-dynein heavy chain (red). The rightmost images demonstrate an overlay of perforin and dynein fluorescence and the smaller image is an enlargement of the region within the white box. The biotinylated anti-tubulin mAb was detected with Pacific Blue-streptavidin, the anti-perforin antibody was directly FITC-conjugated and the anti-dynein antibody was detected with an AlexaFluor-568-conjugated goat anti-rabbit antibody. (F)

demonstrated dynein heavy chain, the p150^{Glued} subunit of dynactin, the dynein intermediate chain, and p50 dynamitin (Figure 2.15B). To determine if dynein was colocalized with lytic granules in intact NK cells, dynein heavy chain was evaluated before and after target cell conjugation by microscopy (Figure 2.15C-E). Colocalization between the lytic granule area and dynein was evaluated quantitatively through measurement of colocalization coefficients (Figure 2.15F). When considering the total visualized dynein or lytic granules, some degree of baseline colocalization between the two was identified. When considering total dynein fluorescence, the colocalization with lytic granules did not change after conjugation. When considering lytic granule regions, however, the colocalization with dynein did increase after conjugation. This could be a feature of lytic granule convergence leading to increased colocalized dynein fluorescent intensity in a unit area, or could represent some additional recruitment of dynein to lytic granules. The consistent colocalization coefficient when evaluating total dynein, however, suggests the former.

Dynein works in concert with dynactin to move cargo along microtubules in a processive minus-end-directed manner (Gill et al., 1991; King and Schroer, 2000). Thus, we hypothesized that the dynein/dynactin complex was the motor responsible for lytic granule traffic to the MTOC in NK cells. To evaluate this hypothesis, the function of the dynein/dynactin complex was disrupted by overexpression of either p50 dynamitin or the first coiled coil domain of p150^{Glued} (CC1). Transient expression of these has been previously described to block dynein function (Burkhardt et al., 1997; Waterman-Storer et al., 1995). Thus, p50 or CC1 GFP fusion proteins were expressed in YTS cells. The use of GFP fusion proteins facilitated identification of cells that had received the overexpression constructs and would have reduced dynein function. This however, precluded the use of GFP-tubulin to define the MTOC, and instead the distance of lytic granules from the centroid of the region bounded by all individual lytic granule regions was

---

Quantitative analyses of colocalization between perforin and dynein fluorescent regions as measured by the colocalization coeficient between the total cellular lytic granule area (black) or total cellular dynein area (gray) as a feature of conjugation time. Data are representative of 3 separate repeats in which 37-64 cells were evaluated per condition. Error bars show ± SD. Mass spectrometry was performed with the assistance of the Stokes Protein Core Facility at Children's Hospital of Philadelphia. Western blots were performed with the assistance of Mariko Tokito.

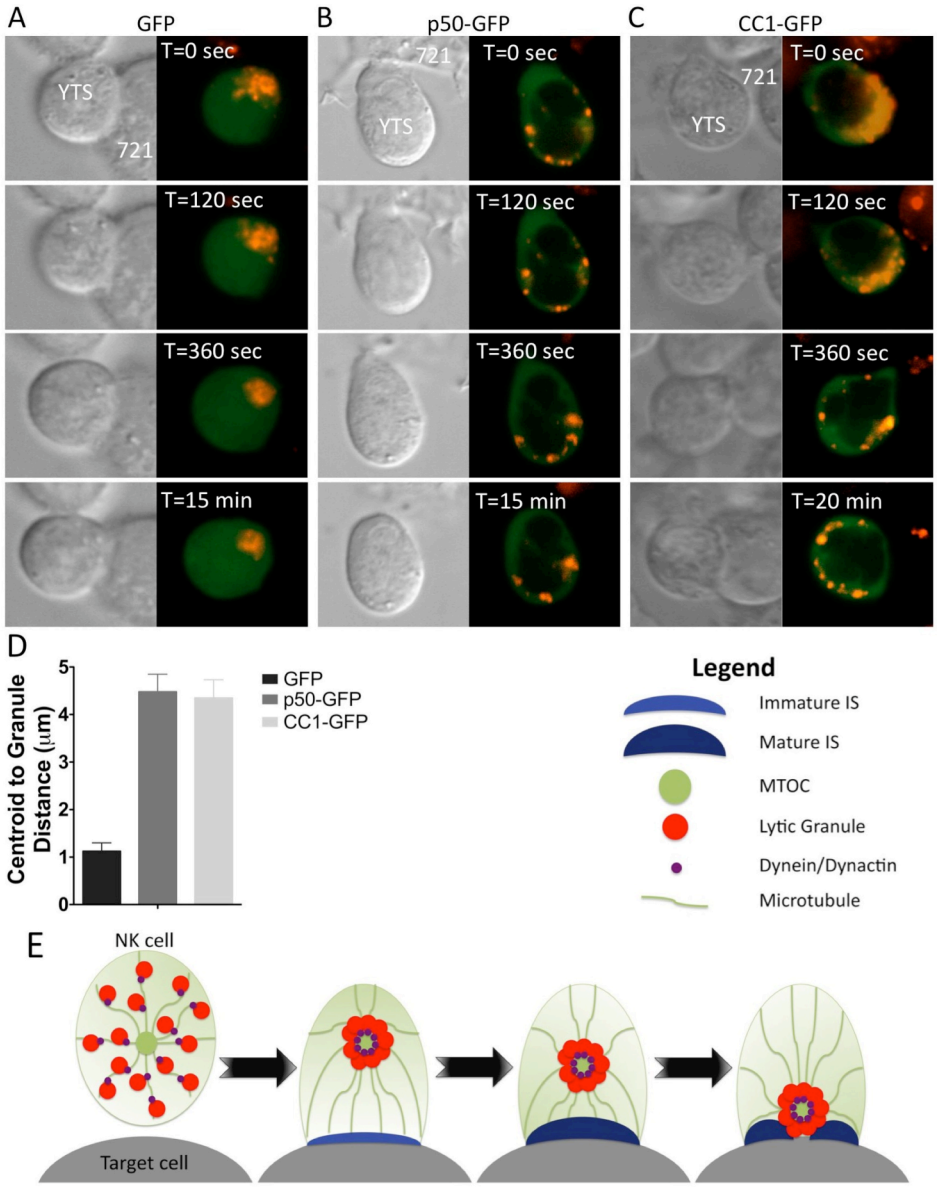

Figure 2.16. Dynein/Dynactin activity is required for rapid lytic granule traffic to the MTOC in NK cells.

Time-lapse images of YTS cells nucleofected with: (A) GFP, (B) p50-GFP or (C) CC1-GFP and in conjugation with susceptible 721.221 target cells (Video 12). In each pair of images, confocal immunofluorescence in the plane of lytic granules (left; green: overexpressed GFP fusion protein, and red: LysoTracker-loaded acidified lysosomes) and DIC (right) are shown. T = 0 refers to the time acquisition began which was between 0 and 2 min after the NK cells were added to the imaging chamber. (D) Lytic granule movement relative to the centroid of the granules as measured by mean centroid to granule distance over time ± SD in control GFP-, p50-GFP-, or CC1-GFP-nucleofected YTS cells conjugated with susceptible 721.221 target cells (n = 5). All distances of lytic granules from the granule centroid in p50- or CC1-nucleofected conjugates were significantly greater than those in GFP-nucleofected conjugates ($p < 0.05$). (E) Model of the linear sequence of events leading to directed secretion of lytic granule contents in an NK cell. In the first step, an NK cell recognizes a target cell and the dynein/dynactin complex rapidly transports lytic granules to the MTOC. Next, the lytic granules converge to the MTOC independently of microtubule dynamics or actin reorganization at the IS. Finally, the MTOC gradually polarizes along with the lytic granules to the IS where their contents can be directed onto the target cell.

measured. When cells received a GFP only construct, lytic granules converged rapidly after target cell conjugation (Figure 2.16A). In cells receiving either p50-GFP or CC1-GFP, however, lytic granules were localized at the cell periphery and did not move centrally over the course of the experiment (Figure 2.16B,C; and Video 6). To quantify these observations, the lytic granule to centroid distance was measured and mean values calculated for each time point. The mean distance of the lytic granules from their centroid over time was significantly greater in p50- and CC1-overexpressing cells when compared to those expressing GFP only (Figure 2.16D). The lytic granules in the p50- and CC1-overexpressing cells also demonstrated little movement, as their mean total displacement was only 68% and 66%, respectively, of that recorded in GFP control cells. Thus, lytic granules depend upon dynein for their early rapid MTOC-directed movement. This defines dynein and lytic granule convergence to the MTOC as an early prerequisite step required for lytic granule polarization to the NK cell IS, but one that does not commit the NK cell to cytotoxicity. The dynein-dependent MTOC-directed compression of a cargo to enable precise delivery, represents a novel form of directed secretion.

DISCUSSION

NK cell cytotoxicity occurs through a series of highly regulated linear stages enabling maturation and function of the IS (Orange, 2008). Previously, MTOC polarization was defined as a crucial step in delivering lytic granules to the target cell (Banerjee et al., 2007; Stinchcombe et al., 2001; Stinchcombe et al., 2006; Wulfing et al., 2003). We have quantified MTOC dynamics and found that in cell lines and *ex vivo* NK cells polarization occurs gradually and requires lytic triggering (Figures 2.2 and 2.11). MTOC polarization did not occur when microtubules were stabilized or when F-actin reorganization was inhibited (Figure 2.12B,E). Without MTOC polarization, there was no reorientation of lytic granules to the IS. Thus, MTOC polarization is required for the IS-directed dynamics of lytic granules and supports previous data in fixed CTL defining this requirement for the MTOC (Jenkins et al., 2009; Stinchcombe et al., 2006). We have previously identified critical regulation of MTOC polarization for cytotoxicity (Banerjee et al.,

2007), which is a desirable point for controlling the NK cell IS in light of the lethal nature of the directed secretion of lytic granule contents. The gradual MTOC polarization to the NK cell IS, however, suggested the existence of other MTOC-related steps prerequisite to function.

The gradual movement of the MTOC was defined in contrast to an unexpected and rapid movement of lytic granules to the MTOC after NK cell activation and before MTOC polarization to the IS. The distance of lytic granules from the MTOC rapidly decreased in NK cells engaged in cytolytic, or non-cytolytic interactions (Figure 2.3). This was further explored using immobilized antibodies against specific receptors and those with activating potential (CD28 and CD11a) induced lytic granule convergence compared to non-activated cells, while a non-activating receptor (CD45) did not (Figure 2.8). While CD28 can trigger cytotoxicity in YTS cells (Chen et al., 2006), CD11a can only trigger under certain circumstances (Barber et al., 2004). Thus, granule convergence to the MTOC occurs after some activation, but does not commit the cell to cytotoxicity. This was further demonstrated in non-cytolytic conjugates in that CD11a was required for convergence of lytic granules to the MTOC (Figure 2.14). For this reason it appears that lytic granule convergence is prerequisite to, but not defining of, cytotoxicity in NK cells. In this light, rapid lytic granule convergence may represent a mechanism with which an NK cell prepares itself for cytotoxicity while activation signals are being further integrated. This would serve the purpose of centrally localizing the lytic granules after a successful adhesion event to ensure that they are not secreted until gradual MTOC polarization brings them into proximity with the IS where their contents can be secreted as a targeted bolus. Thus, in a complex tissue lytic granule convergence could protect healthy surrounding cells from cytotoxicity while directing function at the IS formed with a diseased cell.

Lytic granule convergence still occurred even if microtubules were stabilized or F-actin reorganization inhibited (Figure 2.12). This underscores that the MTOC-directed movement of lytic granules is an extremely early event in formation and function of the NK cell lytic IS. Microtubule stabilization defines that MTOC movement is not required for lytic granule convergence and that it can occur before any reorientation to the synapse has been initiated. This

identifies MTOC-directed lytic granule movement as an initial step in synapse function relevant to the microtubule network.

Actin accumulation at the IS has been defined to occur rapidly and is required for cell surface receptor clustering and lytic granule polarization as shown using Cytochalasin D-treated cells (Orange et al., 2003). Thus, it was surprising that lytic granule convergence still occurred in cells treated with Cytochalasin D, or Latrunculin A. This suggests that signals required for lytic granule convergence, while downstream of activating receptors, are at least partially independent of those requiring significant actin reorganization at the IS. Thus far, few signaling events upstream of F-actin reorganization in NK cells have been defined at a cellular level (Butler et al., 2008; Riteau et al., 2003). These include LFA-1 signaling (Riteau et al., 2003), which was defined as required for granule convergence in non-cytolytic conjugates (Figure 2.14) where the granule convergence was present, but the MTOC did not polarize. Thus, the engagement of LFA-1 can represent a minimal, but potentially non-exclusive requirement for lytic granule convergence to the MTOC. While complete independence from actin reorganization, however, cannot be confirmed with the inhibitor-based experiments presented here, a divergence in signaling would presumably occur after NK cell activation to drive granule convergence. The signal leading to lytic granule convergence would be one that could enable dynein activity and function in granule traffic without needing to promote microtubule or actin reorganization.

Lytic granule convergence to the MTOC was defined initially in our work using the immortalized cell lines YTS or NK92. These are derived from an NK cell lymphoma and leukemia, respectively. While they are therefore, by definition, abnormal, they both effectively mediate cytotoxicity and form a lytic IS. It was reassuring that these two cell lines derivative from different individuals both demonstrated similar characteristics of lytic granule polarization and convergence. It was still possible, however, that the phenomenon was representative of some baseline cell activation, one that would not be present *in vivo* under routine conditions of NK cell-mediated immunosurveillance. Thus, NK cells were directly evaluated *ex vivo* without culture in interleukin-2 or other growth-inducing cytokines (Figure 2.11). While this approach presented

technical limitations, the lytic granule polarization to the IS and convergence to the MTOC reproduced that found in the cell lines. Thus, without exogenous activation signals, gradual MTOC polarization and rapid lytic granule convergence to the MTOC appear as generalizable phenomena in NK cells.

Characteristics of lytic granules in NK cells may be different from in T cells, something suggested by previous observations of MTOC movement and Ca^{++} flux in murine NK cells when compared to murine T cells (Wulfing et al., 2003). Lytic granules are preformed in the vast majority of human NK cells, which enables the constant ability to kill. This, however, potentially puts the onus of more opportunity for regulation of killing upon the NK cell and may relate to the role of NK cells in innate immunity and surveillance versus clonal expansion and the ready effector function of T cells. The difference in functions is also suggested by the potentially greater strength of signals downstream of the T cell receptor complex as compared to NK cell activating receptors. TCR signal strength has been described to dictate the path of lytic granules to specific locations within the IS (Beal et al., 2009). In NK cells, however, lytic granule convergence to the MTOC was found even in non-cytolytic interactions (Figure 2.3), suggesting convergence as an NK cell paradigm for managing granules that may reflect differences between innate and adaptive immunity. Thus, a potentially more regimented control of lytic granules in NK cells may result from a need to manage the abundance of granules present in all mature human NK cells and provide additional checkpoints in cytotoxicity.

Aside from CTLs, other cell types traffic lysosome-related organelles along microtubules in minus-end-directed fashion. Lysosomal tracking toward the MTOC has been observed in normal rat kidney cells after interphase, but the specific motors responsible were not defined (Matteoni and Kreis, 1987). Melanosomes in melanophores are responsive to rapid changes in requirement for distribution of pigment and have also been described to aggregate (Nilsson and Wallin, 1997). Here, kinesins and dynein work in concert to disperse or aggregate pigment, respectively (Nascimento et al., 2003; Nilsson and Wallin, 1997). Minus-end-directed melanosome transport, however, is not a means for directed secretion as it is in NK cells, but

serves to aggregate melanosomes at the MTOC to prevent secretion of pigment (Nascimento et al., 2003). The utility of minus-ended microtubule traffic prior to MTOC reorientation to facilitate directed secretion that we have described is novel, and may represent a useful paradigm in generating precision and regulation of secretory function.

In our experiments, the average instantaneous velocity of individual lytic granules was 1.8 μm/sec in activated YTS cells and 1.4 μm/sec in activated NK92 cells (Figure 2.9F,G). This is more rapid than measured velocity of dynein-dependent minus-end-directed movements of melanosomes, which are recorded at 0.250 μm/sec (Levi et al., 2006). Very rapid melanosome aggregation to the MTOC (7.4 to 11.6 μm/sec), however, has been identified although not attributed to dynein specifically (McNamara and Ribeiro, 2000). In other cell types including fibroblasts, normal rat kidney cells, and in *C. elegans* as well as in *in vitro* assays, minus-ended movements have also been measured at high speeds between 0.7 and 2.5 μm/sec (King and Schroer, 2000; Lye et al., 1987; Matteoni and Kreis, 1987; Presley et al., 1997; Schroer and Sheetz, 1991; Toba et al., 2006). Distance traveled by lytic granules was also evaluated (Figure 2.10D,E) and was found to have run lengths of 4.3 μm on average for activated YTS cells and 2.9 μm on average for NK92 cells. In published results, fluorescent beads or pre-Golgi vesicles have been observed to travel from 1.5 to 5.0 μm at one time (King and Schroer, 2000; Presley et al., 1997). Thus, in our single granule analyses in streaming videos, the minus-end-directed movement in NK cells was commensurate with that observed in other cell types as well as with dynein motor function.

Lytic granule traffic and dynein function was evaluated in NK cells by overexpression of dynactin components. Dynein processivity requires dynactin for the majority of dynein-dependent cargo transport in cytoplasm (Waterman-Storer et al., 1997). When the p50 subunit is overexpressed, it competitively binds to $p150^{Glued}$ and Arp1, thereby blocking proper binding of dynactin subunits and causing dissolution of the complex (Burkhardt et al., 1997). Overexpression of $p150^{Glued}$ subunits such as CC1 cause dissociation of dynactin from dynein and interruption of function (Waterman-Storer et al., 1995). Using either of these approaches, NK

cell lytic granules were found dispersed at the cell periphery (Figure 2.16). Dynein, therefore, which is associated with lytic granules (Figure 2.15), is required for this initial step in lytic granule traffic. Other potential minus-end directed motors exist, specifically among the kinesin14 family (Endres et al., 2006), but these were not identified in our mass spectrometry analysis (data not shown). Dynein has been previously defined at the IS in T cells and is required for facilitating MTOC polarization (Combs et al., 2006; Martin-Cofreces et al., 2008), but was not evaluated with regards to a cargo-based motor function in those studies. Thus, to our knowledge our work represents the first demonstration of this role for a microtubule minus-end-directed motor in immune cells.

Dynein-dependent, MTOC-directed lytic granule transport represents an early checkpoint in NK cell cytotoxicity and may act as an initial enabling step in NK cell function prerequisite to a commitment to cytotoxicity (Figure 2.16E). In this way, the NK cell prepares for cytotoxicity after it recognizes a target cell but needs further signals to mature the IS and commit to the cytotoxic event. This sequence also defines a novel paradigm in a linear series of events leading to directed secretion, one in which an actin-independent signal first aggregates organelles to the MTOC. Here, dynein-dependent minus-ended microtubule transport compresses a cargo prior to it being relocalized to the point of secretion. This enables a simplistic efficiency mobilizing a cellular resource for a precision function.

CHAPTER 3: RAPID ACTIVATION RECEPTOR- OR IL-2-INDUCED LYTIC GRANULE CONVERGENCE IN HUMAN NATURAL KILLER CELLS REQUIRES SRC BUT NOT DOWNSTREAM SIGNALING[2]

SUMMARY

Natural killer (NK) cells participate in host defense by surveying for and ultimately killing virally infected or malignant target cells. NK cell cytotoxicity is a tightly regulated process that proceeds stepwise from adhesion and activation to the secretion of preformed lytic granule contents onto a diseased or stressed cell. We have previously characterized rapid dynein-dependent lytic granule convergence to the microtubule-organizing center (MTOC) as an early, prerequisite step in NK cell cytotoxicity. While multiple activating receptors can trigger granule convergence, the specific signal or signals responsible remained unknown. Using live cell confocal microscopy, NK cell lytic granule movement following NK cell activation was captured and measured. Using inhibitors of common early signaling mediators, we show that Src kinases are required for lytic granule convergence but downstream signals that promote actin rearrangement, MTOC polarization and calcium mobilization are not. Exposure to interleukin 2 (IL-2) was also sufficient to induce lytic granule convergence, which required non-canonical Src-dependent signaling. Thus, NK cell lytic granule convergence, prompted by specific receptor-mediated and soluble cytokine signals, depends upon a directly downstream early Src kinase-dependent signal and emphasizes the importance of this step in readying NK cells for cytotoxicity.

INTRODUCTION

Natural killer (NK) cells play important roles in the innate immune system, defending the host against viral infection and cancer (Orange and Ballas, 2006; Sun and Lanier, 2011). NK cell

[2] A version of this chapter has been submitted for publication as:

Mentlik James A, Uzel G, Banerjee PP, Orange JS.
Rapid activation receptor- or IL-2-induced lytic granule convergence in human natural killer cells requires Src, but not downstream signaling.

killing occurs in a precise, step-wise fashion culminating in secretion of the contents of preformed lysosome-related organelles, known as lytic granules, onto a stressed or infected target cell (Davis and Dustin, 2004; Orange, 2008). NK cell activation occurs through engagement of germline-encoded receptors; immediately thereafter, NK cell F-actin accumulates at the site of contact to support an organized signaling and secretory platform termed the immunological synapse (IS). During synapse formation, lytic granules converge to a randomly located microtubule organizing center (MTOC) (Mentlik et al., 2010). Convergence is rapid and occurs within minutes of target cell contact or activating receptor ligation. The lytic granules utilize dynein-dependent transport to reach the MTOC, but the process is independent of actin reorganization and microtubule dynamics (Mentlik et al., 2010). Once granules have converged, the MTOC and associated lytic granules polarize to the IS, where granules pass through the actin network to fuse with the NK cell membrane, and their contents are secreted (Rak et al., 2011). Each of these intercellular events along the path to directed secretion – granule convergence, IS formation, MTOC polarization, granule penetration and exocytosis – is tightly regulated allowing NK cells to precisely target their deadly effects to virally infected, stressed or tumorigenic cells.

If a target cell is diseased, activating receptor engagement predominates the NK-target cell interaction, facilitating receptor clustering, which in turn triggers downstream intracellular signaling pathways (Chen et al., 2006; Chen et al., 2007; Jiang et al., 2000; Li et al., 2008). Activation of integrin leukocyte function-associated antigen-1 (LFA-1, composed of CD11a and CD18) leads to Vav1 activation (Watzl and Long, 2010), which in turn recruits F-actin to the IS (Riteau et al., 2003; Sanchez-Martin et al., 2004). Similarly, phosphoinositide 3-kinase (PI3K) is activated and recruited to newly engaged triggering receptors thus promoting MEK/Erk signaling and MTOC polarization (Chen et al., 2006; Jiang et al., 2000). Activation of phospholipase C-γ (PLCγ) by ligated immunoreceptors stabilizes synapse formation and triggers release of intracellular calcium stores (Bryceson et al., 2006b; March and Long, 2011). Later, store-operated calcium entry (SOCE) is crucial for exocytosis of lytic granule contents (Billadeau et al., 2003; Upshaw et al., 2005).

If, on the other hand, the target cell is healthy, inhibitory receptor engagement predominates the cell-cell interaction. Killer immunologlobulin receptors (KIRs) recognize major histocompatibility complex (MHC) class I molecules on healthy cells. Once ligated, they become tyrosine phosphorylated and recruit Src homology domain containing-phosphatase 1 (SHP-1). SHP-1 in turn dephosphorylates key downstream proteins (including Vav1) to deactivate activation pathways and halt the cytotoxic response (Cuevas et al., 1999; Faure et al., 2003; Stebbins et al., 2003). F-actin accumulation still occurs at the beginning of the NK-target cell interaction, stabilizing further recruitment of KIR receptors (Standeven et al., 2004). Actin accumulation is required for clustering of activation receptors, but because KIR also clusters, activation can be inhibited directly after initiation by limiting actin function (Schleinitz et al., 2008). Even if activation is initiated, KIR-mediated termination of activation signaling ensures healthy cells will not be lysed.

NK cells are also influenced by cytokines. T cell-secreted interleukin-2 (IL-2) enhances NK cytotoxicity by increasing surface expression of activating receptors and production of additional inflammatory cytokines (Bonnema et al., 1994; Fehniger et al., 2003; Michon et al., 1988). Once IL-2 encounters the IL-2 receptor on the NK cell surface, canonical signaling through Janus activated kinases (JAKs) leads to association with and activation of signal transducers and activators of transcription (STATs) (Johnston et al., 1996). IL-2 receptor signaling, however, also can proceed via a non-canonical pathway which links the Src homology-2 domain-containing transforming protein C (SHC) with mitogen activated protein kinase (MAPK) signaling (Merida et al., 1991; Ravichandran and Burakoff, 1994). Together, these signaling events result in heightened NK cell activation after IL-2 exposure (Figure 1.3).

Src family kinase phosphorylation is ubiquitous in NK cell signaling. Most NK cell surface receptors, both activating and inhibitory, contain tyrosine residues in intracellular domains. Once a receptor recognizes a cognate ligand on a target cell, Src phosphorylation of tyrosines initiates downstream signaling events (Augugliaro et al., 2003; Billadeau et al., 2003; Faure et al., 2003; Mason et al., 2006; Ting et al., 1991; Watzl and Long, 2010). As alluded to above, even soluble

cytokine stimulation utilizes Src kinase-mediated phosphorylation, which can serve to propagate function via maintaining active levels of Jak/STAT signaling (Shuh et al., 2011).

Lytic granule convergence to the MTOC is an early step in directed secretion for cytolysis. Convergence can potentially enable efficient delivery of large numbers of granules to the IS to achieve maximal cytotoxicity, prevent collateral damage to bystander cells from non-directed degranulation, and prepare NK cells for more efficient subsequent kills. Previous work in our laboratory has shown that convergence is rapid, dynein-dependent, and independent of actin reorganization and microtubule dynamics (Mentlik et al., 2010). Furthermore, it follows activation, but not inhibitory receptor ligation (Mentlik et al., 2010). While signaling involved in other aspects of NK cell cytotoxicity is well understood, to date, no signaling molecules have been linked to this process of lytic granule convergence. Using specific inhibitors and soluble cytokines, we herein define the signaling requirements for lytic granule convergence in NK cells. We found that lytic granule convergence follows adhesion, triggering receptor engagement, and IL-2 stimulation. It requires Src family kinases but not PI3K, MEK, PLCγ activation, nor canonical IL-2 signaling. Furthermore it is not inhibited by KIR engagement. We propose, therefore, that lytic granule convergence is initiated very early after receptor engagement, lies directly downstream of Src kinase and is a common feature of NK cell activation.

RESULTS

β2 integrin is required for lytic granule convergence in human NK cells

Lytic granule convergence to the MTOC is rapid and independent of many checkpoints in the process of IS maturation. In our previous studies of granule dynamics, we noted that blocking the β2 integrin LFA-1 adhesion receptor with anti-CD11a antibody interrupted granule convergence (Mentlik et al., 2010). LFA-1 is required for optimal NK cell adhesion (Kohl et al., 1984) and, like granule convergence itself, certain signals downstream of LFA-1 are independent of F-actin reorganization (Riteau et al., 2003). To confirm and extend the role of LFA-1, we examined granule convergence in leukocyte adhesion deficiency-1 (LAD-1) patients who lack the

CD18 subunit of LFA-1 (Anderson and Springer, 1987). NK cells from LAD-1 patients form poor conjugates and have poor cytotoxic efficiency. It is assumed that deficiency in conjugation is responsible for decreased cytotoxicity in these patients. Ex vivo NK (eNK) cells from control and LAD-1 patients were incubated with susceptible K562 target cells, fixed and then stained for lytic granules, the MTOC and F-actin. LAD-1 eNK cells demonstrated reduced but demonstrable conjugates with target cells. In fixed cell conjugates, lytic granules in control eNK cells converged to the MTOC (Figure 3.1A). In contrast, LAD-1 eNK cell lytic granules failed to converge to the

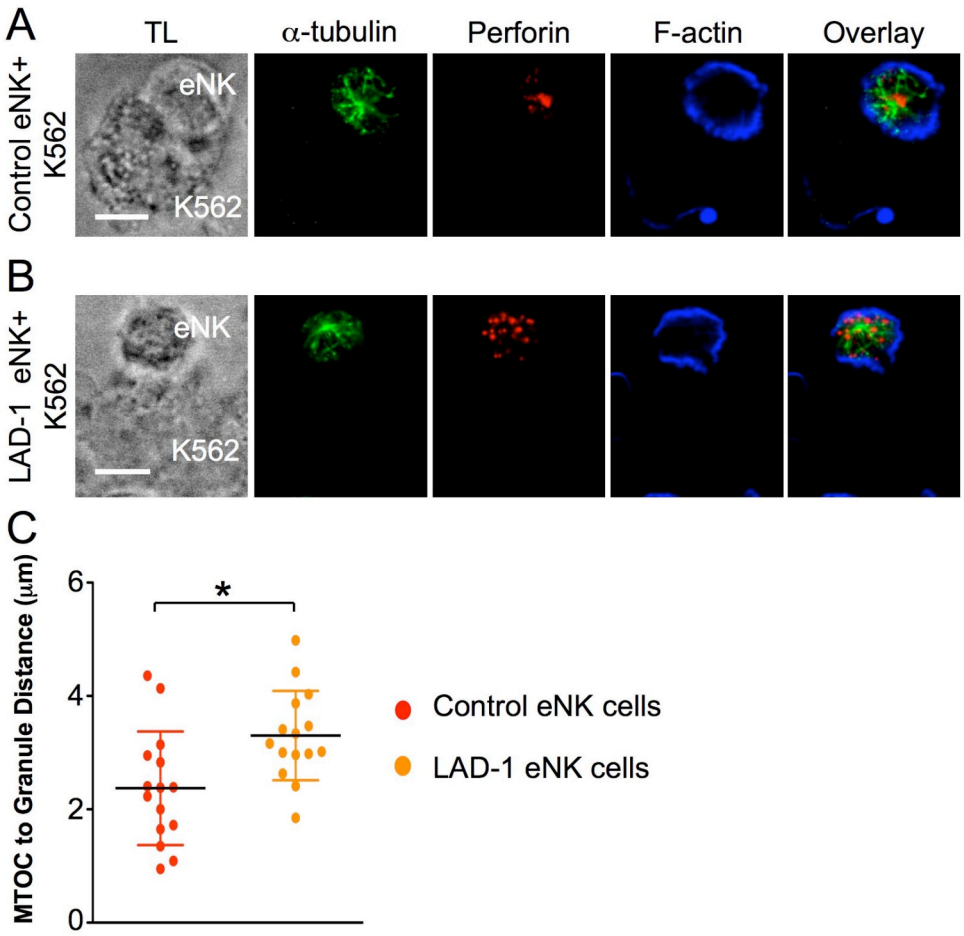

Figure 3.1. β2 integrin is necessary for lytic granule convergence to the MTOC in human ex vivo NK cells.
Microscopy of fixed cells showing immunofluorescence images of a healthy eNK cell (A) and a LAD-1 eNK cell (B) conjugated to a susceptible K562 cell. After 30 minutes at 37°C on poly-L-lysine-coated glass slides, conjugates were fixed and stained with anti-α-tubulin (green), anti-perforin (red) and Phalloidin-F-actin (blue). (C) Lytic granule distance from the MTOC in 15 cells; error bars, ± SD. Mean distance of lytic granules from the MTOC in LAD-1 eNK cells was significantly different from that in control eNK cells ($p < 0.05$). Microscopy was performed with the assistance of Pinaki Banerjee.

MTOC after target cell conjugation (Figure 3.1B,C). These data confirm that signaling through the LFA-1 complex is necessary for lytic granule convergence in NK cells. Furthermore they suggest an additional NK cell impairment other than target cell conjugation in these rare patients.

Src kinase activity is required for human NK cell lytic granule convergence

In addition to LFA-1, activating receptors like CD28 but not non-specific, non-activating receptors like CD45, can promote convergence (Mentlik et al., 2010). We hypothesize that most NK cell activation signaling promotes lytic granule convergence as a way to prime and prepare NK cells for eventual directed secretion. With this in mind, we focused on signals downstream of NK cell activating receptors, prioritizing those involved in key stages of NK cell cytotoxicity.

Src family kinases are redundant tyrosine kinases responsible for phosphorylation of many NK cell receptors (Augugliaro et al., 2003; Mason et al., 2006; Watzl and Long, 2010). Once phosphorylated, the intracellular phosophotyrosine residues attract SH2-domain containing signaling proteins, thereby facilitating downstream signaling. In NK cells, this downstream signaling is crucial for activation and includes Vav1, (required for actin accumulation at the IS (Riteau et al., 2003)) PI3K (necessary for MTOC and granule polarization (Chen et al., 2006; Jiang et al., 2000)) and PLCγ (required for eventual release of granule contents (Upshaw et al., 2005)).

In order to determine whether blocking Src kinases, and thereby interrupting downstream NK cell activation signaling, would preclude lytic granule convergence, we pretreated the GFP-α-tubulin-expressing YTS NK cell line with the Src kinase inhibitor PP2. Following inhibition, cells were loaded with LysoTracker Red, placed live into imaging chamber slides coated with antibody specific for the YTS triggering receptor CD28 or the CD11a subunit of LFA-1 and observed over time. Compared to DMSO-treated control cells, which showed normal lytic granule convergence after activation on either surface (Video 7A), PP2-treated cells failed to demonstrate lytic granule convergence after activation on anti-CD28- (Figure 3.2A,B) or anti-CD11a- (Figure 3.2A,C) coated surfaces (Video 7B). In inhibitor-exposed cells, granules remained diffusely localized

around the cell, even after 15min of activation on antibody-coated surfaces. In DMSO-treated control cells, the average distance of lytic granules from the MTOC at the end of the assay was approximately 2μm versus 5μm in PP2-treated cells (Figure 3.2D,E). Thus, Src kinase activity is required for lytic granule convergence to the MTOC in NK cells.

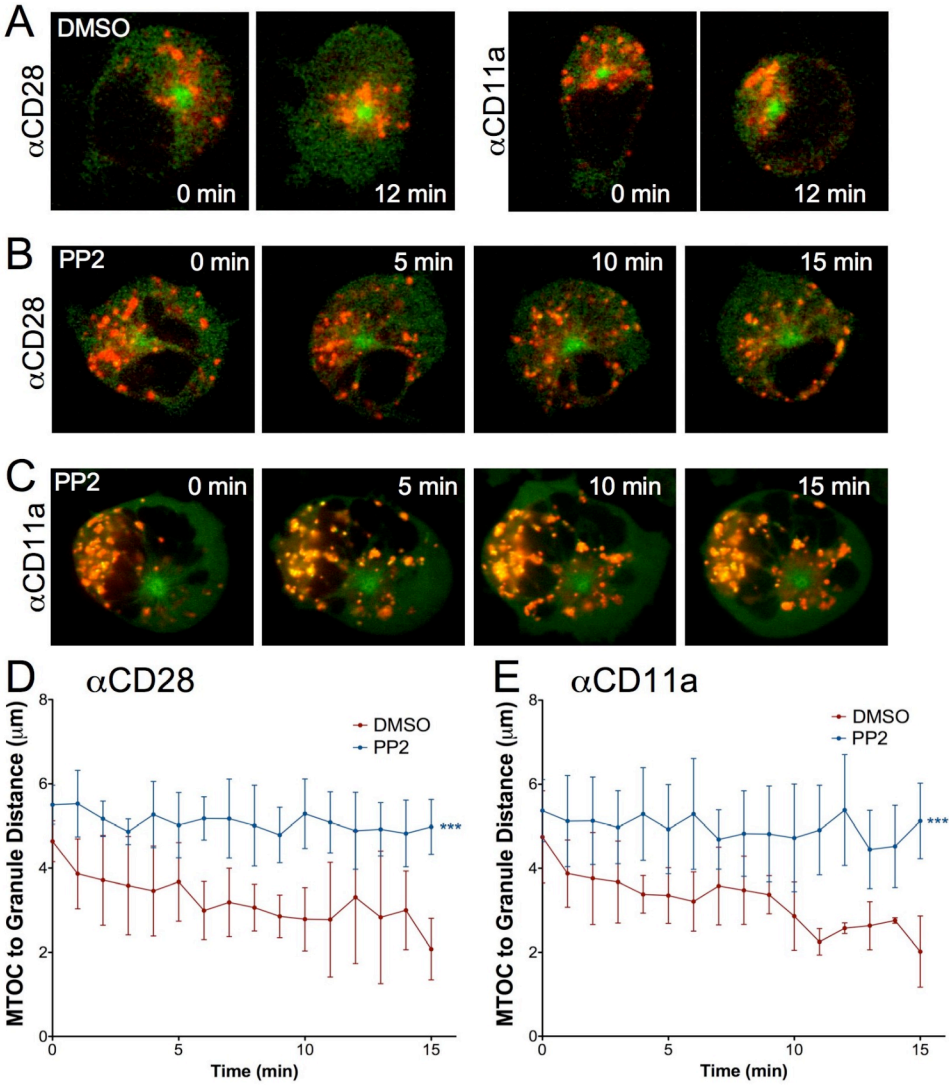

Figure 3.2 Signaling for lytic granule convergence requires Src kinase activity.
Time-lapse frames of lytic granule movement in YTS GFP-tubulin cells pretreated with DMSO (A) or PP2 on an anti-CD28- (B) or an anti-CD11a-coated surface (C). In each image, confocal immunofluorescence in the plane of the MTOC is shown. Green, GFP-tubulin; red, LysoTracker-loaded acidified organelles. Zero minutes represents the time at which the NK cell appeared to contact the glass surface. Quantitative analyses of lytic granule distance from the MTOC of PP2- or DMSO-treated YTS GFP-tubulin cells on anti-CD28- (D) or anti-CD11a- (E) coated surfaces as a function of time as measured by mean MTOC to granule distance in 9 cells per condition; error bars show ± SD. Mean distance of lytic granules from the MTOC was significantly greater in PP2-treated NK cells than in DMSO-treated NK cells ($p < 0.05$).

Lytic granule convergence, but not NK cell cytotoxicity, occurs independently of PI3K, MEK, and PLCγ

Downstream of triggering receptor engagement, PI3K is an early signaling mediator of the MEK/Erk pathway required for MTOC and granule polarization (Chen et al., 2006; Jiang et al., 2000). We questioned whether PI3K is responsible for initiating transport of lytic granules to the MTOC before polarization occurs. Using LY294002 or Wortmannin to block PI3K, we treated YTS GFP-tubulin cells, labeled them with LysoTracker Red and added them to anti-CD28- or anti-CD11a-coated imaging chambers (Figure 3.3A, Videos 8A and 8B). Lytic granule convergence measured over time demonstrated that neither inhibitor disrupted convergence compared to

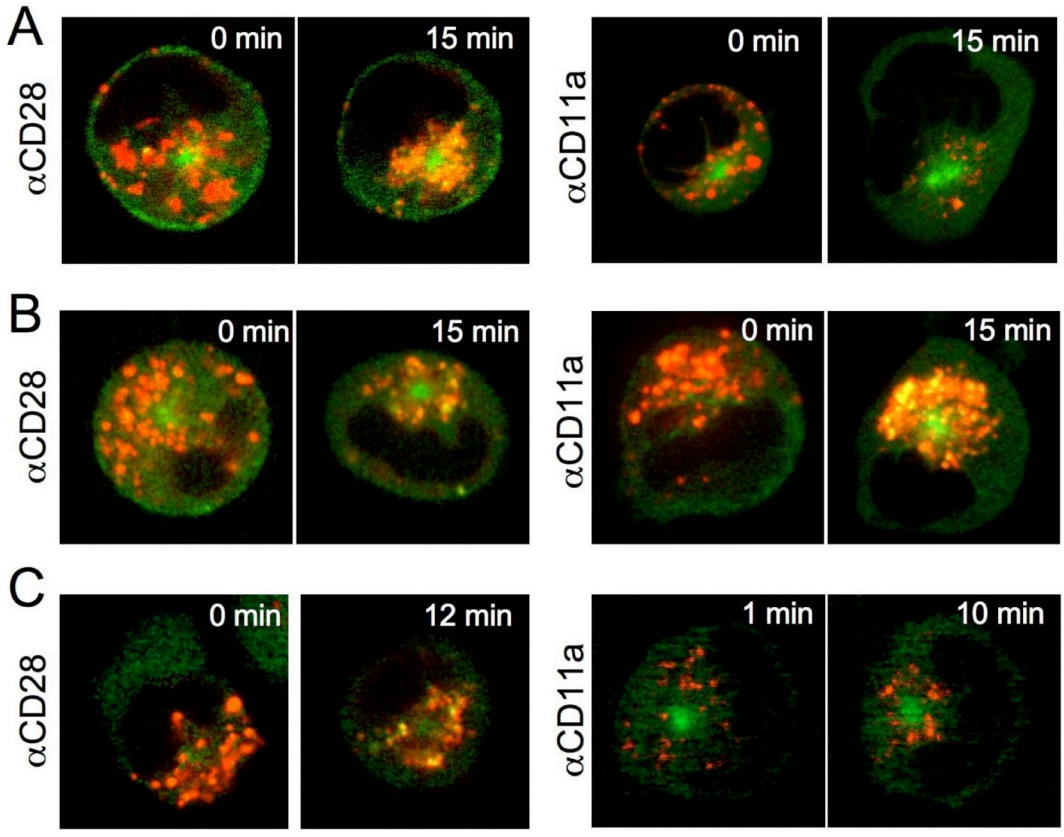

Figure 3.3. Inhibition of PI3K, MEK and PLCγ impairs NK cell cytotoxicity but does not block lytic granule convergence to the MTOC.
Time-lapse frames of lytic granule movement in YTS GFP-tubulin cells pretreated with LY294002 (A), PD98059 (B) or U73122 (C) on an anti-CD28- or an anti-CD11a-coated surface. In each image, confocal immunofluorescence in the plane of the MTOC is shown. Green, GFP-tubulin; red, LysoTracker-loaded acidified organelles. Zero minutes represents the time at which the NK cell appeared to contact the glass surface.

DMSO-treated control cells, irrespective of the triggering receptor engaged (Figure 3.4A,B). At the end of the assay, average granule distance from the MTOC after PI3K inhibition was 2-2.5μm, and was not significantly different from controls. From this we conclude that lytic granule convergence is initiated independently of PI3K signaling.

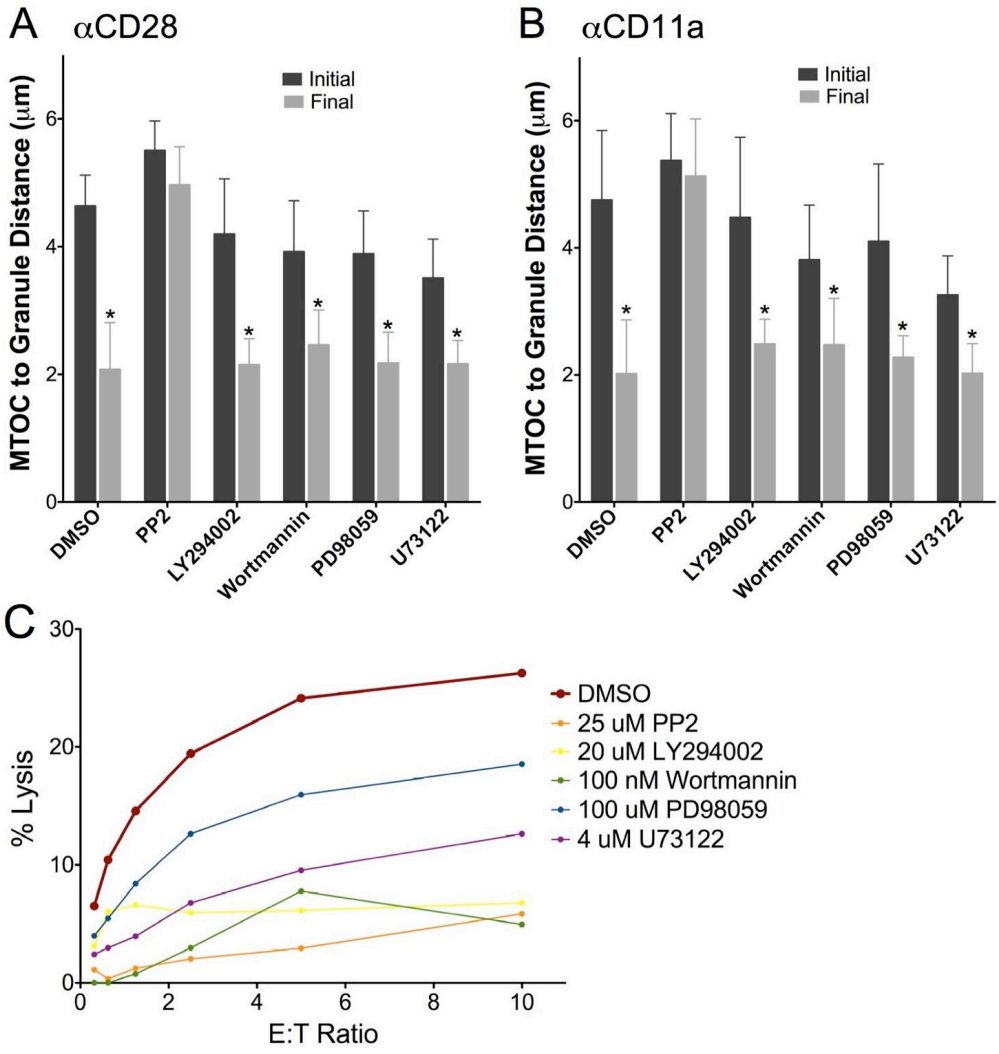

Figure 3.4. Lytic granule convergence depends upon Src kinase but occurs independently of PI3K, MEK and PLCγ activity.

Quantitative analyses of lytic granule distance from the MTOC as a function of time as measured by mean MTOC to granule distance in 9-10 cells per condition in DMSO-treated or inhibitor-treated YTS GFP-tubulin cells on anti-CD28- (A) or anti-CD11a- (B) coated surfaces. Error bars show ± SD. Excepting PP2, mean distance of lytic granules from the MTOC was not significantly different in any inhibitor-treated NK cells compared to DMSO-treated NK cells; distance of lytic granules from the MTOC was significantly greater at time point zero than at the end of the assay ($p < 0.05$). (C) Cytotoxicity assay (4hr ^{51}Cr-release) of YTS cells against 721.221 target cells showing loss of function after treatment with various inhibitors.

We next used the selective MEK inhibitor PD98059 to confirm that lytic granule convergence is not triggered from elsewhere in the signaling pathway downstream of CD28 or CD11a (Li et al., 2008). YTS GFP-tubulin cells were pretreated with PD98059 or DMSO, stained with LysoTracker Red and again placed into anti-CD28- or anti-CD11a-coated imaging chambers (Figure 3.3B, Video 8C). We measured lytic granule convergence over time and found no defect after MEK inhibitor treatment (Figure 3.4A,B). Lytic granules converged to within approximately 2μm of the MTOC when cells were treated with PD98059, essentially the same as control. This confirms that lytic granule convergence does not rely on PI3K or the MEK/Erk pathway but rather exists as an early activation step distinct from MTOC polarization.

PLCγ activation occurs early in NK cell activation and facilitates calcium flux, maintenance of surface receptor levels required to promote NK cell activation, and release of lytic granule contents (Upshaw et al., 2005). In order to deduce the role of PLCγ in granule convergence, we pretreated YTS GFP-tubulin NK cells with the PLCγ inhibitor U73122, and then evaluated lytic granules in antibody-coated imaging chambers as above (Figure 3.3C, Video 8D). Blocking PLCγ had no effect on lytic granule convergence (Figure 3.4A,B); lytic granules were on average 2μm away from the MTOC by the end of the assay. Thus, while PLCγ function is essential to MTOC polarization, sustained signaling and lytic granule contents release, it is not required for granule convergence to the MTOC.

We performed cytotoxicity assays to confirm that each of the above inhibitors was indeed functioning at the selected concentrations in blocking NK cell function. As expected, all inhibitors decreased NK cell cytotoxicity significantly compared to control (Figure 3.4C). Thus while PI3K, MEK, and PLCγ inhibitors block NK cell killing, none of these blocked pathways contributing to NK cell lytic granule convergence. Convergence to the MTOC is therefore either entirely upstream or independent of PI3K, MEK, and PLCγ activity.

Lytic granule convergence occurs in NK cells forming an inhibitory synapse

NK cells utilize a balance of inhibitory and activating receptors and will ultimately only kill a target cell if a threshold for inhibition is exceeded. In this case the IS can mature in order to enable the secretion of lytic granule contents (Orange, 2008). When encountering a healthy cell, killer cell immunoglobulin-like receptors (KIRs) recognize MHC-I molecules and relay the message that this particular cell is not a target for lysis (Faure et al., 2003). After ligation, cytoplasmic tyrosines within KIRs are phosphorylated thus recruiting the phosphatase SHP-1. KIR-associated SHP-1 can dephosphorylate Vav1 to interrupt actin reorganization (Stebbins et al., 2003), thus interrupting maturation of the IS and ultimately release of lytic granule contents.

In order to study the effect of inhibitory synapse formation on granule convergence, we performed live cell confocal microscopy using YTS cells stably expressing a KIR2DL1-GFP fusion protein. As target cells, the 721.221 B lymphoblastoid cell line stably expressing either the cognate ligand for KIR2DL1, HLA-Cw4, or the non-cognate ligand, HLA-Cw3 were utilized. KIR2DL1-GFP cells conjugated with both 721.221 target cells but can kill HLA-Cw3-expressing, but not HLA-Cw4-expressing cells (Figure 3.5A). YTS KIR2DL1-GFP cells form an activating synapse with 721.221 HLA-Cw3 cells with uniformly localized GFP around the NK cell surface, and compacted LysoTracker Red-loaded lytic granules (Figure 3.5B, Video 9A). In contrast, when YTS KIR2DL1-GFP cells conjugated to 721.221 HLA-Cw4 cells, GFP clustered at the synapse (Figure 3.5C, Video 9B) a known signature of the inhibitory synapse (Davis et al., 1999). Interestingly, lytic granules still converged to central locations in these inhibitory conjugates. Whether an activating or inhibitory synapse was formed, lytic granules converged to within 2μm of the centroid of the granules and there was no significant difference in the kinetics of convergence (Figure 3.5D). Thus, inhibitory signaling, which can block Vav1 function, did not interfere with lytic granule convergence after target cell contact.

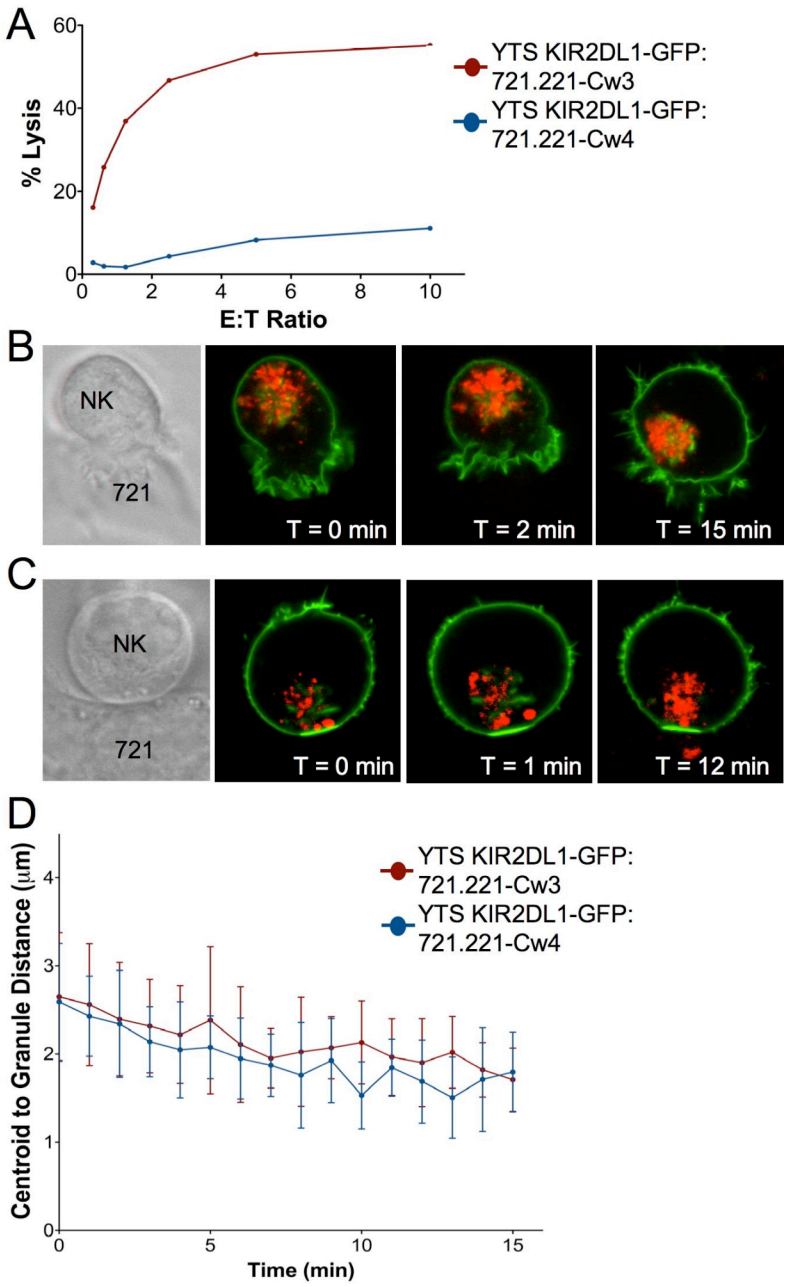

Figure 3.5. Lytic granule convergence occurs despite inhibitory synapse formation and is not reversed by KIR clustering.
721.221 cells expressing HLA-Cw3 or HLA-Cw4 were used as susceptible or non-susceptible target cells for YTS GFP-KIR2DL1, respectively. (A) 4hr ^{51}Cr-release cytotoxicity assay of YTS GFP-KIR2DL1 cells against 721-Cw3 (red) or 721-Cw4 (blue) target cells. Time-lapse frames of lytic granule movement in YTS GFP-KIR2DL1 cells conjugated to 721-Cw3 (B) or 721-Cw4 (C) cells. The leftmost panel shows transmitted light images of the conjugate pictured and each confocal image demonstrates immunofluorescence in the plane of converged lytic granules; green, GFP-KIR2DL1; red, LysoTracker-loaded acidified organelles. T = 0 refers to the time that image acquisition began, which was between 1-5 minutes after YTS GFP-KIR2DL1 cells were added to the imaging chamber. (D) Quantitative analyses of lytic granule distance from the centroid of the granules as a function of time as measured by mean centroid to granule distance in 9 cells; error bars, ± SD. Mean distance of lytic granules from the centroid of the granules in YTS GFP-KIR2DL1:721.221-Cw4 conjugates was not significantly different from that in YTS GFP-KIR2DL1:721.221-Cw3 conjugates.

Soluble interleukin-2 promotes lytic granule convergence independently of JAK3, but not Src

NK cells can be induced by inflammatory cytokines secreted by infected/stressed target cells or nearby CD4+ T cells (Bonnema et al., 1994; Fehniger et al., 2003; Michon et al., 1988). One potent inducer of NK cell activity is IL-2, which canonically functions through IL-2 receptor-associated JAK3. Ligation of the IL-2 receptor also promotes association of SHC, which in turn

activates the MAPK and PI3K pathways to enable non-canonical signaling and further enhances NK cell activation (Johnston et al., 1996; Merida et al., 1991; Ravichandran and Burakoff, 1994). Thus we wanted to determine if IL-2 stimulation would be sufficient to induce lytic granule convergence as a way to potentially heighten the activation state of the NK cell.

As a first step, resting YTS cells were treated with IL-2, fixed, and stained for tubulin and perforin (Figure 3.6A). Lytic granules were diffuse in resting NK cells but markedly converged in

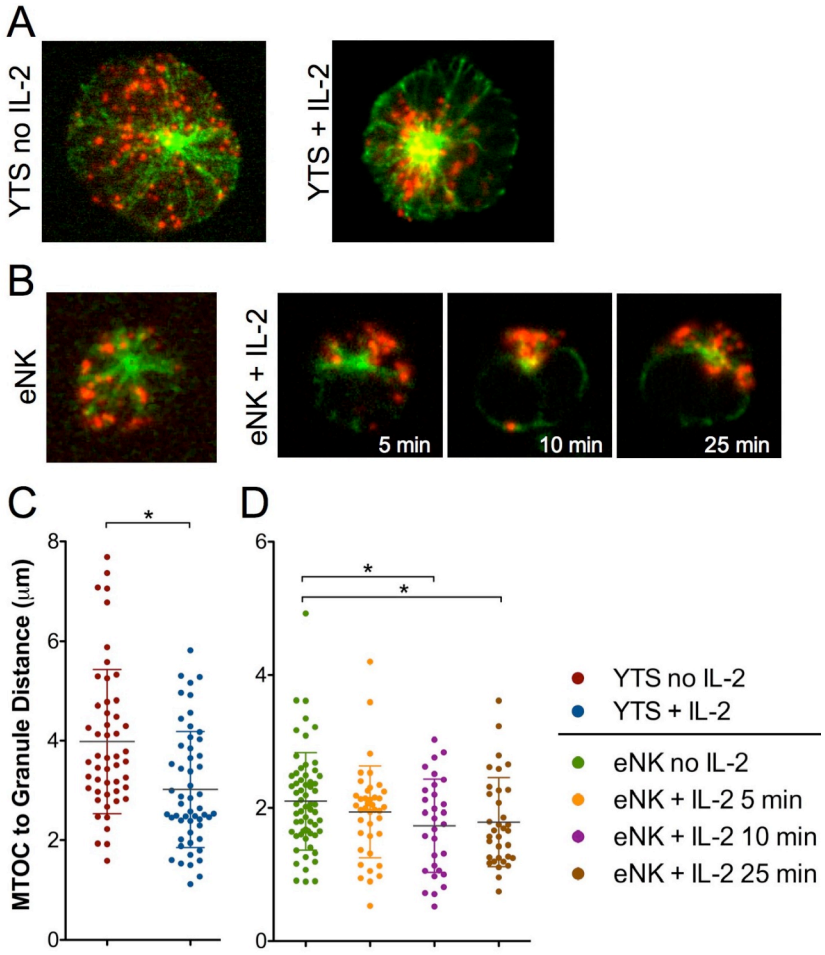

Figure 3.6. Interleukin-2 promotes lytic granule convergence in NK cells.
Confocal immunofluorescent microscopy of fixed cells showing anti-α-tubulin (green) and anti-perforin (red) in YTS cells resting (left) or treated with IL-2 for 15min (right, A) or eNK cells resting (leftmost) or treated with IL-2 for 5, 10 or 25 min (as indicated, B) before fixation on poly-L-lysine-coated glass slides for 5min at 37°C. The biotinylated anti-tubulin mAb was detected with Pacific Blue-streptavidin; the anti-perforin antibody was directly FITC-conjugated. Quantitative analyses of lytic granule distance from the MTOC as a feature of IL-2 treatment in YTS cells (C) and eNK cells (D). Data are representative of two separate experiments in each of which at least 30 cells were evaluated per condition. Error bars, ± SD. Mean distance of lytic granules from the MTOC was significantly less in YTS cells after IL-2 treatment ($p < 0.05$) and in eNK cells after at least 10 minutes of IL-2 treatment ($p < 0.05$ at 10 and 25 min).

IL-2-treated cells. We confirmed this observation using eNK cells isolated from healthy donor peripheral blood in which IL-2 promoted convergence of the lytic granules towards the MTOC (Figure 3.6B). To quantify the effect of IL-2, we measured average lytic granule distance from the MTOC in at least 30 cells in each condition (Figure 3.6C,D) and found that there was a significant decrease in lytic granule distance from the MTOC in both the NK cell line and eNK cells after IL-2 stimulation.

As IL-2-induced lytic granule convergence has not been previously studied, we further characterized this effect of IL-2 using live cell confocal imaging. Resting GFP-tubulin YTS cells with LysoTracker Red-dyed lytic granules were imaged in plain glass-bottomed imaging chambers without any adhesive or antibody coating. After 2-4 minutes, IL-2 was added to the media in the imaging chamber and granule traffic was observed over time (Figure 3.7A, Video 10A). As predicted by the above fixed cell imaging, addition of soluble IL-2 promoted decreased average granule distance from the MTOC (Figure 3.7D), which was comparable to that seen in NK cells triggered through an activating receptor or conjugated to a target cell. As an additional control for the addition of IL-2, parallel experiments were performed using IL-10 instead of IL-2. Here lytic granule convergence was not observed over the >15 minutes of observation after IL-10 addition (Figure 3.7D, Video 10B), thus demonstrating specificity to the IL-2 signal. It is important to reiterate that IL-2 is a soluble signal, thus distinguishing it from all of the surface receptor-, immobilized antibody- or target cell contact-induced signals. Even though we had previously shown that CD45 crosslinking does not trigger convergence (Mentlik et al., 2010), these results with IL-2 further illustrate that convergence is unlikely to represent an artifact of antibody-coated surfaces or physical NK cell deformation.

In order to begin to dissect out which IL-2-mediated signaling pathway contributes to lytic granule convergence, we first blocked canonical IL-2 signaling. The inhibitor ZM449829 potently and selectively prevents JAK3 from phosphorylating STAT molecules and in NK cells can completely block IL-2 induced STAT5 phosphorylation (Orange et al., 2011). If canonical IL-2 signaling is required for IL-2 mediated lytic granule convergence, we would expect ZM449829

pretreatment to block lytic granule convergence. To this end, we pretreated YTS GFP-tubulin cells with ZM449829, stained with LysoTracker Red, and again placed cells into plain glass live

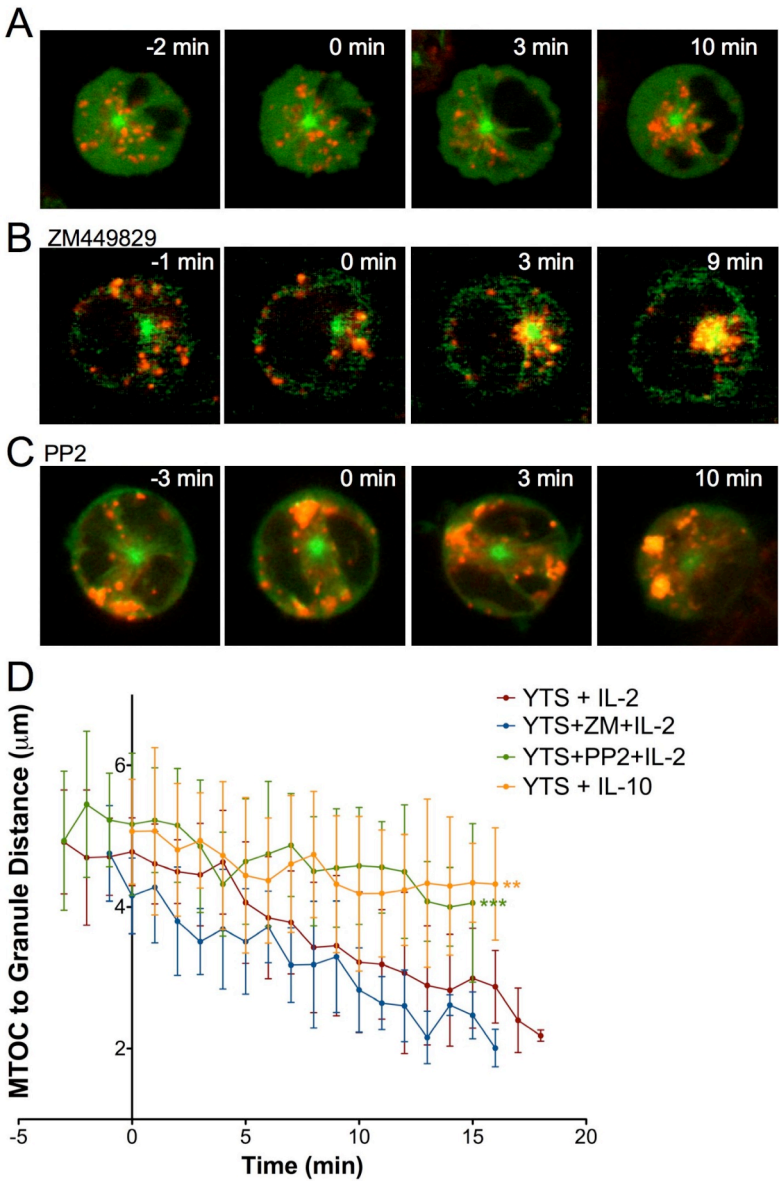

Figure 3.7. Cytokine-induced lytic granule convergence does not occur through canonical signaling but depends on Src kinase activity.

Time-lapse frames of lytic granule movement in YTS GFP-tubulin cells stimulated with IL-2 after DMSO (A), ZM449829 (B), or PP2 (C) treatment. In each image, confocal immunofluorescence in the plane of the MTOC is shown. Green, GFP-tubulin; red, LysoTracker-loaded acidified organelles. Cells were imaged for 2-4 minutes before IL-2 was added. 0 min represents the time at which IL-2 was added. (D) Quantitative analyses of lytic granule distance from the MTOC as a function of time as measured by mean MTOC to granule distance in 5-10 cells per condition; error bars, ± SD. Mean distance of lytic granules from the MTOC after IL-2 addition was not significantly different in ZM449829-treated NK cells compared to DMSO-treated NK cells, but was significantly greater in PP2-treated NK cells ($p < 0.05$). Mean distance of lytic granules from the MTOC after IL-10 addition was also significantly greater than after IL-2 addition ($p < 0.05$).

cell imaging chambers. After 2-4 minutes, IL-2 was added (Figure 3.7B, Video 10C). No defect in lytic granule convergence after JAK3 inhibition was observed. Lytic granules converged to approximately 2μm from the MTOC, which is the same distance measured in uninhibited, IL-2-stimulated cells (Figure 3.7D). Furthermore the kinetics of lytic granule convergence in inhibitor-treated cells tracked over time was also statistically indistinguishable from control (Figure 3.7D). Thus, canonical IL-2 signaling does not appear to be necessary for lytic granule convergence in IL-2-stimulated NK cells.

Src kinases can also play a role in IL-2 receptor signaling by maintaining receptor tyrosine phosphorylation (Shuh et al., 2011). Because Src kinases are necessary for lytic granule convergence after activating receptor triggering, we asked whether this non-canonical Src kinase signaling pathway downstream of the IL-2 receptor also contributes to IL-2-mediated lytic granule convergence. Thus, we treated GFP-tubulin YTS cells with PP2, stained with LysoTracker Red, and placed them into plain glass imaging chambers for 2-4 minutes prior to the addition of IL-2 in solution. Interestingly, lytic granules clumped around the cell-cortical plus ends of microtubules but did not move in a minus-ended direction to the MTOC (Figure 3.7C, Video 10D). Granules remained approximately 4.5μm from the MTOC, comparable to PP2-treated cells upon antibody-coated glass surfaces shown earlier. Importantly, this distance was significantly greater than in uninhibited, IL-2 stimulated cells (Figure 3.7D). Thus, PP2 specifically blocks the mechanism that drives lytic granules to the MTOC. In the case of IL-2 stimulation, as in activating receptor signaling, Src kinases are required for lytic granule convergence in NK cells. This further establishes convergence as an early Src-dependent step in NK cell activation and suggests its role in preparing the NK cell for precise killing.

DISCUSSION

Lytic granule convergence is a rapid, early event in NK cell cytotoxicity. While not all NK receptors trigger convergence, at the least, adhesion receptor engagement is sufficient (Mentlik et al., 2010). We confirmed this finding using NK cells from patients with a naturally occurring

human deficiency of β2 integrin CD18, LAD-1 (Figure 3.1). Here, lytic granule convergence as measured by average distance of lytic granules from the MTOC was abnormal. LAD-1 patients lack the CD18 β2 integrin and cannot form proper αβ pairs necessary for integrin signaling and thus LFA-1 function on NK cells is impaired resulting in defective adherence to infected cells or tissue (Anderson and Springer, 1987). Thus despite the engagement of target cells by LAD-1 NK cells, the convergence of lytic granules to the MTOC was abnormal. This emphasizes LFA-1 as a requirement for target cell-induced NK cell lytic granule convergence. It also defines a potentially important abnormality in this rare human congenital immunodeficiency disorder. Defective NK cell cytotoxicity in these patients was described many years ago, but the mechanism was always thought to be a lack of adherence to target cells. While this is still likely to represent a major defect in LAD-1 NK cells, the role of defective lytic granule convergence warrants further consideration.

Immediately following NK cell receptor engagement, intracellular domain tyrosines common to most activating and inhibitory receptors are phosphorylated by Src kinases (Watzl and Long, 2010). Importantly, the conformational changes that occur with receptor engagement (e.g. the change of CD11a and CD18 into an open conformation) is prerequisite to this phosphorylation. The resulting phosphotyrosine residues recruit SH2-domain-containing signaling molecules that, in turn, facilitate downstream signaling and eventual effector function (Upshaw et al., 2006). Our experiments show that inhibiting Src kinase function blocks lytic granule convergence (Figure 3.2), recapitulating NK cells in which CD11a has been blocked or in those from the LAD-1 patients (Mentlik et al., 2010). More work is necessary, however, to determine which specific Src family kinase is responsible for lytic granule convergence. More than one may be involved, as they appear to serve redundant functions and most have been implicated in the phosphorylation of NK cell activating and inhibitory receptors (Augugliaro et al., 2003; Mason et al., 2006).

Lytic granule convergence depends upon receptor activation but not F-actin function or microtubule dynamics (Mentlik et al., 2010). Given the signals that follow actin organization at the

IS in NK cells, this presents a limited range of signals that are likely to be required for granule convergence. We therefore dissected the signaling pathways downstream of receptor activation using specific inhibitors in an effort to discern the signaling prerequisites for convergence. We evaluated signals that are required for cytotoxicity attesting to their critical involvement in NK cell activation (Figure 3.3). PI3K, a well studied signaling mediator, is required for NK cell MTOC polarization (Jiang et al., 2000), but was not needed for granule convergence (Figures 3.3A and 3.4). MEK, which interfaces with the PI3K signaling pathway and is also required for MTOC polarization (Chen et al., 2006), was similarly not necessary for granule convergence (Figures 3.3B and 3.4). Thus, the mechanism controlling lytic granule convergence is distinct from the PI3K pathway needed for MTOC polarization. This confirms that granule convergence and MTOC polarization are distinct events on the path to directed secretion of lytic granules and underscores that multiple regulatory pathways and checkpoints are involved in the precise control of NK cytotoxicity.

In NK cells, calcium flux is required for cell adhesion, lytic granule maturation, effective cell signaling and release of lytic granule contents (March and Long, 2011). Interestingly, PLCγ activation via phosphorylation, which is needed for calcium flux is present in both activating and inhibitory NK cell signaling pathways (Valiante et al., 1996). In our experiments, inhibiting PLCγ in NK cells had no effect on MTOC-directed lytic granule movement following NK cell activation (Figures 3.3C and 3.4). Though PLCγ plays a role in the development of NK cell lytic granules and the release of their contents, it does not appear to contribute to granule traffic towards the MTOC.

We also examined lytic granule convergence in inhibitory synapses since much of activation signaling is specifically curtailed in this setting by the function of the inhibitory receptor. Interestingly, granule convergence in inhibitory synapses was comparable to that in activating synapses (Figure 3.5). Thus, downstream targets of KIR receptor signaling, including SHP-1-mediated dephosphorylation, appear to have no effect on convergence (Figure 3.5). Vav1, which is necessary for actin reorganization at the IS (Stebbins et al., 2003), is one such target of KIR-

driven SHP-1-mediated dephosphorylation. We had previously shown that lytic granule convergence does not require actin reorganization; finding that convergence occurs in inhibitory synapses – a scenario where actin reorganization is blocked – dovetails with our previous conclusion that granule convergence is an early and actin-independent step (Mentlik et al., 2010).

Our experiments suggest that the signaling requirement for lytic granule convergence lies downstream of the initial adhesion signal, but upstream of signaling pathways as would be truncated by inhibitory receptor signaling or the specific inhibitors we had utilized. Our use of Src inhibition, which did abrogate lytic granule convergence, demonstrates that at least signals from this family of kinases are required. Thus, in the case of inhibitory receptors, the Src-mediated tyrosine phosphorylation of KIR intracellular domains that occurs upstream of SHP-1 recruitment may trigger lytic granule convergence itself (Faure et al., 2003) or it may be derivative from distinct initial activation signaling stemming from Src signaling before SHP-1-mediated dephosphorylation has had its effect (Schleinitz et al., 2008). In either case the potential importance of Src kinase function upon this preparatory NK cell activation step is distinguished.

Soluble IL-2 is a potent NK cell activating factor (Ljunggren and Malmberg, 2007), that may also help to further prepare NK cells for cytotoxicity by promoting lytic granule convergence (Figure 3.6). This was specific to IL-2, and not a feature of simply adding cytokine to the cells since IL-10 did not promote this function (Figure 3.7). This additionally substantiates that granule convergence is not simply a feature of cell deformation via target cell engagement or activating receptor crosslinking via antibody-coated surfaces. It also provided the opportunity to further dissect the signal requirement for lytic granule convergence. While blocking the canonical JAK3-dependent IL-2 signaling pathway did not block convergence, Src kinase inhibition did. These results suggest a role for either non-canonical IL-2 signaling or Src kinase-dependent signaling upstream of JAK3 in triggering lytic granule convergence. Non-canonical IL-2 signaling is mediated through SHC (Ravichandran and Burakoff, 1994; Shuh et al., 2011), whereas only certain Src family kinases lie upstream of JAK3.

Taken together, our results point to a direct effector mechanism for dynein-directed traffic of lytic granules that lies downstream of Src kinase-mediated phosphorylation and upstream or independent of effectors such as PI3K, MEK and PLCγ. One possibility is that Src kinases directly facilitate lytic granule convergence, however, there is little evidence that Src kinases participate in cargo transport. We instead hypothesize that Src kinases are indirectly responsible for lytic granule traffic through an adaptor that is common to both activation receptor and cytokine signaling pathways. One candidate is the adaptor protein growth factor receptor-bound protein 2 (Grb2), a signaling mediator downstream of Src kinase phosphorylation but upstream of Vav1, PI3K, MEK, and PLCγ (Carlier et al., 2000; Cussac et al., 1999; Graham et al., 2006; Okkenhaug and Rottapel, 1998; She et al., 1997). Intriguingly, Grb2 also participates in IL-2 signaling (Gadina et al., 1999). In immune cells, Grb2 is upstream or independent of PI3K (Upshaw et al., 2006), contributes to Erk signaling through the Ras pathway (Cussac et al., 1999) (also true in non-canonical IL-2 signaling (Ravichandran and Burakoff, 1994)), links Vav1 with activating receptors (Upshaw et al., 2006), and facilitates actin polymerization through an interaction with WASp (She et al., 1997). Further, its interaction with PLCγ is disrupted in inhibitory signaling (Valiante et al., 1996). Surprisingly, Grb2 has also been found to interact with the $p150^{Glued}$ subunit of dynactin (Sahni et al., 1996) and in B cells, Grb2 is required for dynein-mediated transport of microclusters (Schnyder et al., 2011). These observations suggest that following receptor or cytokine activation, Grb2 could act as an adaptor to facilitate interactions between lytic granules and dynein/dynactin. Alternatively, Grb2 could help to recruit signaling proteins downstream of Src family kinases to receptor intracellular domains. Future studies are needed to evaluate physical interactions between Grb2, dynactin and lytic granules.

Our findings document for the first time the key signaling requirements for lytic granule convergence to the MTOC. They suggest specific and early signaling that drives microtubule-directed, dynein-dependent lytic granule traffic. While lytic granule convergence is rapid, it likely represents one of the very first steps an NK cell takes to prepare for a cytotoxic event through enabling the focused directed secretion of lytic granule contents.

CHAPTER 4: DISCUSSION

SUMMARY

Natural killer cell cytotoxicity is the first line of defense upon challenge by infection, cancer, or stress. While NK cells are primed and ready to kill, this deadly process must be regulated so that the effector function is not improperly targeted towards healthy cells and tissues. As such, NK cell cytotoxicity is controlled at each step, from the initial contact to the final secretion of the lytic granule contents onto the target cell (Figure 2.16E). At each step, abnormalities in any of the proteins or processes involved lead to a halt in cytotoxicity progression and severe immunodeficiencies (reviewed in (Topham and Hewitt, 2009)). As reviewed in Chapter 1 and (Orange, 2008), the NK cell progresses through a series of stages in the formation of the lytic IS (Figure 1.1). First, in the initiation stage, the NK cell must adhere to the target cell and activation signaling must dominate the interaction (Masilamani et al., 2006). Next, F-actin begins accumulating at the cell-cell contact zone and must remain intact throughout the effector stage (Orange et al., 2002). Lytic granules must converge to the MTOC before the MTOC polarizes to the IS (Mentlik et al., 2010) and (AMJ – manuscript in review). The MTOC must dock at the IS (Banerjee et al., 2007). F-actin must clear from certain places in the IS in order for lytic granules to pass through to the plasma membrane (Rak et al., 2011; Sanborn et al., 2009). Finally the lytic granules must dock with, be primed at, and fuse with the plasma membrane (Bryceson et al., 2007; Feldmann et al., 2003; Menasche et al., 2000).

While other immune cells also have many regulatory steps, few are capable of responding to as many forms of stimuli as NK cells. Contact with affected cells as well as circulating cytokines can activate NK cells (reviewed in Chapter 1, Figures 1.2 and 1.3), and NK cells are then responsible for responding by directed secretion of lytic granule contents and by secretion of cytokines that will influence the innate and adaptive immune systems.

In Chapter 2, we characterized a new step in NK cell cytotoxicity regulation. We showed that lytic granule convergence occurs after engagement of activating receptors on NK cells, even

before the cell has committed to cytotoxicity of the target. Even in cytolytic conjugates, MTOC polarization can take over 30 minutes but lytic granule convergence is complete within 4 minutes of conjugate formation (Figures 2.2, 2.3 and 2.8). We tracked individual lytic granules and found that they move rapidly towards the MTOC when the NK cell becomes activated (Figure 2.9). Importantly, we showed that this event is not unique to our cell lines; using primary NK cells, we showed that lytic granule convergence is physiologically relevant (Figure 2.11). Finally, we demonstrated that this process occurs independently of actin and microtubule dynamics but requires LFA-1 engagement and dynein/dynactin function (Figures 2.12 and 2.14-2.16). While lytic granule convergence to the MTOC does not signify that cytotoxicity will occur, cytotoxicity does not progress normally without it. Thus, the importance of lytic granule convergence is that it represents yet another step regulating NK cell directed secretion.

In Chapter 3, we further characterized lytic granule convergence by studying the signals that contribute to the event. Using LAD-I patient cells, we confirmed that lytic granule convergence requires LFA-1 (specifically CD18), and that without proper adhesion cytotoxicity cannot proceed (Figure 3.1). We further characterized the signaling requirements for lytic granule convergence. Src kinases are necessary for convergence, but PI3K, MEK, and PLCγ are dispensable (Figures 3.2-3.4). We also found that lytic granule convergence still occurs in the presence of an inhibitory synapse, signifying that the signal for convergence is not affected by inhibition of cytotoxicity or recruitment of a phosphatase (Figure 3.5). Here again, we confirmed that lytic granule convergence occurs before the NK cell commits to cytotoxicity. Lastly, we demonstrated that even soluble cytokine (in this case, IL-2) can trigger lytic granule convergence, and that IL-2-mediated lytic granule convergence depends not upon canonical IL-2 signaling but also requires Src kinases (Figures 3.6 and 3.7). Therefore, lytic granule convergence occurs very early in NK cell activation and is a common preparatory step in lytic and inhibitory IS formation as well as cytokine stimulation.

In this discussion, I will discuss areas where our new understanding in NK cells diverges from the current accepted paradigms in CTLs as well as how lytic granule convergence fits in with

our existing knowledge of minus-end directed traffic of cargo in other cell systems. Several questions remain about MTOC polarization after lytic granule convergence as well as the importance of convergence prior to MTOC polarization in NK cells and the signaling required for that convergence. I will also consider models for future studies. To conclude, I will discuss the implications and significance of our findings.

LYTIC GRANULE CONVERGENCE IN CYTOTOXIC T CELLS VS NK CELLS

It has been assumed that lytic granule behavior in NK cells would mimic that in CTLs. In truth, much of our understanding of NK cell-mediated cytotoxicity was first studied in CTLs or is accepted as identical between the two types of lymphocytes. Both destroy virally infected, stressed, and cancerous cells, both by delivery of the contents of lytic granules. However, CTL receptors require somatic recombination in order to create immunological memory towards specific antigens while NK cells contain only germline-encoded receptors (Lanier, 2005). Because of their repertoire of inhibitory receptors, NK cells are associated with higher stem cell transplantation success while T cells are known to be more alloreactive (Bishara et al., 2004). Once mature, the NK cell does not need further development in order to be capable of recognizing and killing a target cell while CTLs are late responders, requiring differentiation and proliferation before being able to kill. On the other hand, NK cell cytotoxicity is a slower process because of less efficient immune recognition and more reliance upon coactivation while CTLs are able to mediate cytotoxicity more rapidly due to higher specificity of T cell receptor-antigen pairing (Wulfing et al., 2003). Signal strength does seem to play a role even in CTLs, however, as weak signal results in slow or incomplete MTOC polarization here as well (Jenkins et al., 2009). Most relevant to this work is that the accepted paradigm for directed secretion is rapid MTOC polarization to the IS and subsequent lytic granule polarization to the docked MTOC (Kupfer and Dennert, 1984; Kupfer et al., 1983). In fact, NK cells show more gradual MTOC polarization while lytic granule convergence to the MTOC precedes polarization to the IS (Mentlik et al., 2010).

Very few have studied CTLs and NK cells side by side and so not all researchers use the same methods when studying the two types of lymphocytes separately. In order to characterize the differences between CTLs and NK cells, it will be important to study both in parallel, using the same techniques. Most current studies involve IL-2-activated CTLs while present NK cell studies involve either resting or IL-2-stimulated cells. For this work, all studies were done in resting NK cells. However, it can be difficult to study MTOC and lytic granule behavior in resting CTLs because they have very few preformed lytic granules and without differentiation, polarize weakly (Orange, 2008). Unfortunately, if we use IL-2-activated NK cells and CTLs, the results will also likely not be illuminating. We show in Chapter 3 that once IL-2 is added to NK cells, lytic granules converge to the MTOC. Prolonged studies over a series of days might show that lytic granules re-disperse, and if this is the case, then this may become a reasonable area of study. Additionally, we should still study MTOC polarization kinetics in parallel more thoroughly. Perhaps the large difference we have noted in MTOC polarization time – 30+ minutes for NK cells versus 5 minutes for CTLs – might be a feature of IL-2. In fact, preliminary results in our lab reveal a possible decrease in MTOC polarization time in NK cells after IL-2 is added to conjugates (AMJ – unpublished).

As a result of our research and that of so many others, it has become more and more apparent that while very alike, it is unfair to assume that NK cell behavior will always follow CTL behavior. Just to name a few, MTOC docking, granule docking and priming, and the existence of a separate pSMAC and cSMAC are not as well studied in NK cells as CTLs (Orange, 2008). With the development of newer and better techniques, such as total internal reflection microscopy (TIRF) and super resolution microscopy (STED, PALM, STORM) some groups are beginning to get a better look at NK cell IS structure and finding differences in distribution of actin (Brown et al., 2011; Rak et al., 2011), while others are studying the role of docking, priming, and fusion proteins in NK cells and finding similarities with CTLs (Dabrazhynetskaya et al., 2011; Liu et al., 2010; Wood et al., 2009). These NK cell-specific studies will continue to be useful in hashing out differences or similarities between the two types of lymphocytes.

MINUS-END-DIRECTED TRAFFIC IN OTHER CELL SYSTEMS

NK cell and CTL lytic granule convergence do not represent the only form of minus-end directed traffic. Lysosome-related organelles and other cargo move along microtubules with the aid of dynein in many cell systems in the body. In neurons, it has been long known that dynein is necessary for retrograde transport across the long distances found in axons (Vallee et al., 1989). Without dynein/dynactin-mediated transport of cargo and survival signals towards the cell body, motor neurons begin to degenerate, leading to numerous neurodegenerative diseases (LaMonte et al., 2002). In particular, cytoplasmic dynein is required for transport of transcription factor nuclear factor-κB (NF-κB) to the nucleus in neurons but not in other cell types (Mikenberg et al., 2006, 2007). Most recently, it has been discovered that in neurons, dynein contributes to more than just retrograde transport. Dynein disruption leads to enhanced endocytosis, causing perturbations in synaptic vesicle docking (Kimura et al., 2012). Those disorders linked to improper dynein function in neurons include Huntington's disease, Alzheimer's disease and Parkinson's disease, to name a few (reviewed in (Eschbach and Dupuis, 2011)). All in all, dynein retrograde transport has already been established as an important feature of proper neuronal function.

Furthermore, dynein-mediated lysosome-related organelle trafficking towards a perinuclear localization has been observed in kidney, liver, and pancreatic cells (Hamm-Alvarez and Sheetz, 1998; Kraemer et al., 1999). In kidney and liver cells, dynein is necessary for transport of ion channels and transporters from the cell surface to lysosomes for degradation, while in pancreatic cells, the role of dynein is still unclear. In macrophages, dynein is responsible for transporting newly internalized phagosomes in a minus-end-directed manner towards the center of the cell (Blocker et al., 1997). Pigment-containing granules called melanosomes in melanophores are capable of rapid changes in distribution of pigment and several groups have long observed dynein distribution on melanosomes (Byers et al., 2000; Rogers et al., 1997; Vancoillie et al., 2000). Here, kinesin-2, dynein and myosin V work in concert to disperse, aggregate or bring pigment to the plasma membrane, respectively (Kural et al., 2007). Interestingly, certain disorders in lytic granule traffic in the immune system are also accompanied

by respective disorder in skin pigmentation because the mechanisms that regulate formation and secretion of antigen-processing compartments and melanosomes are similar (reviewed by (Griffiths, 2002; Marks et al., 2003)). In Chediak-Higashi syndrome, patients display a mutation in a gene called Lyst. The disease is characterized by abnormally large lysosomes in lymphocytes and melanosomes that cannot be secreted. In Griscelli's syndrome, small GTPase Rab27a is defective and the disease is characterized by a lack of lysosomal docking at the plasma membrane, so neither melanosomes nor lytic granules can secrete their contents. Finally, in Hermansky Pudlak syndrome, the adaptor protein AP3 is mutated, leading to improper sorting of membrane proteins from Golgi to lysosome and from minimal to complete albinism and immunodeficiency. In melanocytes, minus-end directed melanosome transport is not a means for directed secretion as it is in NK cells, but serves to sort proteins from the plasma membrane to early pigment granules so they can be transported to a perinuclear location to prevent premature release of immature melanosomes (Watabe et al., 2008). Therefore, the minus-end directed lytic granule traffic that we describe in NK cells for the purpose of directed secretion is a novel regulatory process that aids in precision of a lethal function.

MTOC POLARIZATION AND CELL POLARITY IN NK CELLS

In Chapter 2, we measured MTOC polarization time in NK cells. While MTOC polarization is not fully understood in immune cells, there have been significant studies characterizing it, with similarities and differences between CTLs and NK cells. In CTLs, MTOC polarization requires signaling components Lck, Fyn, ZAP-70, linker for activation in T cells (LAT), SLP-76, Vav1, Cdc42 and DAG (Ardouin et al., 2003; Kuhne et al., 2003; Martin-Cofreces et al., 2008; Quann et al., 2009; Stowers et al., 1995). In NK cells, signaling components required for MTOC polarization include Src kinases, Erk, Vav1 and Pyk2 (Chen et al., 2007; Graham et al., 2006; Sancho et al., 2000). Physically, MTOC polarization in CTLs requires an intact F-actin-rich IS as well as intact microtubules (Stinchcombe et al., 2006). MTOC polarization may be regulated by actin nucleators called formins, by DAG, and by the sliding of microtubules through dynein anchored in the IS via

the adhesion- and degranulation-promoting adaptor protein (ADAP) (Combs et al., 2006; Gomez et al., 2007; Kuhn and Poenie, 2002; Quann et al., 2009). In NK cells, an intact IS and microtubules are also required as well as MTOC anchoring into the IS via CIP4 and formin hDia1 (Banerjee et al., 2007; Butler and Cooper, 2009; Orange et al., 2003). Given all this data, in the case of both CTLs and NK cells, we do not have a perfectly clear understanding of how MTOC polarization is accomplished. However, by bridging the signaling and physical requirements for MTOC polarization with cell polarity, we can gain more insight into the process.

Cell polarity in immune cells refers to the positioning of cell systems relative to a motility axis (reviewed in (Krummel and Macara, 2006)). The F-actin cytoskeleton is polarized to produce a leading and trailing edge. The microtubule network represents another level of polarity as the MTOC is normally positioned behind the nucleus. Surface proteins and receptors also polarize depending on the extracellular cues that the cell is experiencing. Activating receptors are kept from the leading edge until needed so that the cell is not activated for cytotoxicity or cytokine release before experiencing a danger. Polarity proteins are also specifically localized so as to orchestrate polarity of the other cell systems, but we will not be introducing individual polarity proteins here because they are not the focus of this discussion. To accomplish polarity in immune cells, actin polymerizing proteins such as Rho GTPases and Vav1 are thought to work with polarity complexes to control cell directedness so the leading edge of the cell is facing the danger (reviewed in (Stinchcombe and Griffiths, 2007)). Additionally, ADAP has been found in complexes with Fyn, SLP-76 and Vav1 among others and may bridge activation pathways with dynein distribution in the IS (reviewed in (Miletic et al., 2003)). The purpose of ADAP here is probably to orient microtubules to position the MTOC for polarization (Combs et al., 2006).

It has long been known that pushing forces exerted by growing microtubules can control MTOC and nuclear positioning in *Schizosaccharomyces pombe* (Tran et al., 2001). However, mounting evidence in other cell systems supports the model wherein microtubules become anchored to the cell cortex via cortical dynein and then slide or ratchet through the cytoplasm via pulling forces exerted by dynein to position the MTOC (Burakov et al., 2003; Carminati and

Stearns, 1997; Koonce et al., 1999; Laan et al., 2012; Levy and Holzbaur, 2008; Moore et al., 2009; Nguyen-Ngoc et al., 2007; Palazzo et al., 2001; Yamamoto et al., 2001). Further, the capture of microtubule plus ends by cortical dynein actually stabilizes microtubules, which may provide better tracks for vesicular transport (Hendricks et al., 2012). In migratory cells, the MTOC and microtubules are reoriented in the direction of migration (Gotlieb et al., 1981; Kupfer et al., 1982; Malech et al., 1977). Recently, it has been discovered that dynein interacts with polarity proteins at the leading edge to accomplish this (Manneville et al., 2010). In macrophages, the MTOC and microtubules are reoriented in the direction of phagocytosis in a dynein-dependent manner (Eng et al., 2007). In CTLs and NK cells, the MTOC is most likely pulled through the cytoplasm towards the IS and target cell (Combs et al., 2006; Martin-Cofreces et al., 2008). Here, Src kinases and proteins that interact with both actin and microtubules, such as hDia1 and CIP4 may provide an anchor for the MTOC to dock with the IS in order to remain stationary while cargo is unloaded (Banerjee et al., 2007; Butler and Cooper, 2009; Tsun et al., 2011). Unfortunately, the presence of dynein in the IS has not been confirmed in NK cells, and so our future work will be focused on determining if this model is valid and applicable to NK cells.

PURPOSE OF LYTIC GRANULE CONVERGENCE IN NK CELLS

We characterized lytic granule convergence in terms of cytoskeletal requirements in Chapter 2 and in Chapter 3, in terms of signaling requirements. The discovery and characterization of this process is a novel finding not reported or described previously. However, since it is a novel and unexpected finding, its purpose in NK cell directed secretion is unclear. The main evidence that may shed some light on the purpose of lytic granule convergence is studies done on the contribution of individual receptors by Eric Long and colleagues (Bryceson et al., 2005; Bryceson et al., 2006b) (and reviewed in (Bryceson et al., 2006a)). Physiologically, coordination and synergy between different activating receptors on NK cells contribute to successful directed secretion, but when studied individually to determine the contribution of individual receptors, Bryceson, et al. found that some are capable of polarization of lytic granules

(such as integrin LFA-1 and co-activating receptor 2B4), while the receptor CD16 was capable of degranulation without polarization.

CD16 (FcγRIIIA) is a low affinity receptor that is expressed widely in mature NK cells and recognizes the constant regions of antibodies secreted by plasma B cells or added to the system via drug administration. When target cells secrete or become coated with IgG, CD16 is able to mediate killing in a process termed antibody-dependent cellular cytotoxicity (ADCC) (Perussia and Loza, 2000; Thobakgale et al., 2012; Trinchieri and Valiante, 1993). When researchers observed the activity of CD16 against target cells coated with IgG, they found that lytic granule contents were released with no directedness (Bryceson et al., 2005). In other words, the signaling that follows CD16 engagement bypasses or overrides the signal for lytic granule convergence. This may give a clue as to the purpose of convergence because evidently, not every activating receptor causes lytic granules to converge upon engagement, and the result could be less specific killing and no protection to bystanding cells. CD16 signaling is similar to that after engagement of other classical NK cell receptors such as NKG2D and the natural cytotoxicity receptors (NCR). As in NK cell signaling discussed in Chapter 1 that leads to cytotoxicity, release of cytokines and transcriptional regulation, Src kinases are triggered, as well as the PI3K, Erk, SLP-76 and Vav signaling pathways (Chini and Leibson, 2001). There is no speculation as to why CD16 engagement would cause degranulation without convergence or polarization. ADCC is utilized when a large-scale infection or cancer is present. In this case, antibodies are secreted from B cells that become infected or are specifically given to patients that will target virally infected or tumorigenic cells for ADCC (Kohrt et al., 2012; Thobakgale et al., 2012; Wren et al., 2012). It is possible that polarization to one specific target cell is not necessary.

In other instances of host defense, non-directional degranulation may be beneficial. NK cells are able to respond directly to certain bacteria and fungi (Esin et al., 2008; Marr et al., 2009). In this case, the direct function of the NK cell is to secrete perforin to kill the pathogen and the indirect function serves to recruit other immune cells by secretion of cytokines and chemokines (Horowitz et al., 2011). In an environment saturated with pathogens and no true target cell to

bind, neither directed secretion nor lytic granule convergence would be necessary. However, little is known about the path taken by lytic granules upon pathogen recognition. Esin and colleagues discovered that NK cells utilize the NCR family member NKp44 to recognize bacteria. There is no consensus as of yet as to what receptor is engaged by fungi although PI3K/Erk signaling is involved and LFA-1 is not necessary (Esin et al., 2008; Jones et al., 2009; Wiseman et al., 2007). This last is an interesting finding because CD16-mediated degranulation also does not utilize LFA-1-mediated adhesion (Bryceson et al., 2005). However, no evidence of CD16 involvement has been discovered in fungal or bacterial killing as of yet. Further studies into direct pathogen killing by NK cells would lend interesting insight into other mechanisms of degranulation.

In physiological conditions, the purpose of directed secretion is for a circulating NK cell encountering an infected target cell to only kill that susceptible target cell so as not to cause harm to potentially healthy surrounding cells. In this case, MTOC polarization to the IS is the essence of directed secretion, and we theorize that lytic granule convergence is a failsafe mechanism to ensure that undirected degranulation does not occur before MTOC polarization is complete. Even though lytic granules do not converge to the MTOC in CTLs, this mechanism may not be a necessity in CTLs because very few CTLs normally express CD16 and therefore will not experience degranulation without polarization. Even in situations where CD16 expression is detected on CTLs, as in the case of chronic infection, these cells are said to have an NK-like phenotype and receptor repertoire (Bjorkstrom et al., 2008). In this instance, integrin and co-activation receptors are also present and so polarization precedes degranulation in these cells as in a classical NK cell-target cell IS (Bryceson et al., 2005). Further study into this topic with the aid of high-resolution confocal microscopy techniques will allow us to better understand whether lytic granule convergence really does prevent untargeted NK cell-mediated killing.

NK CELL SIGNALING AND CONVERGENCE

Because lytic granule convergence to the MTOC is a recently discovered event, there is still much to learn about the mechanics of the process. While we have determined that lytic

granule convergence occurs directly downstream of Src kinases, upstream of PI3K, MEK, PLCγ, and SHP-1-mediated dephosphorylation, and independently of canonical IL-2 signaling we have not pinpointed the exact signaling requirement for lytic granule convergence triggering. We know that convergence occurs after activating, triggering, and inhibitory receptor engagement as well as after IL-2 stimulation. Our candidate for consideration is the adaptor protein growth factor receptor-bound protein 2 (Grb2) (Chen et al., 2006; Lanier, 2008; Tassi et al., 2006). These processes all share a commonality with Grb2. Grb2 has no enzymatic activity of its own but is crucial for mediating interactions between multiple binding partners with its two Src homology (SH) 3 domains and one SH2 domain. The SH2 domain is capable of binding to phosphotyrosine residues on the cytoplasmic tails of receptors and the two SH3 domains act as scaffolds for signaling platforms downstream (reviewed in (Jang et al., 2009)). While it is not capable of actively contributing signals to lytic granule convergence, there is mounting evidence that Grb2 is an important member of lymphocyte signaling complexes and our evidence makes it a good candidate for a lytic granule convergence promotor. Grb2 is downstream of Src kinase phosphorylation and upstream or independent of Vav1, PI3K, MEK, and PLCγ signaling (Gadina et al., 1999; Graham et al., 2006; Ravichandran and Burakoff, 1994; Upshaw et al., 2006; Yamasaki et al., 2001). Also, interaction between Grb2 and PLCγ is disrupted in inhibitory signaling (Valiante et al., 1996). Further, Grb2 participates in Ras signaling downstream of IL-2 receptor stimulation (Ravichandran and Burakoff, 1994). Surprisingly, Grb2 may also interact with the $p150^{Glued}$ subunit of dynactin (Sahni et al., 1996) and in B cells, Grb2 is required for dynein-mediated transport of microclusters (Schnyder et al., 2011). Because of these observations, we propose that Grb2 facilitates the interaction between lytic granules and the dynein/dynactin complex only in situations of NK cell initial activation (Figure 4.1).

However, CD16 engagement also activates Grb2 (Galandrini et al., 1996) but does not trigger convergence (Bryceson et al., 2005). Future studies will be focused on the exact contribution of Grb2 to lytic granule convergence and the action of CD16 on NK cell degranulation. It may be that since CD16 activation does not occur alone in classical NK cell-

target cell interactions; integrin and co-activating receptor engagement would also be present, it is not a factor in NK cell lytic granule convergence. It is also possible that Grb2 does not trigger lytic granule convergence but Src kinases are solely involved in convergence with Grb2 only acting as an adaptor. While many Src family kinases are involved in NK cell to target cell interactions, only the lymphocyte-specific Src kinase Lck is common to lytic, inhibitory, and cytokine-stimulated early NK cell signaling but it may be that contribution of other Src family kinases has not been discovered yet (Mason et al., 2006; Treanor et al., 2006; Vitte-Mony et al., 1994). We will continue to devote future studies towards discovering Grb2 and specific Src kinase family member involvement in lytic granule convergence.

If we look to dynein activity for an answer, what is known about dynein-mediated transport comes largely from studies in neurons but can probably be applied here. Dynein requires interaction with dynactin for processivity, organelle binding and fast transport (Waterman-Storer et al., 1997). In fact, dynactin may be required for initiation of retrograde motion as it facilitates the interaction between dynein, cargo, and microtubule (Moughamian and Holzbaur, 2012). It is unknown whether NK cell activation induces dynein-dynactin association, if the dynein/dynactin complex is activated upon NK cell activation, or if only NK cell activation induces association of lytic granules with the dynein/dynactin complex. In a study that specifically examined dynein interaction with lysosomes, dynein light intermediate chains were found to specifically recognize and interact with lysosomes, although the authors did not distinguish whether this was a dynactin-dependent or –independent mechanism (Tan et al., 2011). In B cells, recent evidence suggests that dynein is indirectly recruited to cargo by Grb2 and associated mediators (Schnyder et al., 2011). Alternatively, inactivation of kinesin-based movement that is thought to occur when dynein attaches to cargo may be the driving force behind retrograde transport (Schuster et al., 2011). The importance of discovering the signaling requirement for lytic granule convergence is that even in CTLs, the mechanism utilized by lytic granules to track along microtubules to the MTOC is unknown. The signaling and mechanics behind this process will

shed light on a crucial step in NK cell cytotoxicity regulation and aid in our understanding of immunological disorders of lytic granule transport.

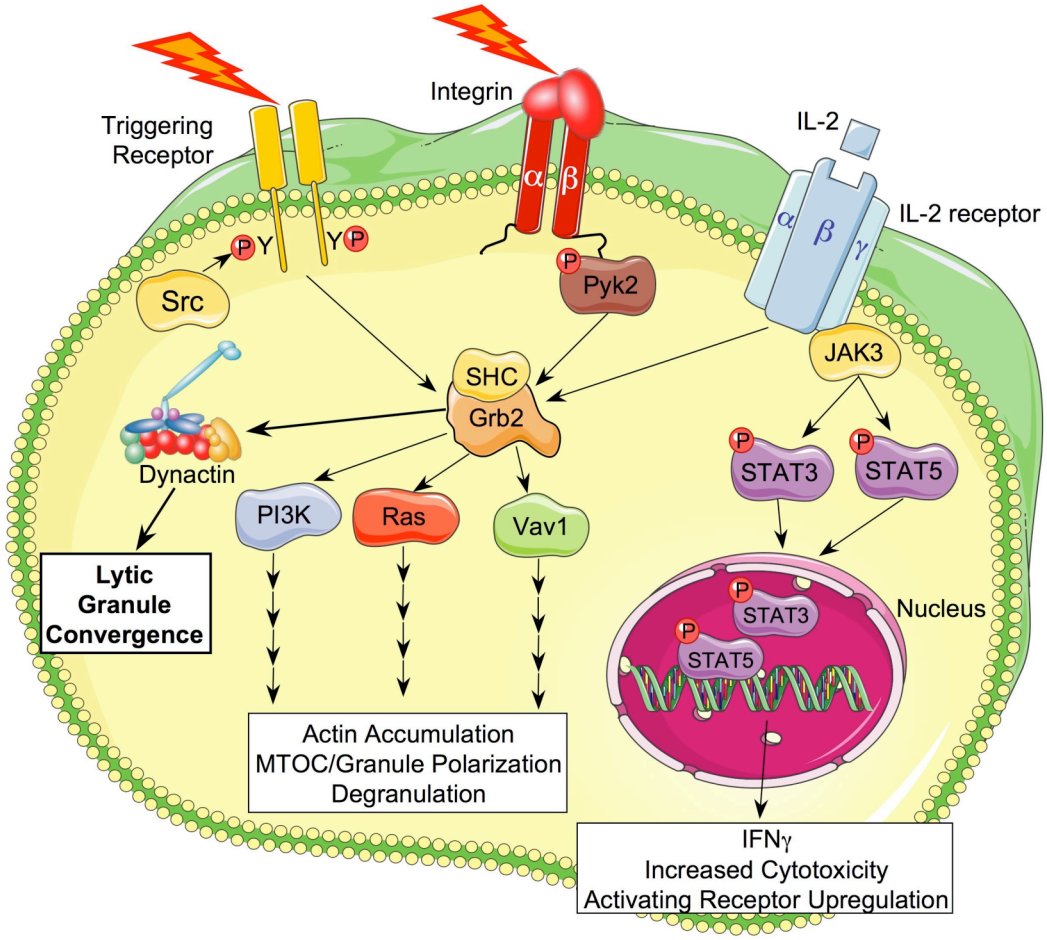

Figure 4.1. Model of lytic granule convergence signaling requirements.
Hypothetical model of an NK cell showing the signaling cascades following multiple receptor engagement, specifically, showing common signaling intersections that may be responsible for lytic granule convergence. After receptor engagement, Src kinases but not PI3K or Vav1 GEF activity are required for lytic granule convergence. After IL-2 receptor engagement, JAK/STAT signaling is not required but non-canonical IL-2 signaling, which involves Grb2, is required for convergence. Because Grb2 is involved in all the above pathways and interacts with dynactin, this makes it a plausible candidate. Not pictured: KIR engagement also leads to lytic granule convergence and shares commonality with the above signaling pathways at the Src family kinase and Grb2 levels.

MODELS

In continuation of this work, three questions remain. How is MTOC polarization accomplished in NK cells? What is the purpose of lytic granule convergence in NK cells? What is

the signaling mechanism utilized by the NK cell to accomplish lytic granule convergence? I have developed hypotheses, models, and preliminary research plans to address these questions.

How is MTOC polarization accomplished in NK cells?

Chiefly, my hypothesis and model for this question originate from previous work in NK cells (Banerjee et al., 2007; Butler and Cooper, 2009) and in CTLs (Combs et al., 2006; Gomez et al., 2007; Kuhn and Poenie, 2002; Quann et al., 2009). I hypothesize that proteins that interact with both actin and microtubules are necessary for anchoring microtubules in the IS, thus establishing polarity, and that dynein localized to the IS is responsible for exerting pulling forces on microtubules, thus propelling the MTOC through the cytoplasm towards the IS. There is currently significant evidence that dynein aids in establishing cell polarity in several cell systems as discussed above. Initial experiments will use high-resolution microscopy techniques such as STED and TIRFm of NK cell-target cell conjugates or activated NK cells on glass, respectively to measure dynein distribution in the IS. I predict that because the NK cell IS is not thought to be as highly ordered as the CTL IS that there will not be a definitive ring of dynein in the periphery of the IS (Combs et al., 2006). High-resolution three-dimensional microscopy will also allow us to visualize whether microtubules become anchored in the IS in early conjugates and whether they begin to curve past the IS as the MTOC is propelled through the cytoplasm in late conjugates (Kuhn and Poenie, 2002).

In order to examine whether MTOC polarization requires dynein, we must first acknowledge a conundrum: if we remove dynein from NK cells, and MTOC polarization does not proceed, is this because we blocked MTOC polarization or because we blocked lytic granule convergence and convergence is a prerequisite to MTOC polarization? In order to differentiate, we will leave dynein function intact but attempt to disrupt dynein recruitment to the IS because I predict that the pool of dynein that shuttles lytic granules to the MTOC is distinct from the pool of dynein that is recruited to the IS. There is no evidence of dynein recruitment to the NK cell IS, so our preliminary experiments would shadow studies in CTLs. We will measure dynein presence and MTOC polarization in the IS after inhibition or removal of ADAP or DAG (Combs et al., 2006;

Quann et al., 2009). And because they are necessary for MTOC polarization in NK cells, I would also like to evaluate the potential importance of CIP4 and formin hDia1 in dynein recruitment to the IS (Banerjee et al., 2007; Butler and Cooper, 2009).

Mechanistically, I hypothesize that dynein nears the IS as microtubules undergo rescue; lengthening from the plus end, and that enrichment of the anchoring protein as a result of IS formation traps dynein there. This causes microtubule stability and provides better tracks for dynein to pull the MTOC through the cytoplasm (Hendricks et al., 2012; Laan et al., 2012). All the proteins discussed above have been found enriched in the IS of CTL or NK cell conjugates, but I would also test for specific, direct interactions between possible anchoring proteins and dynein via biochemical techniques such as immunoprecipitation and mass spectroscopy.

What is the purpose of lytic granule convergence in NK cells?

To confirm our hypothesis that lytic granule convergence is necessary for precise delivery of lytic granule contents to the target cell conjugated to the NK cell only, we must show that lack of convergence can result in undirected secretion. My hypothesis and model for this question arises from work showing that triggering of the low affinity receptor CD16 (FcγRIIIA) results in degranulation without polarization (Bryceson et al., 2005). CD16 engagement on NK cells may signify a necessity for NK cells to degranulate quickly in an environment that may be flooded with cancerous cells or soluble fungi or bacteria. In order to address this question, we will engage CD16 on the NK cell surface exclusively using a target cell line or beads opsonized with IgG that do not contain ligands for any other NK cell activating receptor. Preliminary results to this effect have already shown that lytic granule convergence is minimal (AMJ – unpublished). Surrounding target cells will also be loaded with a dye that indicates cell death so that we can monitor if surrounding target cells are killed by unpolarized lytic granule secretion.

It will also be pivotal to observe MTOC behavior to establish whether the MTOC will polarize towards a bound target cell. CD16 triggers signaling pathways that have been found to be necessary for MTOC polarization, such as PI3K and PLCγ signaling (Chen et al., 2007; Chini and Leibson, 2001). If the MTOC polarizes towards a bound target cell but surrounding target

cells are still killed by unpolarized degranulation, then we will conclude that lytic granule convergence is not a prerequisite for the physical act of MTOC polarization but that it is required for precise lytic granule delivery. If surrounding target cells are killed and the MTOC does not polarize, then our conclusion will be that lytic granule convergence is a prerequisite for both the physical act of polarization and for precise delivery of lytic granule contents.

What is the signaling mechanism utilized by the NK cell to accomplish lytic granule convergence?

We have established that dynein is required for lytic granule convergence, but have not established the mechanism utilized. It is logical that the mechanism for lytic granule convergence is either direct dynein/dynactin activation or indirect kinesin inactivation. Based on our work in Chapter 3 and previous discussion above, I hypothesize that the direct mechanism requires Grb2. Alternatively, I hypothesize that the indirect mechanism utilizes activation-induced self-inhibition of kinesin.

It is important to note that Grb2 is likely anchored at the cell membrane where it interacts with phosphorylated activating receptor intracellular domains while lytic granules are dispersed throughout the cell. However, as discussed in the MTOC polarization model, microtubules are constantly growing and shrinking from the plus ends during the process of dynamic instability (Mitchison and Kirschner, 1984). When microtubules lengthen, bringing dynein/dynactin-bound cargo – in this case, lytic granules – close to the cell cortex, dynactin interacts (probably indirectly (Schnyder et al., 2011)) with Grb2, which facilitates its phosphorylation and preferentially stimulates minus-end-directed movement in a process similar but not identical to melanosome aggregation (Farshori and Holzbaur, 1997; Kumar et al., 2000; Lomakin et al., 2009). Unlike normal endocytic trafficking, lytic granules are already fully mature lysosome-related organelles; they are not being transported to the Golgi network in order for contents to be recycled. The proteins that normally follow newly endocytosed material from the cell membrane into the center of the cell and back outwards to be recycled probably do not apply here, and so we will not initially consider them (reviewed in (Shaw et al., 2001)). Support for this model comes from previous work showing that when dynactin is disrupted, lytic granules are specifically localized to

the cell periphery, not diffusely localized throughout the cytoplasm (Mentlik et al., 2010). The significance of this finding is that it is plausible that lytic granules are stranded at microtubule plus ends in close proximity with the cell membrane, but without dynactin activation, there is no minus-end-directed movement to the MTOC. Additional support for the role of dynactin in this model comes from studies that show that dynactin accumulates at the plus ends of microtubules and aids in initiation of retrograde cargo transport (Moughamian and Holzbaur, 2012; Vaughan et al., 2002).

First and foremost, we must determine whether Grb2 is in any way involved in dynactin activation by microscopy and biochemical techniques. These will include disruption of Grb2 adaptor function via siRNA silencing or a dominant negative mutant followed by confocal microscopy, and immunoprecipitation to test interaction between Grb2 and dynactin. For the latter, using whole cell lysates with mild detergents should allow us to see even indirect interactions. It is likely that the activation of dynactin is facilitated through another signaling molecule because lytic granules from throughout the cell must reach the MTOC, not just the lytic granules along the microtubules that project into the IS. We should examine dynactin phosphorylation, dynein/dynactin association, and perform mass spectroscopy for other proteins associated with dynactin after NK cell activation. Next, high-resolution microscopy should be used when fluorescently tagging Grb2, dynactin, microtubules and lytic granules as current techniques have not resolved movement of individual lytic granules along specific microtubules *in vivo* in NK cells. Better visualization of the process may provide more answers as to the track taken by lytic granules and associated proteins.

Alternatively, if Grb2 proves not to be involved in lytic granule transport, then it is reasonable that inactivation of kinesin drives minus-end-directed transport. It is known that both anterograde and retrograde motors are constitutively bound to organelles (Hendricks et al., 2010). There is evidence that c-Jun N-terminal kinase (JNK) can cause phosphorylation and subsequent self-inhibition of kinesin (Horiuchi et al., 2007; Morfini et al., 2009; Stagi et al., 2006). These studies were performed in neurons but activation of JNK implies an inflammatory pathway

and this can apply to NK cells. Dynactin coordination with dynein might also tip the scale in favor of minus-end-directed movement but this will be difficult to prove because inhibition of dynactin will likely affect all microtubule motor-based movement (Deacon et al., 2003). We should, however, isolate lytic granules from activated and resting NK cells and measure the relative amounts of dynein and kinesin molecules, as differing number of kinesin molecules may signify inhibition of kinesin or disruption of kinesin-cargo binding. We should also attempt blocking kinesin inhibition by blocking JNK activation. All in all, fluorescently tagging or staining these proteins and viewing NK cell-target cell conjugates at different stages will provide us with a wealth of knowledge about motor contribution to lymphocyte activity. At this time, we still lack the full understanding of the localization dynamics of these motors during all stages of lymphocyte cytotoxicity.

CONCLUSIONS

This body of work has described a new step in NK directed secretion. Lytic granule convergence is a newly characterized preparatory step in NK cell-mediated cytotoxicity, one that is prerequisite to killing. Convergence is rapid, dynein-dependent, and occurs even without cytolytic commitment. It can be triggered by contact with a potential target cell and by cytokine stimulation. Furthermore, lytic granule convergence is prompted very early, directly downstream of Src kinase activation. This phenomenon is a novel mechanism that the NK cell utilizes to prepare for cytotoxicity and ensure precision in delivery of a deadly cargo. This work also demonstrates that while similar, lytic granule convergence is another example of divergence between the process of CTL-mediated and NK cell-mediated killing. Lytic granule convergence represents a unique and efficient extra layer of regulation in a deadly process.

Continuing studies of the checkpoints and steps regulating NK cell directed secretion will continue to increase our understanding of how NK cells kill and how they come to affect inflammation, immunodeficiencies, and autoimmunity. Specifically, how F-actin is cleared from the IS and how NK cells detach from lysed target cells remain elusive areas of study. My future

studies of lytic granule convergence would ideally focus on the purpose of convergence first and foremost. We believe that the purpose of lytic granule convergence is precise delivery of lytic granule contents, but this hypothesis still needs to be tested further. Also, the signaling required for lytic granule convergence needs to be further canvassed as well as the differences between NK cell and CTL lytic granule approximation and MTOC polarization. However, we should continue to learn from other cell systems, as much of what we know now about lytic granule behavior in NK cells was first discovered in other cell types. Understanding the mechanics and purpose of lytic granule convergence in NK cells will aid in our comprehension of those immunological disorders that display impaired directed secretion.

CHAPTER 5: MATERIALS AND METHODS

NK CELL LINES, TARGET CELL LINES, AND *EX VIVO* NK CELLS

The immortalized NK cell lines YTS and NK92 were used as model NK cell systems. YTS cells were retrovirally transduced with a GFP-α-tubulin or a GFP-KIR2DL1 expression construct and sorted by flow cytometry for uniform stable expression as described (Banerjee et al., 2007). The NK92 GFP-α-Tubulin-expressing cell line was the kind gift of Dr. Kerry Campbell (Fox Chase Cancer Center). 721.221 EBV-transformed B-lymphoblastoid cells and K562 erythroleukemia cells were used as susceptible target cells for YTS and NK92 cells, respectively, and K562 cells were used as a non-susceptible target cells for YTS cells. 721.221 cells expressing HLA-Cw3 or HLA-Cw4 were used as susceptible or non-susceptible target cells for YTS GFP-KIR2DL1, respectively. Cell lines were maintained as previously described (Banerjee et al., 2007).

Whole blood PBMCs were obtained from volunteer donors and LAD-1 patient donors using centrifugation through Ficoll-Paque Plus lymphocyte isolation medium (Amersham Biosciences). For chapters 2 and 3, *ex vivo* NK (eNK) cells were prepared from PBMCs by negative depletion using the human NK cell isolation kit (Miltenyi Biotec) or the RosetteSep human NK cell isolation reagent (StemCell Technologies, Inc.) and were used for experiments immediately after preparation. K562 cells were used as susceptible target cells for eNK cells. All human samples were obtained after informed donor consent and were used with approval of the Institutional Internal Review Board for the Protection of Human Subjects of the Children's Hospital of Philadelphia and/or National Institutes of Allergy and Infectious Diseases.

LIVE CELL CONFOCAL MICROSCOPY

For MTOC polarization studies and lytic granule convergence studies, GFP-tubulin-expressing cells were suspended in media at a concentration of $3x10^6$ cells/mL, incubated with 5 μM LysoTracker Red DND-99 (Molecular Probes) for 30 min at 37°C, washed, and resuspended in media at $3x10^6$ cells/mL. 721.221 or K562 cells were suspended in media at a concentration of

$4x10^5$ cells/mL and adhered for 30 min at 37°C to ΔT dishes (Bioptechs) pre-coated with antibodies against CD48 (BD) or CD58 (BD) respectively, after which NK cells were added. Conjugates were imaged in a single *z*-axis plane using either an Olympus IX-81 DSU spinning disk confocal microscope outfitted with a Hamamatsu EM-CCD, C9100-02 camera and Olympus PlanApo N oil immersion, 60x, 1.45 NA objective or a Zeiss Axio Observer Z1 outfitted with Yokogawa CSU10 spinning disc, Hammamatsu Orca-AG camera, and Zeiss 63x 1.43 NA oil immersion objective every 10-15 s over 10-40 min. A major difference between the two systems was that the Olympus system used an Olympus halogen illumination system with narrow bandpass filters (Chroma), whereas the Zeiss sytem utilized solid state 488nm (Melles Griot) and 561nm (Crystal) lasers managed through a LMM5 laser combiner unit (Spectral Imaging).

In experiments without target cells, for lytic granule convergence studies, LysoTracker-loaded NK cells were added to ΔT dishes pre-coated with antibodies against CD28 (BD), CD11a (clone TS1/22, ATCC (Sanchez-Madrid et al., 1982)), CD45 (BD), or NKp30 (Beckman-Coulter) and imaged in a single *z*-axis plane at intervals ranging from 1-10 s. In IL-2 experiments, ΔT dishes were uncoated and IL-2 (NIH AIDS reagents program) was added 2-4min after imaging began. Zero minutes represents the time at which IL-2 was added. In IL-10 experiments, IL-10 (AbD Serotec) was added at time zero. In experiments evaluating the role of LFA-1, a previously described murine IgG1 anti-CD11a monoclonal antibody (TS1/22, ATCC), or murine IgG1 control monoclonal antibody (clone MOPC21, BD) were added to NK cells (22.5μg/ml final concentration) prior to their addition to target cells. The TS1/22 was used as a highly purified IgG derived from hybridoma culture supernatant. In all live cell experiments temperature was maintained at 37°C using ΔT dish and objective environmental control units (Bioptechs).

FIXED CELL MICROSCOPY

YTS cells alone, or conjugated to 721.221 target cells at a ratio of 2:1 for 0 min and 25 min were adhered to poly-L-lysine-coated glass slides (PolyPrep; Sigma-Aldrich) for 5 min at 37°C. Healthy or LAD-1 eNK cells conjugated to K562 cells at a ratio of 2:1 for 20 minutes were

adhered to poly-L-lysine-coated glass slides for 15 minutes at 37°C. YTS cells resting or treated with IL-2 for 15 min or eNK cells resting or treated with IL-2 for 5, 10 or 25 min were adhered to poly-L-lysine-coated glass slides for 5 min at 37°C. Fixing, permeabilization, and staining were performed as described (Banerjee et al., 2007), except the following reagents were used in the specified sequence: 1) polyclonal rabbit anti-dynein heavy chain (Santa Cruz, Figure 2.15 only), or anti-pericentrin (rabbit, Abcam, Figure 2.2A only), or pre-immune rabbit IgG (Sigma-Aldrich); 2) AlexaFluor 568-conjugated goat-anti-rabbit (Invitrogen, Figure 2.15 only), or AlexaFluor 647-conjugated goat-anti-rabbit (Invitrogen, Figure 2.2A only); 3) biotinylated monoclonal mouse anti-tubulin (Molecular Probes) or biotinylated mouse IgG control (BD); 4) Streptavidin-Pacific Blue (Molecular Probes); 5) FITC-conjugated mouse anti-perforin clone ΔG9 or FITC-conjugated mouse IgG control (BD) and 647-conjugated Phalloidin (Molecular Probes, Figure 3.1 only). Slides were covered with 0.15 mm coverslips (VWR Scientific) using ProLong Gold or ProLong AntiFade mounting medium (Invitrogen). Imaging was acquired using the same microscope, camera, and objective as in live cell imaging.

IMAGE ANALYSIS

Image sequences were analyzed using Volocity software (Improvision), and images for presentation were contrast enhanced uniformly using Adobe Photoshop. For analysis, unadjusted images were evaluated in Volocity for fluorescence corresponding to the MTOC (GFP maximal intensity), or lytic granules (LysoTracker Red maximal intensity) selected based on 2-5 SD above mean intensity. In time-lapse experiments, 1 image per minute was utilized for quantitative analysis while in streaming video experiments, all were used. To measure MTOC polarization, a line connecting the MTOC to the center of the IS was obtained over time using Volocity software. To measure granule convergence to the MTOC, *x* and *y* coordinates of the MTOC and all lytic granule regions in the plane of the MTOC were obtained. The length of the shortest line connecting the MTOC and each granule region was calculated as if it were the hypotenuse of the triangular region defined by the individual object coordinates, thus representing the MTOC to

granule distance (MGD). The MGD was determined for each lytic granule region present in an individual image from a single time point and mean MGD determined. Thus, the MGD was calculated using the equation below in which x and y were the coordinates of the MTOC centroid and x_i and y_i were the coordinates of the centroid of an individual lytic granule region.

$$MGD = \frac{\left(\sum_{i=1}^{n} \sqrt{(x - x_i)^2 + (y - y_i)^2} \right)}{n}$$

Where specified, the mean MGD over all time points recorded was averaged to provide an estimate of the distances throughout the entire observation of the cell. In experiments where cells did not contain a GFP-tubulin construct (Figures 2.16, 3.5) the *x,y* coordinates of the centroid of the entire region outlined by all lytic granules within the cell was determined and used instead of the MTOC to provide a measure of proximity of lytic granule regions.

To account for any differences in the area of individual lytic granule regions in a single *z*-axis plane, which could bias the MGD away from the actual mean distance of all lytic granules from the MTOC, a modified use of *Shepard's Method* was applied (Shepard, D., "A two-dimensional interpolation function for irregularly-spaced data." Proceedings of the 1968 ACM National Conference. pp. 517-524. 1968). This was considered important as clusters of lytic granules could inappropriately be discerned as an individual granule, therefore inappropriately considering the number of granules at a particular distance from the MTOC. This modification allowed weighting of the distances by the area of the lytic granule regions and was noted area-weighted distance (AWD). The equation for AWD is below in which A_i = the area of the lytic granule region being measured, A = the total lytic granule area in that particular image, and MGD_i = the MGD for the lytic granule region A_i.

$$AWD = \sum_{i=1}^{n} MGD_i * \left[\frac{A_i}{\sum_{i=1}^{n} A} \right]$$

Because the lytic granule region weighted area was normalized to the total present in each plane, measured AWD values could be compared across time points and evaluated irrespective of the total area of granules that may be present in the particular confocal plane.

To ensure that the granule regions in the plane of the MTOC were representative of the distance of all of the granules throughout the volume of the cell the, AWD calculation was performed using granule volume and 3 dimensional reconstructions of cells. Here the z-axis measurements of granule regions from the MTOC were included in the MGD calculation by adding $(z-z_i)^2$ to the x and y coordinates to allow localization of the granule regions relative to the MTOC throughout the reconstruction. Mean AWD values obtained in the plane of the MTOC vs. the volume weighted distances throughout 3-dimensions were similar (data not shown).

In streaming video, Volocity 'Track objects' function was used to identify individual lytic granules over time and define the velocity, displacement, and displacement rate of their tracks. The total velocity of the lytic granule track was calculated using the initial and final position of the lytic granule relative to the MTOC and instantaneous velocity was calculated as the slope of the lytic granule track over the continuous minus-end-directed portion of a lytic granule track. To calculate instantaneous velocity, minus-ended movement was arbitrarily defined as at least 1 μm and was considered to have stopped when three consecutive time points showed no MTOC-directed movement.

Fixed cell images were analyzed for colocalization of dynein and perforin (Figure 2.15). In three individual experiments, 5-20 images of each condition were acquired and from these 37-64 cells in which the MTOC, perforin, and dynein was present were identified and colocalization coefficients determined using Volocity software.

^{51}CR RELEASE CYTOTOXICITY ASSAYS

Cytolytic activity was measured by ^{51}Cr release assays as described (Orange *et al*., 2002), either between YTS and 721.221 cells, NK92 and K562 cells, YTS and K562 cells (Figure

2.1), YTS GFP-tubulin cells plus specific inhibitors and 721.221 cells (Figure 3.3), or YTS GFP-KIR2DL1 cells and 721.221 HLA-Cw3 or HLA-Cw4 cells (Figure 3.5).

INHIBITORS AND CYTOKINES

Where applicable, YTS cells were pre-incubated with 10μM Paclitaxel (Sigma-Aldrich), 10μM Nocodazole (Sigma-Aldrich), 5μM Cytochalasin D (MP Biomedicals, Inc.), 10μM Latrunculin A (Sigma-Aldrich), 25μM PP2 (Sigma-Aldrich), 20μM LY294002 (Sigma-Aldrich), 100nM Wortmannin (Sigma-Aldrich), 100μM PD98059 (Sigma-Aldrich), 4μM U73122 (Sigma-Aldrich) or 1μM ZM449829 (Tocris Bioscience) for 30 min and then washed. Imaging of inhibitor pre-treated cells was performed in the presence of inhibitors.

Where specified, soluble IL-2 (NIH AIDS reagents program) was added prediluted in media at a concentration of 125U/mL to YTS cells or 1000U/mL to eNK cells, only after the time indicated. For Figure 2.14, 100ng/mL soluble IL-10 (AbD Serotec) prediluted in media was added to YTS cells at time zero.

NUCLEOFECTION ASSAYS

Isolated eNK cells were nucleofected with the EGFP-tubulin expressing vector using an Amaxa Nucleofector; program O-017, and the human cell reagent (Amaxa). Nucleofected cells were incubated in RPMI media supplemented with 20% FCS overnight before use. GFP-fused p50 dynamitin or CC1 subunit of $p150^{Glued}$ constructs in pEGFP-N1 vector (LaMonte et al., 2002; Ligon et al., 2003) were nucleofected into $1x10^7$ YTS cells using program O-017 with reagent R after which cells were cultured for 24 hours.

LYTIC GRANULE ISOLATION AND ANALYSIS

Lytic granules were isolated from disrupted YTS cells and used for either mass spectrometry or Western blot analysis, performed as previously described (Sanborn et al., 2009).

Western blot analysis using lytic granules and other cellular fractions for identification of dynein complex members was performed as described elsewhere (LaMonte et al., 2002).

STATISTICAL ANALYSIS

The minimum number of cells evaluated was determined using sample size calculations based upon preliminary data, with α and β error levels of 1%. Differences over time between non-activated and activated NK cells, inhibitor-treated and DMSO-treated NK cells, or between resting and IL-2-treated NK cells were evaluated using unpaired Mann-Whitney U tests. Individual point means were compared using unpaired two-tailed Student's t test. Differences were considered significant when $p \leq 0.05$.

APPENDIX 1 – VIDEO LEGENDS

Video 1A: MTOC polarization in a YTS GFP-tubulin cell conjugated to a susceptible target cell.

Time-lapse movie of MTOC polarization in a YTS GFP-tubulin cell (right) conjugated to a susceptible 721.221 cell (middle, outlined; Figure 2.2B) showing differential interference contrast (top) and fluorescent acquisition sequences (bottom). The 721.221 target cell to which the YTS GFP-tubulin cell (right) is conjugated, is outlined with white dashes every few frames in the fluorescent imaging sequence to facilitate orientation recognition. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity as defined in the text. Images were obtained at a rate of 4 frames per minute and at least one image per minute is shown from T = 4 min to T = 24 min.

Video 1B: MTOC polarization in an NK92 GFP-tubulin cell conjugated to a susceptible target cell.

Time-lapse movie of MTOC polarization in an NK92 GFP-tubulin cell (top) conjugated to a susceptible K562 cell (bottom; Figure 2.2C). Differential interference contrast imaging is shown on top and fluorescence on the bottom. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity as defined in the text. In selected frames, the target cell is outlined with a dashed line. In some frames the NK-92 cell is extending superior to the target and thus the dashes are discontinuous. Images were obtained at a rate of 4 frames per minute and at least one image per 90 s is shown from T = 1 min to T = 24 min.

Video 1C: MTOC positioning in a YTS cell conjugated to a non-susceptible target cell.

Time-lapse movie of MTOC dynamics in a YTS GFP-tubulin cell (left) conjugated to a non-susceptible K562 cell (right; Figure 2.2D) showing differential interference contrast (top) and fluorescent acquisition sequences (bottom). Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity as defined in the text. Images were obtained at a rate of 4 frames per minute and at least one image per two minutes is shown from T = 0 min to T = 34 min.

Video 2: Lytic granule movement relative to the MTOC in YTS cells exposed to immobilized antibodies.

Time-lapse movies of lytic granule dynamics in a YTS GFP-tubulin cell on an anti-CD28- (left), anti-CD11a- (middle), or anti-CD45-coated (right) imaging chambers (Figure 2.8). Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity as defined in the text. Images were obtained at a rate of 6 frames per minute and at least one image per 90 s is shown from T = 0 min to T = 6, 9 or 15 min.

Video 3A: Lytic granule dynamics in a resting YTS cell.

Streaming video of a resting YTS GFP-tubulin cell in an uncoated imaging chamber. Red fluorescence represents LysoTracker-loaded acidified lysosomes. Images were obtained at a streaming rate of 4 frames per second and the video is shown at 5 times real time.

Video 3B: Lytic granule dynamics in a resting NK92 cell.

Streaming video of a resting NK92 GFP-tubulin cell in an uncoated imaging chamber. Red fluorescence represents LysoTracker-loaded acidified lysosomes. Images were obtained at a rate of 4 frames per second and the video is shown at 5 times real time.

Video 3C: Lytic granule dynamics in an activated YTS cell.

Streaming video of an activated YTS GFP-tubulin cell in an anti-CD28- and anti-CD11a-coated imaging chamber. Red fluorescence represents LysoTracker-loaded acidified lysosomes. Images were obtained at a rate of 4 frames per second and the video is shown at 10 times real time.

Video 3D: Lytic granule dynamics in an activated NK92 cell.

Streaming video of an activated NK92 GFP-tubulin cell in an anti-NKp30- and anti-CD11a-coated imaging chamber. Red fluorescence represents LysoTracker-loaded acidified lysosomes. Images were obtained at a rate of 4 frames per second and the video is shown at 5 times real time.

Video 4: MTOC and lytic granule dynamics in an ex vivo NK cell conjugating with a susceptible target cell.

Time-lapse movie of an eNK cell nucleofected with GFP-tubulin (small cell) shown during conjugation with a susceptible K562 target cell (large cell; Figure 2.11). Differential interference contrast imaging is shown on top and fluorescence on the bottom. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity as defined in the text. Images were obtained at a rate of 4 frames per minute and and at least one image per 90 s is shown from T = 0 min to T = 23 min.

Video 5A: Effect of Taxol treatment on MTOC dynamics and lytic granule convergence.

Time-lapse movie of a YTS GFP-tubulin cell pre-treated with Taxol in conjugation with a susceptible 721.221 target cell (Figure 2.12A-C). Differential interference contrast imaging is shown on top and fluorescence on the bottom. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity as defined in the text. Images were obtained at a rate of 4 frames per minute and at least two images per minute are shown from T = 0 min to T = 12.5 min. Taxol was present in the imaging chamber media throughout the duration of the experiment.

Video 5B: Effect of Cytochalasin D treatment on MTOC dynamics and lytic granule convergence.

Time-lapse movie of a YTS GFP-tubulin cell pre-treated with Cytochalasin D in conjugation with a susceptible 721.221 target cell (Figure 2.12D-F). Differential interference contrast imaging is shown on top and fluorescence on the bottom. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity as defined in the text. Images were obtained at a rate of 6 frames per minute and at least one image per two minutes is shown from T = 0 min to T = 8.5 min. Cytochalasin D was present in the imaging chamber media throughout the duration of the experiment.

Video 6: Effect of p50 dynamitin, or $p150^{Glued}$ dynactin first coiled-coil domain (CC1) overexpression on lytic granule dynamics relative to a granule centroid.

Time-lapse movie of a YTS cell nucleofected with p50-GFP (left), or with CC1-GFP (right) in conjugation with a susceptible 721.221 target cell (Figure 2.16). Differential interference contrast imaging is shown on top and fluorescence on the bottom. Green fluorescence represents the p50-GFP, or with CC1-GFP fusion

protein and red LysoTracker-loaded acidified lysosomes. Images were obtained at a rate of 6 frames per minute and at least one image per minute is shown from T = 0 min to T = 15 min (left) or one image per four minutes from T = 0 min to T = 22 min (right).

Video 7A: Lytic granule behavior in control DMSO-treated NK cells after CD28 or CD11a triggering.

Time-lapse video of lytic granule dynamics in a DMSO-treated YTS GFP-tubulin cell on anti-CD28- (left) or anti-CD11a- (right) coated imaging chambers (Figure 3.2A). Green fluorescence demonstrates GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC was defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 15min.

Video 7B: Lytic granule behavior in PP2-treated NK cells after CD28 or CD11a triggering.

Time-lapse video of lytic granule dynamics in a PP2-treated YTS GFP-tubulin cell on anti-CD28- (left) or anti-CD11a- (right) coated imaging chambers (Figure 3.2B,C). Green fluorescence represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 15min.

Video 8A: Lytic granule behavior in LY294002-treated NK cells after CD28 or CD11a triggering.

Time-lapse video of lytic granule dynamics in a LY294002-treated YTS GFP-tubulin cell on anti-CD28- (left) or anti-CD11a- (right) coated imaging chambers (Figure 3.3A). Green fluorescence represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 15min.

Video 8B: Lytic granule behavior in Wortmannin-treated NK cells after CD28 or CD11a triggering.

Time-lapse video of lytic granule dynamics in a Wortmannin-treated YTS GFP-tubulin cell on anti-CD28- (left) or anti-CD11a- (right) coated imaging chambers (Figure 3.3, not shown). Green fluorescence

represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 15min.

Video 8C: Lytic granule behavior in PD98059-treated NK cells after CD28 or CD11a triggering.

Time-lapse video of lytic granule dynamics in a PD98059-treated YTS GFP-tubulin cell on anti-CD28- (left) or anti-CD11a- (right) coated imaging chambers (Figure 3.3B). Green fluorescence represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 15min.

Video 8D: Lytic granule behavior in U73122-treated NK cells after CD28 or CD11a triggering.

Time-lapse video of lytic granule dynamics in a U73122-treated YTS GFP-tubulin cell on anti-CD28- (left) or anti-CD11a- (right) coated imaging chambers (Figure 3.3C). Green fluorescence represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 15 or 13min.

Video 9A: Lytic granule dynamics relative to a granule centroid in a YTS GFP-KIR2DL1 cell conjugated to a non-cognate KIR ligand-expressing target cell.

Time-lapse movie of lytic granule dynamics in a YTS GFP-KIR2DL1 cell (top) conjugated to a susceptible 721 HLA-Cw3 cell (bottom; Figure 3.5B). Green fluorescence represents GFP-KIR2DL1 and red LysoTracker-loaded acidified lysosomes. Images were obtained at a rate of 6 frames per minute and at least one image per 60 s is shown from T = 0min to 17min.

Video 9B: Lytic granule dynamics relative to a granule centroid in a YTS GFP-KIR2DL1 cell conjugated to a cognate KIR ligand-expressing target cell.

Time-lapse movie of lytic granule dynamics in a YTS GFP-KIR2DL1 cell (top) conjugated to a non-susceptible 721 HLA-Cw4 cell (bottom; Figure 3.5C). Green fluorescence represents GFP-KIR2DL1 and

red, LysoTracker-loaded acidified lysosomes. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 17min.

Video 10A: NK cell lytic granule movement after IL-2 addition.

Time-lapse video of lytic granule dynamics in a resting YTS GFP-tubulin cell on a plain imaging chamber (Figure 3.7A) with IL-2 added at 4 min. Green fluorescence represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 15min.

Video 10B: NK cell lytic granule movement after IL-10 addition.

Time-lapse video of lytic granule dynamics in a resting YTS GFP-tubulin cell on a plain imaging chamber (Figure 3.7, not shown) with IL-10 added at 0min. Green fluorescence represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 17min.

Video 10C: Lytic granule movement in a ZM449829-treated NK cell after IL-2 addition.

Time-lapse video of lytic granule dynamics in a ZM449829-treated YTS GFP-tubulin cell on a plain imaging chamber (Figure 3.7B) with IL-2 added at 2min. Green fluorescence represents GFP-tubulin and red LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 10min.

Video 10D: Lytic granule movement in a PP2-treated NK cell after IL-2 addition.

Time-lapse video of lytic granule dynamics in a PP2-treated YTS GFP-tubulin cell on a plain imaging chamber (Figure 3.7C) with IL-2 added at 4min. Green fluorescence represents GFP-tubulin and red, LysoTracker-loaded acidified lysosomes. The MTOC is defined as the focus of GFP intensity. Images were obtained at a rate of 6 frames per minute and at least one image per 60s is shown from T = 0min to 20min.

REFERENCES

Anderson, D.C., and Springer, T.A. (1987). Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu Rev Med *38*, 175-194.

Andzelm, M.M., Chen, X., Krzewski, K., Orange, J.S., and Strominger, J.L. (2007). Myosin IIA is required for cytolytic granule exocytosis in human NK cells. J Exp Med *204*, 2285-2291.

Ardouin, L., Bracke, M., Mathiot, A., Pagakis, S.N., Norton, T., Hogg, N., and Tybulewicz, V.L. (2003). Vav1 transduces TCR signals required for LFA-1 function and cell polarization at the immunological synapse. Eur J Immunol *33*, 790-797.

Arnon, T.I., Markel, G., and Mandelboim, O. (2006). Tumor and viral recognition by natural killer cells receptors. Semin Cancer Biol *16*, 348-358.

Augugliaro, R., Parolini, S., Castriconi, R., Marcenaro, E., Cantoni, C., Nanni, M., Moretta, L., Moretta, A., and Bottino, C. (2003). Selective cross-talk among natural cytotoxicity receptors in human natural killer cells. Eur J Immunol *33*, 1235-1241.

Banerjee, P.P., Pandey, R., Zheng, R., Suhoski, M.M., Monaco-Shawver, L., and Orange, J.S. (2007). Cdc42-interacting protein-4 functionally links actin and microtubule networks at the cytolytic NK cell immunological synapse. J Exp Med *204*, 2305-2320.

Barber, D.F., Faure, M., and Long, E.O. (2004). LFA-1 contributes an early signal for NK cell cytotoxicity. J Immunol *173*, 3653-3659.

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

Beal, A.M., Anikeeva, N., Varma, R., Cameron, T.O., Vasiliver-Shamis, G., Norris, P.J., Dustin, M.L., and Sykulev, Y. (2009). Kinetics of early T cell receptor signaling regulate the pathway of lytic granule delivery to the secretory domain. Immunity *31*, 632-642.

Beider, K., Nagler, A., Wald, O., Franitza, S., Dagan-Berger, M., Wald, H., Giladi, H., Brocke, S., Hanna, J., Mandelboim, O.*, et al.* (2003). Involvement of CXCR4 and IL-2 in the homing and retention of human NK and NK T cells to the bone marrow and spleen of NOD/SCID mice. Blood *102*, 1951-1958.

Berthou, C., Bourge, J.F., Zhang, Y., Soulie, A., Geromin, D., Denizot, Y., Sigaux, F., and Sasportes, M. (2000). Interferon-gamma-induced membrane PAF-receptor expression confers tumor cell susceptibility to NK perforin-dependent lysis. Blood *95*, 2329-2336.

Billadeau, D.D., Upshaw, J.L., Schoon, R.A., Dick, C.J., and Leibson, P.J. (2003). NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. Nat Immunol *4*, 557-564.

Bishara, A., De Santis, D., Witt, C.C., Brautbar, C., Christiansen, F.T., Or, R., Nagler, A., and Slavin, S. (2004). The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. Tissue Antigens *63*, 204-211.

Bjorkstrom, N.K., Gonzalez, V.D., Malmberg, K.J., Falconer, K., Alaeus, A., Nowak, G., Jorns, C., Ericzon, B.G., Weiland, O., Sandberg, J.K., and Ljunggren, H.G. (2008). Elevated numbers of Fc

gamma RIIIA+ (CD16+) effector CD8 T cells with NK cell-like function in chronic hepatitis C virus infection. J Immunol *181*, 4219-4228.

Blocker, A., Severin, F.F., Burkhardt, J.K., Bingham, J.B., Yu, H., Olivo, J.C., Schroer, T.A., Hyman, A.A., and Griffiths, G. (1997). Molecular requirements for bi-directional movement of phagosomes along microtubules. J Cell Biol *137*, 113-129.

Bonnema, J.D., Rivlin, K.A., Ting, A.T., Schoon, R.A., Abraham, R.T., and Leibson, P.J. (1994). Cytokine-enhanced NK cell-mediated cytotoxicity. Positive modulatory effects of IL-2 and IL-12 on stimulus-dependent granule exocytosis. J Immunol *152*, 2098-2104.

Brandt, C.S., Baratin, M., Yi, E.C., Kennedy, J., Gao, Z., Fox, B., Haldeman, B., Ostrander, C.D., Kaifu, T., Chabannon, C.*, et al.* (2009). The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J Exp Med *206*, 1495-1503.

Brown, A.C., Oddos, S., Dobbie, I.M., Alakoskela, J.M., Parton, R.M., Eissmann, P., Neil, M.A., Dunsby, C., French, P.M., Davis, I., and Davis, D.M. (2011). Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. PLoS Biol *9*, e1001152.

Bryceson, Y.T., March, M.E., Barber, D.F., Ljunggren, H.G., and Long, E.O. (2005). Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. J Exp Med *202*, 1001-1012.

Bryceson, Y.T., March, M.E., Ljunggren, H.G., and Long, E.O. (2006a). Activation, coactivation, and costimulation of resting human natural killer cells. Immunol Rev *214*, 73-91.

Bryceson, Y.T., March, M.E., Ljunggren, H.G., and Long, E.O. (2006b). Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood *107*, 159-166.

Bryceson, Y.T., Rudd, E., Zheng, C., Edner, J., Ma, D., Wood, S.M., Bechensteen, A.G., Boelens, J.J., Celkan, T., Farah, R.A.*, et al.* (2007). Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. Blood *110*, 1906-1915.

Burakov, A., Nadezhdina, E., Slepchenko, B., and Rodionov, V. (2003). Centrosome positioning in interphase cells. J Cell Biol *162*, 963-969.

Burkhardt, J.K., Echeverri, C.J., Nilsson, T., and Vallee, R.B. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J Cell Biol *139*, 469-484.

Burkhardt, J.K., McIlvain, J.M., Jr., Sheetz, M.P., and Argon, Y. (1993). Lytic granules from cytotoxic T cells exhibit kinesin-dependent motility on microtubules in vitro. J Cell Sci *104 (Pt 1)*, 151-162.

Burshtyn, D.N., Shin, J., Stebbins, C., and Long, E.O. (2000). Adhesion to target cells is disrupted by the killer cell inhibitory receptor. Curr Biol *10*, 777-780.

Butler, B., and Cooper, J.A. (2009). Distinct roles for the actin nucleators Arp2/3 and hDia1 during NK-mediated cytotoxicity. Curr Biol *19*, 1886-1896.

Butler, B., Kastendieck, D.H., and Cooper, J.A. (2008). Differently phosphorylated forms of the cortactin homolog HS1 mediate distinct functions in natural killer cells. Nat Immunol *9*, 887-897.

Byers, H.R., Yaar, M., Eller, M.S., Jalbert, N.L., and Gilchrest, B.A. (2000). Role of cytoplasmic dynein in melanosome transport in human melanocytes. J Invest Dermatol *114*, 990-997.

Carlier, M.F., Nioche, P., Broutin-L'Hermite, I., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P., and Pantaloni, D. (2000). GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASp) with actin-related protein (ARP2/3) complex. J Biol Chem *275*, 21946-21952.

Carminati, J.L., and Stearns, T. (1997). Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. J Cell Biol *138*, 629-641.

Carpen, O., Virtanen, I., Lehto, V.P., and Saksela, E. (1983). Polarization of NK cell cytoskeleton upon conjugation with sensitive target cells. J Immunol *131*, 2695-2698.

Chen, X., Allan, D.S., Krzewski, K., Ge, B., Kopcow, H., and Strominger, J.L. (2006). CD28-stimulated ERK2 phosphorylation is required for polarization of the microtubule organizing center and granules in YTS NK cells. Proc Natl Acad Sci U S A *103*, 10346-10351.

Chen, X., Trivedi, P.P., Ge, B., Krzewski, K., and Strominger, J.L. (2007). Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. Proc Natl Acad Sci U S A *104*, 6329-6334.

Chini, C.C., and Leibson, P.J. (2001). Signal transduction during natural killer cell activation. Curr Protoc Immunol *Chapter 11*, Unit 11.9B.

Combs, J., Kim, S.J., Tan, S., Ligon, L.A., Holzbaur, E.L., Kuhn, J., and Poenie, M. (2006). Recruitment of dynein to the Jurkat immunological synapse. Proc Natl Acad Sci U S A *103*, 14883-14888.

Cooper, M.A., Elliott, J.M., Keyel, P.A., Yang, L., Carrero, J.A., and Yokoyama, W.M. (2009). Cytokine-induced memory-like natural killer cells. Proc Natl Acad Sci U S A *106*, 1915-1919.

Cooper, M.A., and Yokoyama, W.M. (2010). Memory-like responses of natural killer cells. Immunol Rev *235*, 297-305.

Cosman, D., Mullberg, J., Sutherland, C.L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N.J. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity *14*, 123-133.

Criado, M., Lindstrom, J.M., Anderson, C.G., and Dennert, G. (1985). Cytotoxic granules from killer cells: specificity of granules and insertion of channels of defined size into target membranes. J Immunol *135*, 4245-4251.

Cuevas, B., Lu, Y., Watt, S., Kumar, R., Zhang, J., Siminovitch, K.A., and Mills, G.B. (1999). SHP-1 regulates Lck-induced phosphatidylinositol 3-kinase phosphorylation and activity. J Biol Chem *274*, 27583-27589.

Cussac, D., Vidal, M., Leprince, C., Liu, W.Q., Cornille, F., Tiraboschi, G., Roques, B.P., and Garbay, C. (1999). A Sos-derived peptidimer blocks the Ras signaling pathway by binding both Grb2 SH3 domains and displays antiproliferative activity. FASEB J *13*, 31-38.

Dabrazhynetskaya, A., Ma, J., Guerreiro-Cacais, A.O., Arany, Z., Rudd, E., Henter, J.I., Karre, K., Levitskaya, J., and Levitsky, V. (2011). Syntaxin 11 marks a distinct intracellular compartment recruited to the immunological synapse of NK cells to colocalize with cytotoxic granules. J Cell Mol Med *16*, 129-141.

Davis, D.M., Chiu, I., Fassett, M., Cohen, G.B., Mandelboim, O., and Strominger, J.L. (1999). The human natural killer cell immune synapse. Proc Natl Acad Sci U S A *96*, 15062-15067.

Davis, D.M., and Dustin, M.L. (2004). What is the importance of the immunological synapse? Trends Immunol *25*, 323-327.

Deacon, S.W., Serpinskaya, A.S., Vaughan, P.S., Lopez Fanarraga, M., Vernos, I., Vaughan, K.T., and Gelfand, V.I. (2003). Dynactin is required for bidirectional organelle transport. J Cell Biol *160*, 297-301.

Eischen, C.M., and Leibson, P.J. (1997). Role for NK-cell-associated Fas ligand in cell-mediated cytotoxicity and apoptosis. Res Immunol *148*, 164-169.

Endres, N.F., Yoshioka, C., Milligan, R.A., and Vale, R.D. (2006). A lever-arm rotation drives motility of the minus-end-directed kinesin Ncd. Nature *439*, 875-878.

Endt, J., McCann, F.E., Almeida, C.R., Urlaub, D., Leung, R., Pende, D., Davis, D.M., and Watzl, C. (2007). Inhibitory receptor signals suppress ligation-induced recruitment of NKG2D to GM1-rich membrane domains at the human NK cell immune synapse. J Immunol *178*, 5606-5611.

Eng, E.W., Bettio, A., Ibrahim, J., and Harrison, R.E. (2007). MTOC reorientation occurs during FcgammaR-mediated phagocytosis in macrophages. Mol Biol Cell *18*, 2389-2399.

Eschbach, J., and Dupuis, L. (2011). Cytoplasmic dynein in neurodegeneration. Pharmacol Ther *130*, 348-363.

Esin, S., Batoni, G., Counoupas, C., Stringaro, A., Brancatisano, F.L., Colone, M., Maisetta, G., Florio, W., Arancia, G., and Campa, M. (2008). Direct binding of human NK cell natural cytotoxicity receptor NKp44 to the surfaces of mycobacteria and other bacteria. Infect Immun *76*, 1719-1727.

Farag, S.S., and Caligiuri, M.A. (2004). Cytokine modulation of the innate immune system in the treatment of leukemia and lymphoma. Adv Pharmacol *51*, 295-318.

Farshori, P., and Holzbaur, E.L. (1997). Dynactin phosphorylation is modulated in response to cellular effectors. Biochem Biophys Res Commun *232*, 810-816.

Faure, M., Barber, D.F., Takahashi, S.M., Jin, T., and Long, E.O. (2003). Spontaneous clustering and tyrosine phosphorylation of NK cell inhibitory receptor induced by ligand binding. J Immunol *170*, 6107-6114.

Fauriat, C., Long, E.O., Ljunggren, H.G., and Bryceson, Y.T. (2010). Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood *115*, 2167-2176.

Fehniger, T.A., Cooper, M.A., Nuovo, G.J., Cella, M., Facchetti, F., Colonna, M., and Caligiuri, M.A. (2003). CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood *101*, 3052-3057.

Feldmann, J., Callebaut, I., Raposo, G., Certain, S., Bacq, D., Dumont, C., Lambert, N., Ouachee-Chardin, M., Chedeville, G., Tamary, H.*, et al.* (2003). Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell *115*, 461-473.

Gadina, M., Sudarshan, C., and O'Shea, J.J. (1999). IL-2, but not IL-4 and other cytokines, induces phosphorylation of a 98-kDa protein associated with SHP-2, phosphatidylinositol 3'-kinase, and Grb2. J Immunol *162*, 2081-2086.

Galandrini, R., Palmieri, G., Piccoli, M., Frati, L., and Santoni, A. (1996). CD16-mediated p21ras activation is associated with Shc and p36 tyrosine phosphorylation and their binding with Grb2 in human natural killer cells. J Exp Med *183*, 179-186.

Gershenfeld, H.K., Hershberger, R.J., Mueller, C., and Weissman, I.L. (1988). A T cell- and natural killer cell-specific, trypsin-like serine protease. Implications of a cytolytic cascade. Ann N Y Acad Sci *532*, 367-379.

Gill, S.R., Schroer, T.A., Szilak, I., Steuer, E.R., Sheetz, M.P., and Cleveland, D.W. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. J Cell Biol *115*, 1639-1650.

Gismondi, A., Jacobelli, J., Mainiero, F., Paolini, R., Piccoli, M., Frati, L., and Santoni, A. (2000). Cutting edge: functional role for proline-rich tyrosine kinase 2 in NK cell-mediated natural cytotoxicity. J Immunol *164*, 2272-2276.

Giurisato, E., Cella, M., Takai, T., Kurosaki, T., Feng, Y., Longmore, G.D., Colonna, M., and Shaw, A.S. (2007). Phosphatidylinositol 3-kinase activation is required to form the NKG2D immunological synapse. Mol Cell Biol *27*, 8583-8599.

Gomez, T.S., Kumar, K., Medeiros, R.B., Shimizu, Y., Leibson, P.J., and Billadeau, D.D. (2007). Formins regulate the actin-related protein 2/3 complex-independent polarization of the centrosome to the immunological synapse. Immunity *26*, 177-190.

Gotlieb, A.I., May, L.M., Subrahmanyan, L., and Kalnins, V.I. (1981). Distribution of microtubule organizing centers in migrating sheets of endothelial cells. J Cell Biol *91*, 589-594.

Graham, D.B., Cella, M., Giurisato, E., Fujikawa, K., Miletic, A.V., Kloeppel, T., Brim, K., Takai, T., Shaw, A.S., Colonna, M., and Swat, W. (2006). Vav1 controls DAP10-mediated natural cytotoxicity by regulating actin and microtubule dynamics. J Immunol *177*, 2349-2355.

Griffiths, G.M. (2002). Albinism and immunity: what's the link? Curr Mol Med *2*, 479-483.

Hamm-Alvarez, S.F., and Sheetz, M.P. (1998). Microtubule-dependent vesicle transport: modulation of channel and transporter activity in liver and kidney. Physiol Rev *78*, 1109-1129.

Hendricks, A.G., Lazarus, J.E., Perlson, E., Gardner, M.K., Odde, D.J., Goldman, Y.E., and Holzbaur, E.L. (2012). Dynein tethers and stabilizes dynamic microtubule plus ends. Curr Biol *22*, 632-637.

Hendricks, A.G., Perlson, E., Ross, J.L., Schroeder, H.W., 3rd, Tokito, M., and Holzbaur, E.L. (2010). Motor coordination via a tug-of-war mechanism drives bidirectional vesicle transport. Curr Biol *20*, 697-702.

Horiuchi, D., Collins, C.A., Bhat, P., Barkus, R.V., Diantonio, A., and Saxton, W.M. (2007). Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. Curr Biol *17*, 1313-1317.

Horowitz, A., Stegmann, K.A., and Riley, E.M. (2011). Activation of Natural Killer Cells during Microbial Infections. Front Immunol *2*, 88.

Jang, I.K., Zhang, J., and Gu, H. (2009). Grb2, a simple adapter with complex roles in lymphocyte development, function, and signaling. Immunol Rev *232*, 150-159.

Jenkins, M.R., Tsun, A., Stinchcombe, J.C., and Griffiths, G.M. (2009). The strength of T cell receptor signal controls the polarization of cytotoxic machinery to the immunological synapse. Immunity *31*, 621-631.

Jiang, K., Zhong, B., Gilvary, D.L., Corliss, B.C., Hong-Geller, E., Wei, S., and Djeu, J.Y. (2000). Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. Nat Immunol *1*, 419-425.

Johnston, J.A., Bacon, C.M., Finbloom, D.S., Rees, R.C., Kaplan, D., Shibuya, K., Ortaldo, J.R., Gupta, S., Chen, Y.Q., Giri, J.D., and et al. (1995). Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15. Proc Natl Acad Sci U S A *92*, 8705-8709.

Johnston, J.A., Bacon, C.M., Riedy, M.C., and O'Shea, J.J. (1996). Signaling by IL-2 and related cytokines: JAKs, STATs, and relationship to immunodeficiency. J Leukoc Biol *60*, 441-452.

Johnston, J.A., Kawamura, M., Kirken, R.A., Chen, Y.Q., Blake, T.B., Shibuya, K., Ortaldo, J.R., McVicar, D.W., and O'Shea, J.J. (1994). Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature *370*, 151-153.

Jones, G.J., Wiseman, J.C., Marr, K.J., Wei, S., Djeu, J.Y., and Mody, C.H. (2009). In contrast to anti-tumor activity, YT cell and primary NK cell cytotoxicity for Cryptococcus neoformans bypasses LFA-1. Int Immunol *21*, 423-432.

Katz, P., Zaytoun, A.M., and Lee, J.H., Jr. (1982). Mechanisms of human cell-mediated cytotoxicity. III. Dependence of natural killing on microtubule and microfilament integrity. J Immunol *129*, 2816-2825.

Kimura, N., Okabayashi, S., and Ono, F. (2012). Dynein dysfunction disrupts intracellular vesicle trafficking bidirectionally and perturbs synaptic vesicle docking via endocytic disturbances a potential mechanism underlying age-dependent impairment of cognitive function. Am J Pathol *180*, 550-561.

King, S.J., and Schroer, T.A. (2000). Dynactin increases the processivity of the cytoplasmic dynein motor. Nat Cell Biol *2*, 20-24.

Kishimoto, T.K., Hollander, N., Roberts, T.M., Anderson, D.C., and Springer, T.A. (1987). Heterogeneous mutations in the beta subunit common to the LFA-1, Mac-1, and p150,95 glycoproteins cause leukocyte adhesion deficiency. Cell *50*, 193-202.

Kohl, S., Springer, T.A., Schmalstieg, F.C., Loo, L.S., and Anderson, D.C. (1984). Defective natural killer cytotoxicity and polymorphonuclear leukocyte antibody-dependent cellular cytotoxicity in patients with LFA-1/OKM-1 deficiency. J Immunol *133*, 2972-2978.

Kohrt, H.E., Houot, R., Weiskopf, K., Goldstein, M.J., Scheeren, F., Czerwinski, D., Colevas, A.D., Weng, W.K., Clarke, M.F., Carlson, R.W.*, et al.* (2012). Stimulation of natural killer cells with a CD137-specific antibody enhances trastuzumab efficacy in xenotransplant models of breast cancer. J Clin Invest *122*, 1066-1075.

Koonce, M.P., Kohler, J., Neujahr, R., Schwartz, J.M., Tikhonenko, I., and Gerisch, G. (1999). Dynein motor regulation stabilizes interphase microtubule arrays and determines centrosome position. EMBO J *18*, 6786-6792.

Kraemer, J., Schmitz, F., and Drenckhahn, D. (1999). Cytoplasmic dynein and dynactin as likely candidates for microtubule-dependent apical targeting of pancreatic zymogen granules. Eur J Cell Biol *78*, 265-277.

Krummel, M.F., and Macara, I. (2006). Maintenance and modulation of T cell polarity. Nat Immunol *7*, 1143-1149.

Kuhn, J.R., and Poenie, M. (2002). Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. Immunity *16*, 111-121.

Kuhne, M.R., Lin, J., Yablonski, D., Mollenauer, M.N., Ehrlich, L.I., Huppa, J., Davis, M.M., and Weiss, A. (2003). Linker for activation of T cells, zeta-associated protein-70, and Src homology 2 domain-containing leukocyte protein-76 are required for TCR-induced microtubule-organizing center polarization. J Immunol *171*, 860-866.

Kumar, S., Lee, I.H., and Plamann, M. (2000). Cytoplasmic dynein ATPase activity is regulated by dynactin-dependent phosphorylation. J Biol Chem *275*, 31798-31804.

Kupfer, A., and Dennert, G. (1984). Reorientation of the microtubule-organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. J Immunol *133*, 2762-2766.

Kupfer, A., Dennert, G., and Singer, S.J. (1983). Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets. Proc Natl Acad Sci U S A *80*, 7224-7228.

Kupfer, A., Louvard, D., and Singer, S.J. (1982). Polarization of the Golgi apparatus and the microtubule-organizing center in cultured fibroblasts at the edge of an experimental wound. Proc Natl Acad Sci U S A *79*, 2603-2607.

Kural, C., Serpinskaya, A.S., Chou, Y.H., Goldman, R.D., Gelfand, V.I., and Selvin, P.R. (2007). Tracking melanosomes inside a cell to study molecular motors and their interaction. Proc Natl Acad Sci U S A *104*, 5378-5382.

Kurzer, J.H., Argetsinger, L.S., Zhou, Y.J., Kouadio, J.L., O'Shea, J.J., and Carter-Su, C. (2004). Tyrosine 813 is a site of JAK2 autophosphorylation critical for activation of JAK2 by SH2-B beta. Mol Cell Biol *24*, 4557-4570.

Laan, L., Pavin, N., Husson, J., Romet-Lemonne, G., van Duijn, M., Lopez, M.P., Vale, R.D., Julicher, F., Reck-Peterson, S.L., and Dogterom, M. (2012). Cortical dynein controls microtubule dynamics to generate pulling forces that position microtubule asters. Cell *148*, 502-514.

LaMonte, B.H., Wallace, K.E., Holloway, B.A., Shelly, S.S., Ascano, J., Tokito, M., Van Winkle, T., Howland, D.S., and Holzbaur, E.L. (2002). Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. Neuron *34*, 715-727.

Lanier, L.L. (2005). NK cell recognition. Annu Rev Immunol *23*, 225-274.

Lanier, L.L. (2008). Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol *9*, 495-502.

Lee, K.H., Dinner, A.R., Tu, C., Campi, G., Raychaudhuri, S., Varma, R., Sims, T.N., Burack, W.R., Wu, H., Wang, J.*, et al.* (2003). The immunological synapse balances T cell receptor signaling and degradation. Science *302*, 1218-1222.

Levi, V., Serpinskaya, A.S., Gratton, E., and Gelfand, V. (2006). Organelle transport along microtubules in Xenopus melanophores: evidence for cooperation between multiple motors. Biophys J *90*, 318-327.

Levy, J.R., and Holzbaur, E.L. (2008). Dynein drives nuclear rotation during forward progression of motile fibroblasts. J Cell Sci *121*, 3187-3195.

Li, C., Ge, B., Nicotra, M., Stern, J.N., Kopcow, H.D., Chen, X., and Strominger, J.L. (2008). JNK MAP kinase activation is required for MTOC and granule polarization in NKG2D-mediated NK cell cytotoxicity. Proc natl Acad Sci U S A *105*, 3017-3022.

Ligon, L.A., Shelly, S.S., Tokito, M., and Holzbaur, E.L. (2003). The microtubule plus-end proteins EB1 and dynactin have differential effects on microtubule polymerization. Mol Biol Cell *14*, 1405-1417.

Liu, D., Bryceson, Y.T., Meckel, T., Vasiliver-Shamis, G., Dustin, M.L., and Long, E.O. (2009). Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. Immunity *31*, 99-109.

Liu, D., Meckel, T., and Long, E.O. (2010). Distinct role of rab27a in granule movement at the plasma membrane and in the cytosol of NK cells. PLoS One *5*, e12870.

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

Ljunggren, H.G., and Malmberg, K.J. (2007). Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol *7*, 329-339.

Lomakin, A.J., Semenova, I., Zaliapin, I., Kraikivski, P., Nadezhdina, E., Slepchenko, B.M., Akhmanova, A., and Rodionov, V. (2009). CLIP-170-dependent capture of membrane organelles by microtubules initiates minus-end directed transport. Dev Cell *17*, 323-333.

Lub, M., van Kooyk, Y., van Vliet, S.J., and Figdor, C.G. (1997). Dual role of the actin cytoskeleton in regulating cell adhesion mediated by the integrin lymphocyte function-associated molecule-1. Mol Biol Cell *8*, 341-351.

Lunemann, A., Lunemann, J.D., and Munz, C. (2009). Regulatory NK-cell functions in inflammation and autoimmunity. Mol Med *15*, 352-358.

Lye, R.J., Porter, M.E., Scholey, J.M., and McIntosh, J.R. (1987). Identification of a microtubule-based cytoplasmic motor in the nematode C. elegans. Cell *51*, 309-318.

Mace, E.M., Monkley, S.J., Critchley, D.R., and Takei, F. (2009). A dual role for talin in NK cell cytotoxicity: activation of LFA-1-mediated cell adhesion and polarization of NK cells. J Immunol *182*, 948-956.

Malech, H.L., Root, R.K., and Gallin, J.I. (1977). Structural analysis of human neutrophil migration. Centriole, microtubule, and microfilament orientation and function during chemotaxis. J Cell Biol *75*, 666-693.

Malek, T.R., and Bayer, A.L. (2004). Tolerance, not immunity, crucially depends on IL-2. Nat Rev Immunol *4*, 665-674.

Manneville, J.B., Jehanno, M., and Etienne-Manneville, S. (2010). Dlg1 binds GKAP to control dynein association with microtubules, centrosome positioning, and cell polarity. J Cell Biol *191*, 585-598.

March, M.E., and Long, E.O. (2011). beta2 integrin induces TCRzeta-Syk-phospholipase C-gamma phosphorylation and paxillin-dependent granule polarization in human NK cells. J Immunol *186*, 2998-3005.

Marks, M.S., Theos, A.C., and Raposo, G. (2003). Melanosomes and MHC class II antigen-processing compartments: a tinted view of intracellular trafficking and immunity. Immunol Res *27*, 409-426.

Marr, K.J., Jones, G.J., Zheng, C., Huston, S.M., Timm-McCann, M., Islam, A., Berenger, B.M., Ma, L.L., Wiseman, J.C., and Mody, C.H. (2009). Cryptococcus neoformans directly stimulates perforin production and rearms NK cells for enhanced anticryptococcal microbicidal activity. Infect Immun *77*, 2436-2446.

Martin-Cofreces, N.B., Robles-Valero, J., Cabrero, J.R., Mittelbrunn, M., Gordon-Alonso, M., Sung, C.H., Alarcon, B., Vazquez, J., and Sanchez-Madrid, F. (2008). MTOC translocation modulates IS formation and controls sustained T cell signaling. J Cell Biol *182*, 951-962.

Masilamani, M., Nguyen, C., Kabat, J., Borrego, F., and Coligan, J.E. (2006). CD94/NKG2A inhibits NK cell activation by disrupting the actin network at the immunological synapse. J Immunol *177*, 3590-3596.

Mason, L.H., Willette-Brown, J., Taylor, L.S., and McVicar, D.W. (2006). Regulation of Ly49D/DAP12 signal transduction by Src-family kinases and CD45. J Immunol *176*, 6615-6623.

Matteoni, R., and Kreis, T.E. (1987). Translocation and clustering of endosomes and lysosomes depends on microtubules. J Cell Biol *105*, 1253-1265.

McCann, F.E., Eissmann, P., Onfelt, B., Leung, R., and Davis, D.M. (2007). The activating NKG2D ligand MHC class I-related chain A transfers from target cells to NK cells in a manner that allows functional consequences. J Immunol *178*, 3418-3426.

McNamara, J.C., and Ribeiro, M.R. (2000). The calcium dependence of pigment translocation in freshwater shrimp red ovarian chromatophores. Biol Bull *198*, 357-366.

Menasche, G., Pastural, E., Feldmann, J., Certain, S., Ersoy, F., Dupuis, S., Wulffraat, N., Bianchi, D., Fischer, A., Le Deist, F., and de Saint Basile, G. (2000). Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nat Genet *25*, 173-176.

Mentlik, A.N., Sanborn, K.B., Holzbaur, E.L., and Orange, J.S. (2010). Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. Mol Biol Cell *21*, 2241-2256.

Merida, I., Diez, E., and Gaulton, G.N. (1991). IL-2 binding activates a tyrosine-phosphorylated phosphatidylinositol-3-kinase. J Immunol *147*, 2202-2207.

Michon, J.M., Caligiuri, M.A., Hazanow, S.M., Levine, H., Schlossman, S.F., and Ritz, J. (1988). Induction of natural killer effectors from human thymus with recombinant IL-2. J Immunol *140*, 3660-3667.

Mikenberg, I., Widera, D., Kaus, A., Kaltschmidt, B., and Kaltschmidt, C. (2006). TNF-alpha mediated transport of NF-kappaB to the nucleus is independent of the cytoskeleton-based transport system in non-neuronal cells. Eur J Cell Biol *85*, 529-536.

Mikenberg, I., Widera, D., Kaus, A., Kaltschmidt, B., and Kaltschmidt, C. (2007). Transcription factor NF-kappaB is transported to the nucleus via cytoplasmic dynein/dynactin motor complex in hippocampal neurons. PLoS One *2*, e589.

Miletic, A.V., Swat, M., Fujikawa, K., and Swat, W. (2003). Cytoskeletal remodeling in lymphocyte activation. Curr Opin Immunol *15*, 261-268.

Mitchison, T., and Kirschner, M. (1984). Dynamic instability of microtubule growth. Nature *312*, 237-242.

Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. Nature *395*, 82-86.

Moore, J.K., Stuchell-Brereton, M.D., and Cooper, J.A. (2009). Function of dynein in budding yeast: mitotic spindle positioning in a polarized cell. Cell Motil Cytoskeleton *66*, 546-555.

Morfini, G.A., You, Y.M., Pollema, S.L., Kaminska, A., Liu, K., Yoshioka, K., Bjorkblom, B., Coffey, E.T., Bagnato, C., Han, D.*, et al.* (2009). Pathogenic huntingtin inhibits fast axonal transport by activating JNK3 and phosphorylating kinesin. Nat Neurosci *12*, 864-871.

Moughamian, A.J., and Holzbaur, E.L. (2012). Dynactin is required for transport initiation from the distal axon. Neuron *74*, 331-343.

Nascimento, A.A., Roland, J.T., and Gelfand, V.I. (2003). Pigment cells: a model for the study of organelle transport. Annu Rev Cell Dev Biol *19*, 469-491.

Nguyen-Ngoc, T., Afshar, K., and Gonczy, P. (2007). Coupling of cortical dynein and G alpha proteins mediates spindle positioning in Caenorhabditis elegans. Nat Cell Biol *9*, 1294-1302.

Nilsson, H., and Wallin, M. (1997). Evidence for several roles of dynein in pigment transport in melanophores. Cell Motil Cytoskeleton *38*, 397-409.

Okkenhaug, K., and Rottapel, R. (1998). Grb2 forms an inducible protein complex with CD28 through a Src homology 3 domain-proline interaction. J Biol Chem *273*, 21194-21202.

Orange, J.S. (2008). Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol *8*, 713-725.

Orange, J.S., and Ballas, Z.K. (2006). Natural killer cells in human health and disease. Clin Immunol *118*, 1-10.

Orange, J.S., Harris, K.E., Andzelm, M.M., Valter, M.M., Geha, R.S., and Strominger, J.L. (2003). The mature activating natural killer cell immunologic synapse is formed in distinct stages. Proc Natl Acad Sci U S A *100*, 14151-14156.

Orange, J.S., Ramesh, N., Remold-O'Donnell, E., Sasahara, Y., Koopman, L., Byrne, M., Bonilla, F.A., Rosen, F.S., Geha, R.S., and Strominger, J.L. (2002). Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. Proc Natl Acad Sci U S A *99*, 11351-11356.

Orange, J.S., Roy-Ghanta, S., Mace, E.M., Maru, S., Rak, G.D., Sanborn, K.B., Fasth, A., Saltzman, R., Paisley, A., Monaco-Shawver, L.*, et al.* (2011). IL-2 induces a WAVE2-dependent pathway for actin reorganization that enables WASp-independent human NK cell function. J Clin Invest *121*, 1535-1548.

Orange, J.S., Wang, B., Terhorst, C., and Biron, C.A. (1995). Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. J Exp Med *182*, 1045-1056.

Palazzo, A.F., Joseph, H.L., Chen, Y.J., Dujardin, D.L., Alberts, A.S., Pfister, K.K., Vallee, R.B., and Gundersen, G.G. (2001). Cdc42, dynein, and dynactin regulate MTOC reorientation independent of Rho-regulated microtubule stabilization. Curr Biol *11*, 1536-1541.

Paust, S., and von Andrian, U.H. (2011). Natural killer cell memory. Nat Immunol *12*, 500-508.

Pende, D., Parolini, S., Pessino, A., Sivori, S., Augugliaro, R., Morelli, L., Marcenaro, E., Accame, L., Malaspina, A., Biassoni, R.*, et al.* (1999). Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med *190*, 1505-1516.

Perricone, R., Perricone, C., De Carolis, C., and Shoenfeld, Y. (2008). NK cells in autoimmunity: a two-edg'd weapon of the immune system. Autoimmun Rev *7*, 384-390.

Perussia, B., and Loza, M.J. (2000). Assays for antibody-dependent cell-mediated cytotoxicity (ADCC) and reverse ADCC (redirected cytotoxicity) in human natural killer cells. Methods Mol Biol *121*, 179-192.

Peterson, M.E., and Long, E.O. (2008). Inhibitory receptor signaling via tyrosine phosphorylation of the adaptor Crk. Immunity *29*, 578-588.

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J., and Lippincott-Schwartz, J. (1997). ER-to-Golgi transport visualized in living cells. Nature *389*, 81-85.

Quann, E.J., Merino, E., Furuta, T., and Huse, M. (2009). Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. Nat Immunol *10*, 627-635.

Rak, G.D., Mace, E.M., Banerjee, P.P., Svitkina, T., and Orange, J.S. (2011). Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. PLoS Biol *9*, e1001151.

Ravichandran, K.S., and Burakoff, S.J. (1994). The adapter protein Shc interacts with the interleukin-2 (IL-2) receptor upon IL-2 stimulation. J Biol Chem *269*, 1599-1602.

Riteau, B., Barber, D.F., and Long, E.O. (2003). Vav1 phosphorylation is induced by beta2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization. J Exp Med *198*, 469-474.

Rogers, S.L., Tint, I.S., Fanapour, P.C., and Gelfand, V.I. (1997). Regulated bidirectional motility of melanophore pigment granules along microtubules in vitro. Proc Natl Acad Sci U S A *94*, 3720-3725.

Sahni, M., Zhou, X.M., Bakiri, L., Schlessinger, J., Baron, R., and Levy, J.B. (1996). Identification of a novel 135-kDa Grb2-binding protein in osteoclasts. J Biol Chem *271*, 33141-33147.

Sanborn, K.B., Rak, G.D., Maru, S.Y., Demers, K., Difeo, A., Martignetti, J.A., Betts, M.R., Favier, R., Banerjee, P.P., and Orange, J.S. (2009). Myosin IIA associates with NK cell lytic granules to enable their interaction with F-actin and function at the immunological synapse. J Immunol *182*, 6969-6984.

Sanchez-Madrid, F., Krensky, A.M., Ware, C.F., Robbins, E., Strominger, J.L., Burakoff, S.J., and Springer, T.A. (1982). Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc Natl Acad Sci U S A *79*, 7489-7493.

Sanchez-Martin, L., Sanchez-Sanchez, N., Gutierrez-Lopez, M.D., Rojo, A.I., Vicente-Manzanares, M., Perez-Alvarez, M.J., Sanchez-Mateos, P., Bustelo, X.R., Cuadrado, A., Sanchez-Madrid, F.*, et al.* (2004). Signaling through the leukocyte integrin LFA-1 in T cells induces a transient activation of Rac-1 that is regulated by Vav and PI3K/Akt-1. J Biol Chem *279*, 16194-16205.

Sancho, D., Nieto, M., Llano, M., Rodriguez-Fernandez, J.L., Tejedor, R., Avraham, S., Cabanas, C., Lopez-Botet, M., and Sanchez-Madrid, F. (2000). The tyrosine kinase PYK-2/RAFTK regulates natural killer (NK) cell cytotoxic response, and is translocated and activated upon specific target cell recognition and killing. J Cell Biol *149*, 1249-1262.

Sandusky, M.M., Messmer, B., and Watzl, C. (2006). Regulation of 2B4 (CD244)-mediated NK cell activation by ligand-induced receptor modulation. Eur J Immunol *36*, 3268-3276.

Schleinitz, N., March, M.E., and Long, E.O. (2008). Recruitment of activation receptors at inhibitory NK cell immune synapses. PLoS One *3*, e3278.

Schnyder, T., Castello, A., Feest, C., Harwood, N.E., Oellerich, T., Urlaub, H., Engelke, M., Wienands, J., Bruckbauer, A., and Batista, F.D. (2011). B cell receptor-mediated antigen gathering requires ubiquitin ligase Cbl and adaptors Grb2 and Dok-3 to recruit dynein to the signaling microcluster. Immunity *34*, 905-918.

Schroer, T.A., and Sheetz, M.P. (1991). Two activators of microtubule-based vesicle transport. J Cell Biol *115*, 1309-1318.

Schuster, M., Lipowsky, R., Assmann, M.A., Lenz, P., and Steinberg, G. (2011). Transient binding of dynein controls bidirectional long-range motility of early endosomes. Proc Natl Acad Sci U S A *108*, 3618-3623.

Shaw, J.D., Cummings, K.B., Huyer, G., Michaelis, S., and Wendland, B. (2001). Yeast as a model system for studying endocytosis. Exp Cell Res *271*, 1-9.

She, H.Y., Rockow, S., Tang, J., Nishimura, R., Skolnik, E.Y., Chen, M., Margolis, B., and Li, W. (1997). Wiskott-Aldrich syndrome protein is associated with the adapter protein Grb2 and the epidermal growth factor receptor in living cells. Mol Biol Cell *8*, 1709-1721.

Shuh, M., Morse, B.A., Heidecker, G., and Derse, D. (2011). Association of SRC-related kinase Lyn with the interleukin-2 receptor and its role in maintaining constitutive phosphorylation of JAK/STAT in human T-cell leukemia virus type 1-transformed T cells. J Virol *85*, 4623-4627.

Smyth, M.J., Cretney, E., Kelly, J.M., Westwood, J.A., Street, S.E., Yagita, H., Takeda, K., van Dommelen, S.L., Degli-Esposti, M.A., and Hayakawa, Y. (2005). Activation of NK cell cytotoxicity. Mol Immunol *42*, 501-510.

Stagi, M., Gorlovoy, P., Larionov, S., Takahashi, K., and Neumann, H. (2006). Unloading kinesin transported cargoes from the tubulin track via the inflammatory c-Jun N-terminal kinase pathway. FASEB J *20*, 2573-2575.

Standeven, L.J., Carlin, L.M., Borszcz, P., Davis, D.M., and Burshtyn, D.N. (2004). The actin cytoskeleton controls the efficiency of killer Ig-like receptor accumulation at inhibitory NK cell immune synapses. J Immunol *173*, 5617-5625.

Stebbins, C.C., Watzl, C., Billadeau, D.D., Leibson, P.J., Burshtyn, D.N., and Long, E.O. (2003). Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. Mol Cell Biol *23*, 6291-6299.

Stinchcombe, J.C., Bossi, G., Booth, S., and Griffiths, G.M. (2001). The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity *15*, 751-761.

Stinchcombe, J.C., and Griffiths, G.M. (2007). Secretory mechanisms in cell-mediated cytotoxicity. Annu Rev Cell Dev Biol *23*, 495-517.

Stinchcombe, J.C., Majorovits, E., Bossi, G., Fuller, S., and Griffiths, G.M. (2006). Centrosome polarization delivers secretory granules to the immunological synapse. Nature *443*, 462-465.

Stowers, L., Yelon, D., Berg, L.J., and Chant, J. (1995). Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related GTPase CDC42. Proc Natl Acad Sci U S A *92*, 5027-5031.

Sun, J.C., Beilke, J.N., and Lanier, L.L. (2009). Adaptive immune features of natural killer cells. Nature *457*, 557-561.

Sun, J.C., and Lanier, L.L. (2011). NK cell development, homeostasis and function: parallels with CD8(+) T cells. Nat Rev Immunol *11*, 645-657.

Tan, S.C., Scherer, J., and Vallee, R.B. (2011). Recruitment of dynein to late endosomes and lysosomes through light intermediate chains. Mol Biol Cell *22*, 467-477.

Tassi, I., Klesney-Tait, J., and Colonna, M. (2006). Dissecting natural killer cell activation pathways through analysis of genetic mutations in human and mouse. Immunol Rev *214*, 92-105.

Thiery, J., Keefe, D., Saffarian, S., Martinvalet, D., Walch, M., Boucrot, E., Kirchhausen, T., and Lieberman, J. (2010). Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis. Blood *115*, 1582-1593.

Thobakgale, C.F., Fadda, L., Lane, K., Toth, I., Pereyra, F., Bazner, S., Ndung'u, T., Walker, B.D., Rosenberg, E.S., Alter, G.*, et al.* (2012). Frequent and strong antibody-mediated natural killer cell activation in response to HIV-1 Env in individuals with chronic HIV-1 infection. J Virol *86*, 6986-6993.

Ting, A.T., Einspahr, K.J., Abraham, R.T., and Leibson, P.J. (1991). Fc gamma receptor signal transduction in natural killer cells. Coupling to phospholipase C via a G protein-independent, but tyrosine kinase-dependent pathway. J Immunol *147*, 3122-3127.

Toba, S., Watanabe, T.M., Yamaguchi-Okimoto, L., Toyoshima, Y.Y., and Higuchi, H. (2006). Overlapping hand-over-hand mechanism of single molecular motility of cytoplasmic dynein. Proc Natl Acad Sci U S A *103*, 5741-5745.

Topham, N.J., and Hewitt, E.W. (2009). Natural killer cell cytotoxicity: how do they pull the trigger? Immunology *128*, 7-15.

Tran, P.T., Marsh, L., Doye, V., Inoue, S., and Chang, F. (2001). A mechanism for nuclear positioning in fission yeast based on microtubule pushing. TJ Cell Biol *153*, 397-411.

Treanor, B., Lanigan, P.M., Kumar, S., Dunsby, C., Munro, I., Auksorius, E., Culley, F.J., Purbhoo, M.A., Phillips, D., Neil, M.A.*, et al.* (2006). Microclusters of inhibitory killer immunoglobulin-like receptor signaling at natural killer cell immunological synapses. J Cell Biol *174*, 153-161.

Trinchieri, G., and Valiante, N. (1993). Receptors for the Fc fragment of IgG on natural killer cells. Nat Immun *12*, 218-234.

Tsun, A., Qureshi, I., Stinchcombe, J.C., Jenkins, M.R., de la Roche, M., Kleczkowska, J., Zamoyska, R., and Griffiths, G.M. (2011). Centrosome docking at the immunological synapse is controlled by Lck signaling. J Cell Biol *192*, 663-674.

Upshaw, J.L., Arneson, L.N., Schoon, R.A., Dick, C.J., Billadeau, D.D., and Leibson, P.J. (2006). NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. Nat Immunol *7*, 524-532.

Upshaw, J.L., Schoon, R.A., Dick, C.J., Billadeau, D.D., and Leibson, P.J. (2005). The isoforms of phospholipase C-gamma are differentially used by distinct human NK activating receptors. J Immunol *175*, 213-218.

Valiante, N.M., Phillips, J.H., Lanier, L.L., and Parham, P. (1996). Killer cell inhibitory receptor recognition of human leukocyte antigen (HLA) class I blocks formation of a pp36/PLC-gamma signaling complex in human natural killer (NK) cells. J Exp Med *184*, 2243-2250.

Vallee, R.B., Shpetner, H.S., and Paschal, B.M. (1989). The role of dynein in retrograde axonal transport. Trends Neurosci *12*, 66-70.

van der Merwe, A.P., Davis, S.J., Shaw, A.S., and Dustin, M.L. (2000). Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition. Semin Immunol *12*, 5-21.

Vancoillie, G., Lambert, J., Mulder, A., Koerten, H.K., Mommaas, A.M., Van Oostveldt, P., and Naeyaert, J.M. (2000). Cytoplasmic dynein colocalizes with melanosomes in normal human melanocytes. Br J Dermatol *143*, 298-306.

Vaughan, P.S., Miura, P., Henderson, M., Byrne, B., and Vaughan, K.T. (2002). A role for regulated binding of p150(Glued) to microtubule plus ends in organelle transport. J Cell Biol *158*, 305-319.

Vitte-Mony, I., Delespine-Carmagnat, M., and Bertoglio, J.H. (1994). Signal transduction of interleukin 2 in human natural killer cells: involvement of the p56lck tyrosine kinase. Mol Immunol *31*, 623-632.

Vivier, E., Nunes, J.A., and Vely, F. (2004). Natural killer cell signaling pathways. Science *306*, 1517-1519.

Vyas, Y.M., Mehta, K.M., Morgan, M., Maniar, H., Butros, L., Jung, S., Burkhardt, J.K., and Dupont, B. (2001). Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated noncytolytic and cytolytic interactions. J Immunol *167*, 4358-4367.

Wald, O., Weiss, I.D., Wald, H., Shoham, H., Bar-Shavit, Y., Beider, K., Galun, E., Weiss, L., Flaishon, L., Shachar, I.*, et al.* (2006). IFN-gamma acts on T cells to induce NK cell mobilization and accumulation in target organs. J Immunol *176*, 4716-4729.

Watabe, H., Valencia, J.C., Le Pape, E., Yamaguchi, Y., Nakamura, M., Rouzaud, F., Hoashi, T., Kawa, Y., Mizoguchi, M., and Hearing, V.J. (2008). Involvement of dynein and spectrin with early melanosome transport and melanosomal protein trafficking. J Invest Dermatol *128*, 162-174.

Waterman-Storer, C.M., Karki, S., and Holzbaur, E.L. (1995). The p150Glued component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1). Proc Natl Acad Sci U S A *92*, 1634-1638.

Waterman-Storer, C.M., Karki, S.B., Kuznetsov, S.A., Tabb, J.S., Weiss, D.G., Langford, G.M., and Holzbaur, E.L. (1997). The interaction between cytoplasmic dynein and dynactin is required for fast axonal transport. Proc Natl Acad Sci U S A *94*, 12180-12185.

Watzl, C., and Long, E.O. (2010). Signal transduction during activation and inhibition of natural killer cells. Curr Protoc Immunol *Chapter 11*, Unit 11.9B.

Watzl, C., and Long, E.O. (2003). Natural killer cell inhibitory receptors block actin cytoskeleton-dependent recruitment of 2B4 (CD244) to lipid rafts. J Exp Med *197*, 77-85.

Wiedemann, A., Muller, S., Favier, B., Penna, D., Guiraud, M., Delmas, C., Champagne, E., and Valitutti, S. (2005). T-cell activation is accompanied by an ubiquitination process occurring at the immunological synapse. Immunol Lett *98*, 57-61.

Wiseman, J.C., Ma, L.L., Marr, K.J., Jones, G.J., and Mody, C.H. (2007). Perforin-dependent cryptococcal microbicidal activity in NK cells requires PI3K-dependent ERK1/2 signaling. J Immunol *178*, 6456-6464.

Wood, S.M., Ljunggren, H.G., and Bryceson, Y.T. (2011). Insights into NK cell biology from human genetics and disease associations. Cell Mol Life Sci *68*, 3479-3493.

Wood, S.M., Meeths, M., Chiang, S.C., Bechensteen, A.G., Boelens, J.J., Heilmann, C., Horiuchi, H., Rosthoj, S., Rutynowska, O., Winiarski, J.*, et al.* (2009). Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity. Blood *114*, 4117-4127.

Wren, L., Parsons, M.S., Isitman, G., Center, R.J., Kelleher, A.D., Stratov, I., Bernard, N.F., and Kent, S.J. (2012). Influence of Cytokines on HIV-Specific Antibody-Dependent Cellular Cytotoxicity Activation Profile of Natural Killer Cells. PLoS One *7*, e38580.

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

Yamamoto, A., Tsutsumi, C., Kojima, H., Oiwa, K., and Hiraoka, Y. (2001). Dynamic behavior of microtubules during dynein-dependent nuclear migrations of meiotic prophase in fission yeast. Mol Biol Cell *12*, 3933-3946.

Yamasaki, M., Arai, H., Ashida, N., Ishii, K., and Kita, T. (2001). Monocyte chemoattractant protein 1 causes differential signalling mediated by proline-rich tyrosine kinase 2 in THP-1 cells. Biochem J *355*, 751-756.

Zychlinsky, A., Joag, S., Liu, C.C., and Young, J.D. (1990). Cytotoxic mechanisms of murine lymphokine-activated killer cells: functional and biochemical characterization of homogeneous populations of spleen LAK cells. Cell Immunol *126*, 377-390.