Aus dem Bereich Med. Physiologie
Theoretische Medizin und Biowissenschaften
der Medizinischen Fakultät
der Universität des Saarlandes, Homburg/Saar

Syntaxin7 is required for lytic granule release from cytotoxic T lymphocytes

Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften

der Medizinischen Fakultät
der UNIVERSITÄT DES SAARLANDES
2009

vorgelegt von: Varsha Pattu
geb.am: 20.07.1982 in Chennai, India

Tag des Promotionskolloquiums: _____

Dekan: _____

Vorsitzender: _____

Berichterstatter: _____

_____

_____

To my parents

LIST OF CONTENTS

Acknowledgements

I would first and foremost like to thank my supervisor Prof. Dr. Jens Rettig for his excellent guidance and support during my entire PhD. Without his encouragement and faith I would not have been able to complete my thesis. I would always be grateful to him and I hope he will continue to advise me throughout my scientific career.

I would also like to thank Prof Dr. Markus Hoth for his constant encouragement and suggestions to improve my project. I would like to thank Dr. Elmar Krause for helping me with my thesis and my project. I would like to Dr. Eva Schwarz for helping me a lot during the beginning of my PhD and teaching me about T cell culture. I would like to thank Bin Qu with whom I had a very good collaboration with during my PhD.

I would like to thank Dr. Ulf Matti for helping me with all the molecular biology and for his help during the beginning of my stay in Germany. I would like to thank Dr. Misty Marshall for helping me with all the biochemistry in my project. I would like to thank Dr. Ute Becherer for helping me with the TIRF experiments and analysis.

I would like to thank Dr. David Stevens, Dr Claudia Schirra and Dr. Detlef Hof for help and advice with my project. I would like to thank Lisa Weins, Monica Dudenhöfer Pfeifer, Mahantappa Halimani, Anneka Bost, Yuan Yuan Liu, Tobias Schmidt, Mathias Pasche, Silke, Sandra Bost, and Quynh for being very nice and making the lab very memorable. I would like to thank Mr. Gries for help with the posters.

I would like to thank Elmar Krause, Misty Marshall, Lisa Weins, Shruti Bhat, and Mahantappa Halimani for helping me with my thesis.

I would also to thank members from the lab of Markus Hoth expecially Christian Junker, Carsten Kummerow and Shruti Bhat for giving good inputs and suggestions during the killing club.

I would like to thank Reiko Trautmann, Caroline Bick, Manuela Schneider, Kathrin Sandmeier, Bettina Strauβ and Anja Ludes for excellent technical help.

I would like to the graduate school and Prof. Dieter Bruns for the guidance and support during my PhD.

Last but not the least I would like to thank my family and friends for their constant encouragement. I would especially like to thank my parents for their love and support. Without their confidence in me I would not have been able to finish my thesis. I would also like to thank Archana Murali and S. Aparna for always being there for me and never making me feel the distance between us.

Zusammenfassung

Die vorliegende Doktorarbeit beschäftigt sich mit zytotoxischen T-Lymphozyten (Andersen et al., 2006), einer Zellpopulation des Immunsystems. Sie gehören zur zellulären Immunabwehr und erkennen mit ihren T-Zellrezeptoren (TCR) körpereigene Zellen, die von Bakterien oder Viren infiziert wurden. Nach der Erkennung erfolgt die Ausbildung einer Immunologischen Synapse zwischen Zielzelle und CTL. Am Ort der Synapse werden aus den CTL lytische Substanzen (i.e. Perforin, Granulysin und verschiedene Granzyme) exozytotisch ausgeschieden, die in der Zielzelle Apoptose auslösen und so die Infektion bekämpfen. Während der Reifung der lytischen Vesikel laufen intrazellulär verschiedene Fusionsereignisse unterschiedlicher Vesikel-populationen ab. Letztlich fusioniert das reife Vesikel mit der Plasmamembran und gibt seine lytischen Substanzen frei. Es wird vermutet, dass, wie in anderen Zellsystemen auch, die Fusionsereignisse von SNARE Proteinen gesteuert werden.

In der vorliegenden Arbeit wird speziell die Funktion des SNARE Proteins Syntaxin7 untersucht. Ein von uns durchgeführter PCR Screen nach verschiedenen SNAREs hatte das Vorhandensein von Syntaxin7 gezeigt. Außerdem wurde die Expression von Syntaxin7 nach Aktivierung der CTLs hochreguliert. Syntaxin7 erschien deshalb als aussichtsreicher Kandidat für eine funktionelle Beteiligung an der Immunfunktion von CTLs. Speziell sollte geklärt werden (1) wo in der Zelle Syntaxin7 lokalisiert ist, (2) ob es tatsächlich an der Exozytose von lytischen Vesikeln beteiligt ist und (3) welchen Schritt bei der Vesikelreifung und Exozytose es steuert.
Im Wesentlichen wurden zur Klärung dieser Fragen zwei experimentelle Ansätze verwendet - Überexpression einer dominant negativen Form von Syntaxin7 und Hemmung der Syntaxin7 Expression mit siRNAs. Daten wurden in der Studie mit Hilfe von Populations- und Einzelzell-Zytotoxizitäts-Assays, gentechnischen (PCR) und proteinbiochemischen (Western Blot) Verfahren und Hochauflösungs-lichtmikroskopie (TIRF, SIM) erhoben.
Es konnte belegt werden, dass die Anreicherung und Freisetzung lytischer Vesikel durch Hemmung der Syntaxin7 Funktion unterbunden werden kann. Lokalisationsstudien haben gezeigt, dass Syntaxin7 überraschender Weise nicht auf den lytischen Vesikeln vorkommt. Weitere Untersuchungen ergaben, dass in Syntaxin7 defizienten CTLs neben dem Sekretionsdefekt auch das übliche Recycling

des TCR zur immunologischen Synapse weitgehend ausblieb. Daraus wurde die Hypothese gebildet, dass Syntaxin7 nicht direkt an der Exozytose lytischer Vesikel beteiligt ist. Vielmehr spielt es eine Rolle bei der Ausbildung eines funktionellen cSMAC, wozu die Migration und das Recycling des TCR Voraussetzung sind. Mit Hilfe hochauflösender Fluoreszenzmikroskopie wurde anschließend der Schritt des TCR Recyclings identifiziert, der durch Syntaxin7 geregelt wird. Dazu wurden verschiedene endosomale und lysosomale Marker verwendet und ihre Kolokalisation zu Syntaxin7 bzw. dem TCR untersucht. Wir fanden, dass Syntaxin7 sowohl in späten Endosomen als auch an der Plasmamembran vorkommt. Außerdem konnte gezeigt werden, dass TCRs mit Rab7, einem Markerprotein für späte Endosomen, kolokalisieren. Diese Kolokalisation nahm nach Transfektion mit Syntaxin7 siRNA ab, während die Kolokalisation mit EEA, einem Marker früher Endosomen, zunahm.
Diese Ergebnisse machen deutlich, dass Syntaxin7 bei der Reifung früher zu späten Endosomen eine entscheidende Rolle spielt. Ist dieser Schritt durch Blockierung oder Herunterregulation von Syntaxin7 inhibiert, wird der Aufbau des cSMAC und damit die Exozytose lytischer Vesikel gestört.

1. Introduction

1.1 The immune system – the basics

The immune system is the defense system that functions to preserve the integrity of the host organism by protecting it against invading pathogenic organisms or any sort of injury. It has evolved in vertebrates and comprises of a dynamic network of a variety of cells that work together with different proteins to specifically recognize and eliminate any foreign antigen. The immune cells are migratory in nature and therefore continuously move between and within tissues for immune surveillance and defense against bacteria, viruses and damage (Gowans and Knight, 1964). The immune system has both non specific (innate immunity) and specific components (adaptive immunity). Components of the innate immune system do not show any specificity to a particular pathogen for its elimination. The cells of the innate immune system therefore offer the first line of defense against invading pathogens. All the specific components of the immune system come under the adaptive immune system. They generate specific immune responses such as the production of antibodies against a particular pathogen. The adaptive immune system sometimes confers lifelong protection to infection against the same pathogen or generates a more heightened and rapid response (memory response) to ensure quicker elimination of the pathogen. Adaptive immunity therefore is different from innate immunity as it occurs during the lifetime of an individual as an adaptation to infection with a pathogen and requires time to generate an immune response. The adaptive immune system consists of a cellular and humoral branch. Innate and adaptive immunity depend on each other to elicit effective immune responses (Eisenbarth and Flavell, 2009).

1.1.1 Cellular components of the immune system

All cells of the immune system arise from haematopoeitic stem cells in the bone marrow. These stem cells divide to generate two populations of stem cells – one is the myeloid progenitor that gives rise to granulocytes, macrophages, dendritic cells and mast cells and the second is the common lymphoid progenitor that gives rise to natural killer cells and lymphocytes. Granulocytes include eosinophils, basophils and neutrophils. They circulate in the blood and act as effector cells at sites of

inflammation and infection and are short lived. Macrophages are phagocytes and are critical for innate immunity. They are distributed widely in body tissues and are the mature form of monocytes which circulate in the blood and differentiate continuously into macrophages. Dendritic cells (DCs) are specialized to take up antigens, process it and display it for recognition by T lymphocytes. During the uptake and processing of antigens dendritic cells are activated and express co stimulatory molecules and migrate to the lymph nodes. Mast cells reside near small blood vessels and upon activation release substances affecting vascular permeability. They are best known for orchestrating allergic responses. Natural killer cells are large, granular cells that lack antigen specific receptors. They are important for innate immunity and can kill virus infected cells. Lymphocytes can generate an immune response against almost any antigen. This is possible because every individual lymphocyte matures with a unique variant of a receptor that can recognize antigens. Therefore collectively lymphocytes bear receptors that can recognize almost every antigen. Upon infection, there is selective activation and expansion of lymphocytes that bear receptors recognizing that particular antigen. This is how adaptive immune system is effective in eliminating all infections.

1.2 Lymphocytes

Lymphocytes are small, having a condensed chromatin indicating minimal transcriptional activity. Naïve lymphocytes have no known function and can only function after activation in adaptive immune responses. An adaptive immune response is generated when a specific antigen presenting DC is recognized by the lymphocyte leading to their activation. The adaptive immune system is mediated largely by lymphocytes and consists of a cellular and humoral branch. The former is mediated by T lymphocytes and the latter by B lymphocytes. B lymphocytes express cell surface immunoglobulin (antibody) molecules that act as receptors for antigen. When they are activated they secrete the soluble antibody that provides protection against the pathogens in the extracellular space. T cells have receptors that recognize peptide fragments of pathogens presented on the surface of antigen presenting by a set of glycoproteins called the Majorhistocompatability complex

(MHC). There are two classes of MHC molecules that present peptides on the surface of antigen presenting cells. The MHC class I molecules are present on almost all nucleated cells in the body and the MHC class II molecules are present on specialized cells such as dendritic cells. T lymphocyes are divided in two major subsets based on the presence of two receptors on their surface the CD4 and CD8 receptor. Cells bearing the CD4 receptor (CD4 T cells) recognize peptides presented by MHC class II molecules and cells bearing the CD8 receptor (CD8 T cells) recognize peptides presented by MHC class I molecules. CD4 T cells when activated are called T helper cells and function in activating other cells of the immune system like macrophages and B cells. CD8 T cells upon activation differentiate into effector cytotoxic T lymphocytes. These cells function in the direct induction of cell death of the target cell and function in combating tumors, bacteria and virus infected cells.

The importance of the adaptive immune system is evident in the form of life threatening diseases which result when the lymphocytes are not functional leading to immunodeficiency syndromes. While the innate immunity provides the immediate defense, it is not sufficient to combat all infections. For thorough and effective elimination of all pathogens in the body the proper functioning of the adaptive immune system is a must. There is still a lot to be discovered about the activation and killing function of CD8T cells which bring about the most direct elimination of infected cells.

1.3. From naïve to effector CD8 T cells

Naïve CD8T cells are generated in the thymus and migrate between the blood and lymph node in search of antigen (Goldschneider et al., 1986). Following their maturation in the thymus they move to the lymph node (Fig. 1) where they first encounter antigen presenting cells. The antigen presenting cells (APCs) include dendritic cells, B cells, macrophages, etc. This first encounter with APCs is essential for their conversion to effector status. Naïve CD8 T cells are much smaller in size and have different calcium signaling in comparison to effector CD8 T cells (Bromley and Dustin, 2002). They interact with antigen presenting dendritic cells in three sequential stages. In the first phase, which constitutes up to approximately 8 h after the entry of

T cells in to the lymph node, there are several short encounters of low signal intensity between the rapidly migrating cells with dendritic cells. These repetitive antigen independent interactions contribute to maintaining the T cell repertoire in the absence of activation (Pannetier et al., 1995). When a specific antigen presented by MHC class I molecules on the surface of dendritic cells is recognized by a naïve T cell a mature synapse between the naïve T cell and dendritic cells is formed. This process requires about 30 minutes (Lee et al., 2002). CD44 and CD69 which are early activation markers of T lymphocytes are upregulated during this phase.

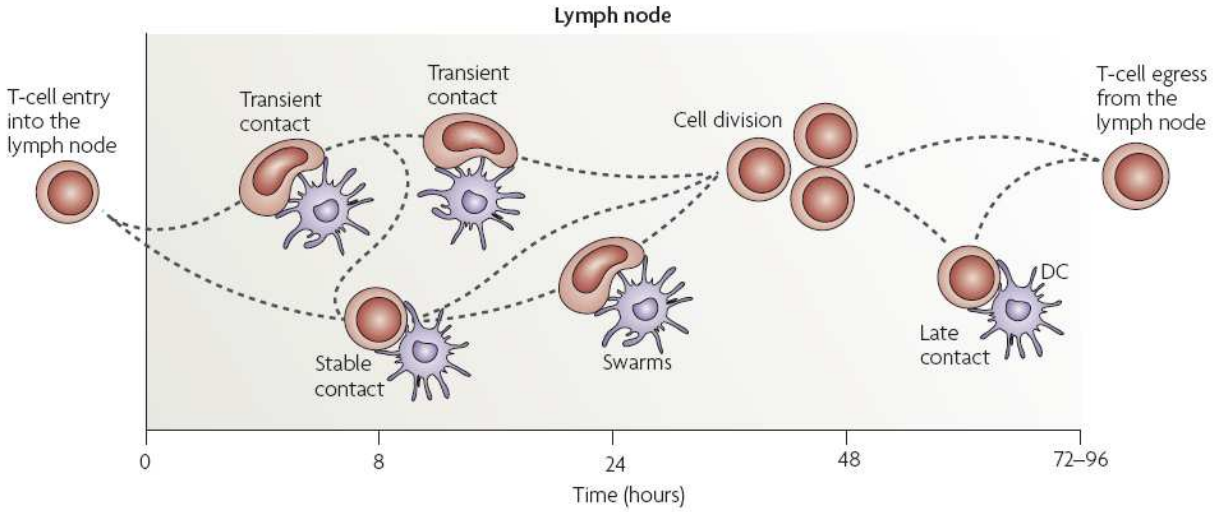

Figure 1. T cells can interact with antigen-bearing dendritic cells (DCs) in lymph nodes in multiple ways.
Antigen recognition by T cells can occur through interactions with DCs that are short-lived, long-lived or in swarms. T cells can collect and integrate signals delivered, such as those from the T-cell receptor and co-stimulatory ligands, during each of these encounters. The collection of histories of T-cell–DC contact dynamics influences both the quality and the heterogeneity of the ensuing T-cell response (Bousso, 2008).

The second phase of naïve T cell activation lasts 8 h until 24 h after T cell migration. During this phase stable T cell-DC cell conjugates are formed that last for about 1 h and there is abundant expression of CD25 (Tugores et al., 1992) but no cell division. The longevity of cell interactions in this phase is consistent with the formation of a mature contact zone. During this phase, clustering of CD8 T cells around single DCs occurs in a manner similar to that seen in CD4 T cells. One day after the initial homing leads to the third phase of T cell activation. This phase generates T cell blasts and comprises of serial engagements with different DCs. Provisional activation

and clonal expansion of CD8 T cells is provided by short lived interactions with immature DCs (Iezzi et al., 1998). A phase of sustained signaling with mature DCs seems to be crucial for supporting the survival of activated T cells and their commitment to full effector potential (Costello et al., 2002; Huppa et al., 2003; Hurez et al., 2003).

When they come in contact with APCs through the MHC peptide that is present on the membrane of APCs an immunological synapse (IS; see also section1.5) is formed. The IS which refers to the contact zone between the two cell types is stable and lasts for several hours. During this time the entire transcription machinery is activated and several genes are up-regulated. Most importantly the calcium signals in the T cells are increased and the cytotoxic machinery required for effector function are synthesized since naïve T cells lack perforin and granzymes (Chattopadhyay et al., 2009). Naïve T cells cannot function as killer cells (O'Keefe et al., 2004) and need to be activated in order to be killing competent, therefore the activation process of naïve T cells is very important in determining the effector function of T cells.

After T cells emigrate from the thymus, their homeostasis involves short lived serial contacts of low signal intensity. Recirculating naïve T cells constitutively interact with high numbers of DCs as they migrate through the perifollicular regions of the lymph node. These repetitive antigen independent interactions could contribute to maintaining the T cell repertoire in the absence of activation and clonal expansion. These short interactions increase calcium signaling and also lead to up-regulation of early activation markers CD25 and CD69. Serial signaling by different APCs induces the provisional activation of T cells that allow stabilization and commitment towards differentiation and effector function. Once T cells are activated, they need low doses of antigen for killing by forming an unsegregated synapse by interactions between CD95 (also known as FAS) and CD95 ligand (Purbhoo et al., 2004). Segregation of the IS that occurs in response to high doses of antigen, leads to the recruitment of components important for the secretory pathway of killing towards the IS enabling the polarized release of granzymes and perforin into the target cell (O'Keefe et al., 2004).

1.4 Effector CD8 T cells- killer Cytotoxic T lymphocytes

When naïve CD8T cells are activated and reach the effector status they are called Cytotoxic T lymphocytes (CTLs) because of their ability to directly induce death in their target cells. Segregation of the immunological synapse (IS; see also section 1.5) that occurs in response to high doses of antigen, leads to the recruitment of components important for the secretory pathway of killing towards the IS enabling the polarized release of granzymes and perforin into the target cell (O'Keefe et al., 2004). Cytotoxic T lymphocytes use different pathways to kill target cells. One pathway is mediated by cytokines (Andersen et al., 2006) such as Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which are produced and secreted as long as the T-cell receptor (TCR; see also section 1.5) stimulation continues. These cytokines affect the opposed target cell or cells distal to the effector T cell. TNF-α engages its receptor on the target cell and triggers the caspase cascade, leading to target cell apoptosis. IFN-γ however induces transcriptional activation of the MHC class I antigen presentation. Two pathways involve cell-cell contact dependent interaction between effector and target cells. They use two different mechanisms to result in apoptosis of target cells (Lowin et al., 1994b). In one case, the Fas ligand which is expressed on the surface of CTLs, binds to the Fas receptor (Fas, CD95) on the target cell. This binding triggers apoptosis through the classical caspase cascade (Nagata, 1996). Low doses of antigen are needed for killing by this pathway. An unsegregated synapse (see 1.5) between the CTL and target cell is formed by interactions between CD95 (also known as FAS) and CD95 ligand (Purbhoo et al., 2004). The Fas pathway is important in CTLs for self tolerance and survival by regulating responses to self and foreign antigens (Van Parijs et al., 1998). It protects T cells against activation induced cell death (Kataoka et al., 1998). In the other case, CTLs release perforin and granzymes into the intercellular cleft between the CTL and target cell to induce target cell death (Lowin et al., 1995). These proteins are highly cytotoxic and the CTLs have devised an elaborate mechanism to protect themselves and neighbouring cells from being killed accidentally (see also section 1.6.1) while still ensuring that the attacked cell can show a rapid and efficient cytotoxic response upon triggering the TCR. CTLs have the major lytic proteins pre-synthesized to

ensure rapid responses upon encountering a target cell. The lytic proteins are stored in regulated secretory lysosomes which polarize themselves to the cell surface and expose their content only upon contact with a target.

1.4.1 Granule mediated cell death pathway using Perforin and Granzymes

Granule mediated cytolysis is the most important effector function of CD8 T cells. After a killer cell recognizes its target cell, the cytotoxic granules move to the immunological synapse, where their membranes fuse with the killer cell plasma membrane and they release their contents to induce target cell apoptosis. The principal death effectors are the serine proteases called granzymes and the membrane perturbing proteins, perforin and granulysin. Studies using a perforin knock out mouse have shown the importance of the granule exocytosis pathway for controlling viral infections and tumors (Kagi et al., 1994; Kojima et al., 1994; Lowin et al., 1994a). Moreover, perforin knock out mice are also easily susceptible to tumors and infections (Smyth et al., 2000; van den Broek et al., 1996). The same basic mechanisms are used by all killer cells whether they are CD8 cytotoxic T cells or natural killer cells. Both cytotoxic T cells and natural killer cells from perforin knock out mice show a great impairment in target cell killing even though the Fas mediated killing is normal.

These cytolytic molecules need to be sorted and stored in secretory lysosomes which are specialized lysosomes. Secretory lysosomes are the only lysosomes present in CTLs and have a dual function (Burkhardt et al., 1990; Peters et al., 1991). They act as the store of acid hydrolases for the digestion of endocytosed macromolecules, and they contain the cytotoxic components that are necessary for killing.

The key soluble component of the lytic granules, perforin was identified by the homology to the C9 of complement system (Shinkai et al., 1988; Tschopp et al., 1986). Perforin was found to form pores of size 15-16 nm in the membrane of red blood cells and so was also functionally similar (Sauer et al., 1991). There are other components that are crucial for target cell apoptosis, the serine proteases called granzymes. The action of both perforin and granzymes is required for target cell killing (Nakajima et al., 1995; Shiver and Henkart, 1991). Independent experiments have shown that the pores made by perforin are needed for the granzymes to enter the target cell. There are several forms of granzymes that mediate different modes of

target cell death. Granzyme B cleaves caspase and brings rapid cell death and Granzyme A generates single stranded nicks in the DNA of the target cell leading to its death (Beresford et al., 2001). Studies using effector cells deficient in Granzyme A have shown that both granular killing and Fas mediated killing is normal (Ebnet et al., 1995). However, effector CTLs deficient in Granzyme B show impaired rapid DNA fragmentation, occurrence of late DNA fragmentation and normal Fas mediated killing (Trambas and Griffiths, 2003).

Perforin activity is acutely dependent on pH and so drops rapidly at a pH of 4.5 (the pH of endosomal compartments). Therefore perforin does not disrupt the membrane of the endosomal compartments where it is stored (Kuta et al., 1989). Upon release in the synaptic cleft where the pH is no longer acidic, the activity of perforin is restored. It integrates into the membrane of the target cell through its lipid binding C2 domain. Another molecule that has been shown to play an important role in self protection is cathepsin B. Cathepsin B is present on the granule membrane of CTLs and upon exocytosis gets incorporated into the membrane of the CTL. This cathepsin B can cleave perforin that binds to the target cell membrane and thereby prevents pore formation in the membrane of the target cell (Balaji et al., 2002).

Killer T cells are also protected from granzyme B mediated killing by the expression of serpins which are serine protease inhibitors on their membrane (Hirst et al., 2003).

1.5 The immunological synapse

Synapse means connection and the term neuronal synapse was used to describe connections between neurons. It is evident that the formation of cell-cell contact is very important for mediating and regulating immune responses as well and so the term immunological synapse (IS) to describe connections between immune cells and their specific target cells was coined in the 1990s by N. Norcoss and also by W. Paul and colleagues (Norcross, 1984; Paul et al., 1987). There are various types and functions of immunological synapses depending on the type of cells they connect. The synapse that is important for us is the one that is formed between CTLs and target cells. It is called the secretory immunological synapse (Fig. 2) and is a variant of the mature or fully segregated synapse whose formation consists of different

phases of signaling (Bossi et al., 2002; Potter et al., 2001; Stinchcombe et al., 2001b).

The first phase leads to a stop signal that causes migrating T cells to come to a transient arrest when they recognize cognate peptide MHC complexes (Dustin et al., 1997). This is followed by the phase of signal induction which is very crucial as it determines the fate of the cell-cell contact. The first few seconds of contact induces calcium signaling followed by recruitment of signaling molecules such as CD3ζ, LCK, ZAP70 and phosphatidylinositol 3-kinase (PI3K), followed by phosphorylation of LCK and ZAP70. TCR, CD8 and CD28 move to the contact area of dynamic signaling within 30-60 s of contact formation (Horejsi et al., 2004; Stradal et al., 2004; Zal et al., 2002). TCR generates signals that induce the activation of several tyrosine kinases of the Src and Syk families, followed by the assembly of a platform of signaling and adaptor proteins. This signaling platform is required for the activation of downstream effectors, including PI3K (Robertson et al., 2005) and Rho GTPases. PI3K via its Src homology 2 domain, binds to phosphotyrosine residues in the platform and generates a local increase in phosphatidyl inositol–triphosphate which clusters in the inner leaflet of the plasma membrane. The Rho GTPases rac1 and Rho control the assembly of actin filaments and cytoskeletal dynamics close to the cell-cell junction (Villalba et al., 2001). After 5-30 minutes of continuous T cell-APC contact defines the third phase of the IS formation which is defined by molecular segregation. A central zone containing TCR, CD3 as well as associated protein kinase C is formed called the central supramolecular activation cluster (cSMAC). It is persistence of TCR signaling at the IS that leads to the accumulation of surface TCRs at the cSMAC. This is mediated by actin and endosomal vesicles (Das et al., 2004). Surrounding this zone is the peripheral Supramolecular activation cluster (pSMAC) comprising Talin, lymphocyte function associated antigen 1 (LFA1), CD2 and CD11a (O'Keefe et al., 2004; Stinchcombe et al., 2001b). Adjacent to the TCR enriched cSMAC is the secretory domain that contains the microtubule organizing centre (MTOC), polymerized tubulin. It is at this secretory domain that the release of lytic granules takes place (Kuhn and Poenie, 2002).

If the segregation of molecules is arrested before the complete clustering of TCR molecules or signaling molecules, an unsegregated IS is formed. In this case, the

TCR and signaling molecules show a diffused pattern at the contact zone. Naïve CD8 T cells form an unsegregated IS (see 1.5) during their initial phase of contact with numerous DCs and have shown to become activated without a discrete cSMAC. An unsegregated synapse was seen in CTLs in the cases of low antigen stimulation and resulted in vesicle exocytosis and target cell killing (Faroudi et al., 2003).

The fourth phase after sustained signaling leads to the internalization of TCR and sorting to the lysosomes for degradation. This downregulation of TCR is thought to be important for terminating the IS thereby leading to IS resolution resulting in the separation of the two cells (Liu et al., 2000). This marks the final phase of CTL-target cell interaction.

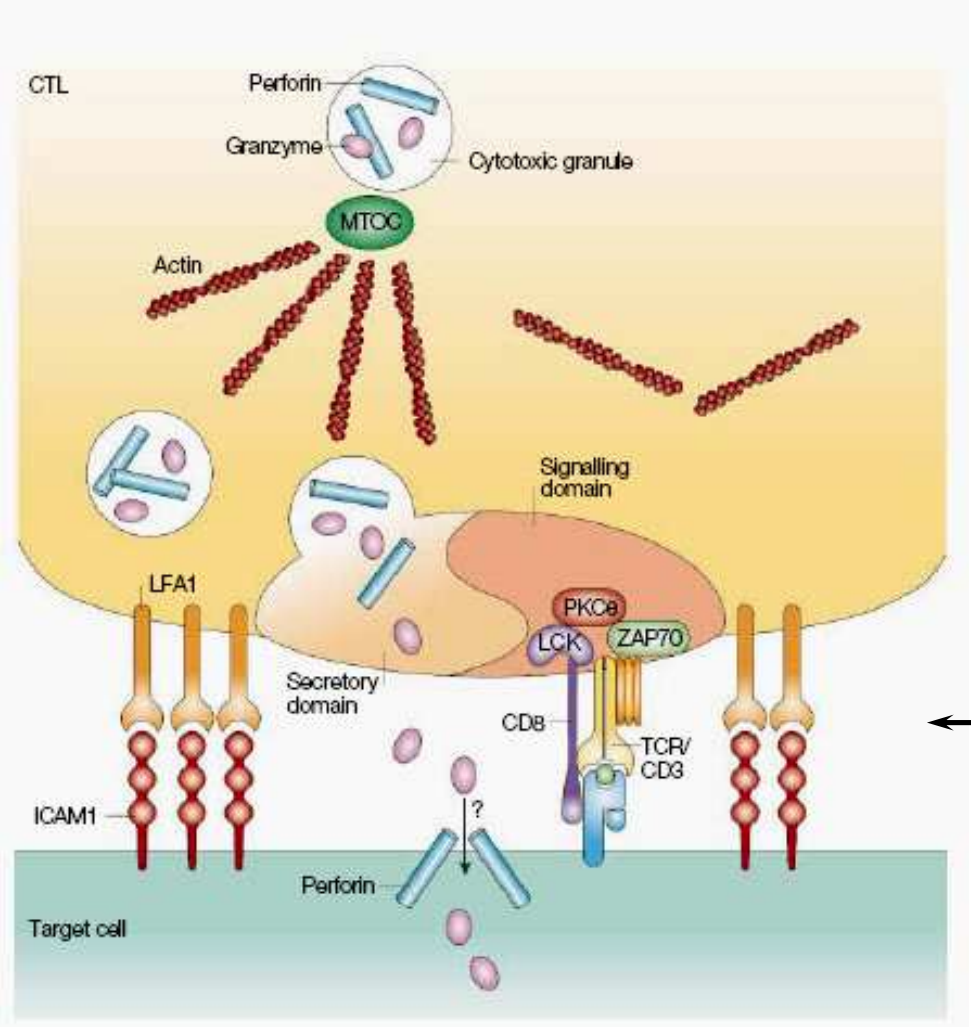

Figure 2. The secretory immunological synapse (IS) in CTLs.
Clustering of the TCR/CD3 complex to form the cSMAC is followed by fusion of lytic granules at the secretory domain adjacent to the immunological synapse (Lieberman, 2003).

1.6 Intracellular trafficking through endosomes

Trafficking in eukaryotic cells involves the transport of cargo from a donor compartment to a target compartment and is essential for the normal functioning of cells. Trafficking includes both the sorting of newly synthesized proteins from the ER and golgi to their respective destination and the transfer of proteins from the plasma membrane either to the lysosomes for degradation or for to recycling endosomes for recycling them back to the plasma membrane. Proteins that are endocytosed from the plasma membrane have three possible destinations – the plasma membrane, the trans-golgi network (TGN) or the lysosomes (Seaman, 2004).

The first branch point from the plasma membrane in the endocytic pathway is the early endosome which matures into the late endosome. The mechanism by which late endosomes are formed from early endosomes has been a subject of dispute. One model suggests that the late endosomes are formed from the early endosomes by gradual addition of late endosome components and removal of early endosome components. Rab5 which is a marker for early endosomes has been shown to gradually disappear with the subsequent acquisition of Rab7 marking the progression from early to late endosomes (Rink et al., 2005). The pH is also supposed to be a factor for marking the progression of early to late endosomes as Bafilomycin A1- a vacuolar H^+-ATPase inhibitor slows the progression from early to late endosomes (Aniento et al., 1996). Late endosomes contain more lumenal vesicles than early endosomes and are often described as multivesicular bodies (MVBs) (Russell et al., 2006) a characteristic that is promoted by lipids (Matsuo et al., 2004).The formation of lysosomes from MVBs has been studied in cell free content mixing assays providing evidence that late endosomes or MVBs fuse directly with the lysosomes (Bright et al., 1997; Mullock et al., 2000; Ward et al., 2000b). Endosome lysosome fusion events have several characteristics. Content mixing was only observed when the organelles were in physical contact. Organelles either transiently fuse (kissing events) or undergo permanent fusion. Kissing events often, but not always, precede full fusion. Third, contents were sometimes exchanged between organelles by tubules that occurred from either type of organelle. Tubules facilitate the exchange of contents between organelles by both kissing and full fusion events (Bright et al., 2005). Fusion of late endosomes and lysosomes as with other fusion events in the

secretory and endocytic pathways requires the presence of SNARE proteins and a small GTPase of the Rab family, Rab7 (Mullock et al., 1998). The route from the sorting endosome back to the plasma membrane can be either by direct fusion (Hao and Maxfield, 2000) or though the endocytic recycling compartment which is a long lived organelle (Sheff et al., 1999).

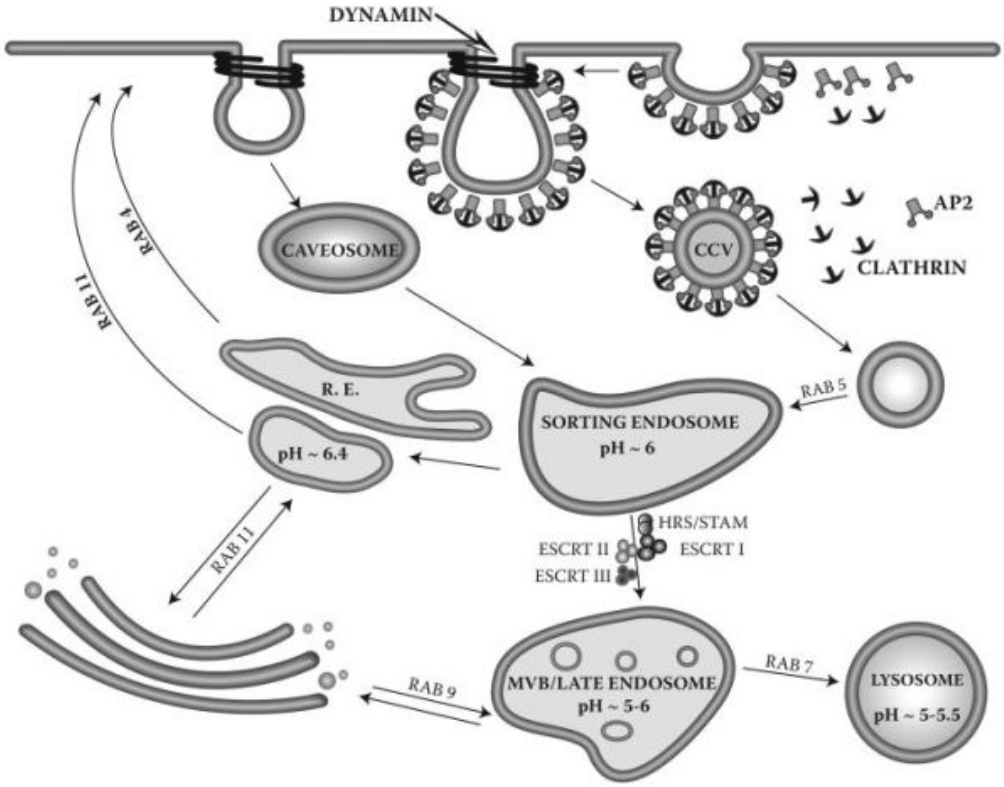

Figure 3. The clathrin mediated endocytic pathway.
Recruitment of AP2 and clathrin to the cell membrane causes this to deform and invaginate. The accessory protein amphiphysin recruits the GTPase dynamin, which acts as a "pinchase" to release the clathrin-coated vesicle. Upon shedding of the clathrin coat, the cargo containing vesicle fuses with the sorting endosome by action of Rab5 and the aid of accessory endosomal proteins such as EEA1. Cargo can then be recycled via the recycling endosome (RE), an event mediated by Rab4 or Rab11, or transported to late endosomes/multivesicular bodies (MVBs) with the aid of Hrs and the ESCRT machinery. From late endosomes proteins can be recycled via the Golgi or degraded in the lysosome. The diagram also shows how proteins internalized in a caveolin dependent manner can join the clathrin endocytic pathway at the level of the sorting endosome and are also dependent on dynamin activity.

1.7 Soluble N-ethylmaleimide Sensitive Factor Attachment Receptors (SNAREs)

SNAREs belong to a family of membrane proteins that have been implicated as the conserved core protein machinery required for all intracellular membrane fusion events that mediate intracellular trafficking (Chen and Scheller, 2001; Jahn et al., 2003). The synaptic proteins syntaxin (STX1), SNAP-25 (25 kDa synaptosome-associated protein) and VAMP1 (vesicle-associated membrane protein) were the first SNAREs to be discovered (Bennett et al., 1992; Oyler et al., 1989; Trimble et al., 1988). The hallmark of all SNARE proteins is that they contain a conserved SNARE motif of about 60 residues that mediates SNARE-SNARE protein interaction. Most SNAREs contain only one SNARE motif near the C-terminus except three SNAREs (SNAP-23, SNAP-25 and SNAP-29) which contain two SNARE motifs separated by a linker region. The crystal structure of the synaptic SNARE complex consisting of one SNARE domain of STX1 and VAMP2 and two SNARE domains of SNAP25 reveal that the four SNARE domains form a twisted parallel 4 helical bundle with each SNARE domain contributing one helix. It is the four helical bundle that drives fusion (Fig. 4). SNAREs were initially classified functionally as v-SNAREs and t-SNARES based on their localisation on the vesicle or target membrane respectively (Sollner et al., 1993a). The terms R SNAREs (arginine containing SNAREs) and Q SNAREs (glutamine containing SNAREs) were introduced later to classify SNAREs based on their structure (Fasshauer et al., 1998b). There are at present 38 known members of the mammalian SNARE family. This core SNARE complex which mediates the SNARE-SNARE protein interactions that are pivotal to the function of these proteins is extremely heat stable up to 90°C, resistant to SDS denaturation, protease digestion and clostridial neurotoxin cleavage (Fasshauer et al., 1998a; Hayashi et al., 1994; Poirier et al., 1998). Within the SNARE complex the four helices are connected by 16 layers of interacting surfaces mediated by the side chain of the residues and which are mostly hydrophobic and are arranged perpendicular to the axis of the four helical bundle. The middle of the bundle (see Figure 4) is characterized by a layer (the 0 layer) of interaction mediated by three Glutamine (Q) residues (one contributed by STX1 and two by SNAP-25), and one Arginine (R) residue (contributed by VAMP2).

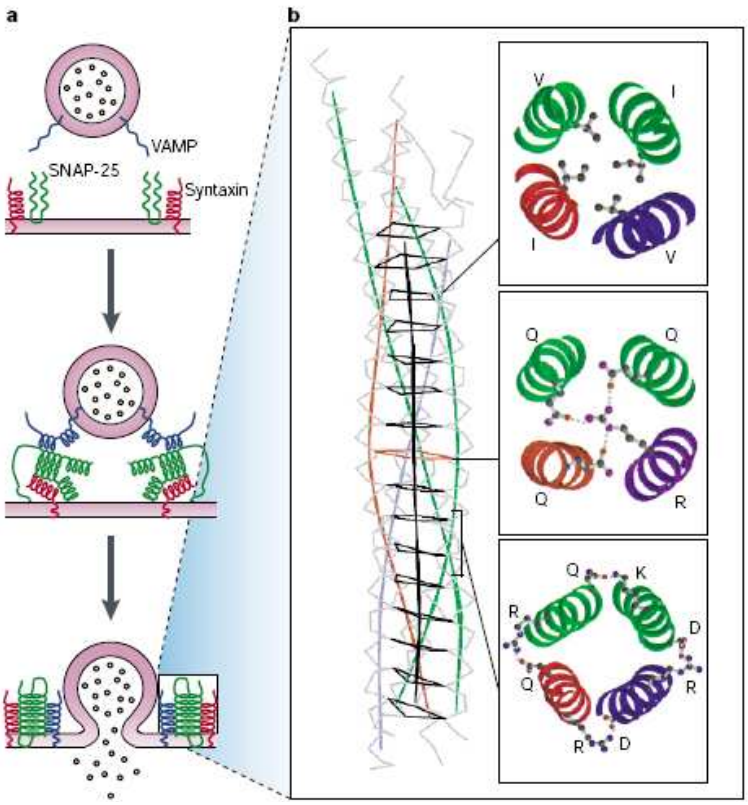

Figure 4. SNARE proteins form a four helical complex that drives membrane fusion.
(a) VAMP (blue) on the vesicle interacts with syntaxin (red) and SNAP-25 (green) on the plasma membrane to form a four helical bundle that zips up concomitant with bilayer fusion. (b) Back bone of the SNARE complex with the central ionic layer (red) and 15 hydrophobic layers (black) that mediate the core interactions. The ball and stick structures represent the indicated amino acids and the dotted lines represent hydrogens or salt bridges stabilizing the interaction (Chen and Scheller, 2001).

SNAREs are thus classified as Q and R SNAREs based on the presence of either Q or R at this position. R SNAREs are single transmembrane proteins that contribute one SNARE motif to the trans SNARE complex. Individual Q SNARE proteins are classified as Qa, Qb, Qc or Qbc SNAREs on the basis of the relative position of their SNARE motifs in the assembled trans SNARE complex. Q SNAREs are present on the target membrane and form a complex with the vesicular R SNARE present on the donor membrane. The Q SNARE functions as a complex that is composed of two or three polypeptides which together contribute three SNARE motifs to the trans SNARE complex. Most of the Q SNAREs are single SNARE motif, transmembrane polypeptides. The main exceptions to this are the Qbc SNAREs (SNAP-23, 25, 29 and 47), which are peripheral membrane proteins that lack a transmembrane domain.

A functional trans SNARE complex therefore draws from the subsets described to form R-Qa-Qb-Qc or R-Qa-Qbc configurations to provide for selective membrane fusion at different sites in the cell. The interaction of R and Q SNAREs in the transient trans SNARE complex serves to pull the vesicle and target membrane together. Crystallographic studies have shown that twisting or zippering of the four alpha helices of the SNARE motifs in an assembled trans SNARE complex has the potential to generate the force required to fuse the lipid bilayers (Fasshauer, 2003; Sutton et al., 1998). Once fusion has occurred, the vesicle membrane is in continuity with the target membrane and the SNAREs are atleast transiently in the formation of the cis SNARE complex in the same membrane. After fusion, the SNAREs are rapidly disassembled so that the R-SNARE and the Q SNARE components can be reused for subsequent membrane fusion events. The R SNAREs must be reloaded into carriers for transport back into their original site on a donor compartment. The disassembly of the cis SNARE complex is mediated by a soluble complex containing the ubiquitous cytoplasmic ATPase NSF (N ethyl maleimide sensitive factor) and alpha SNAP (Furst et al., 2003; Hohl et al., 1998; Sollner et al., 1993b).

1.8 Genetic defects in killing

Many human diseases and their mouse counterparts are caused by defects in the secretory pathway required for cytotoxicity (Arico et al., 2002; Feldmann et al., 2003; Menasche et al., 2000). Several of these diseases show an unusual combination of immunodeficiency and albinism reflecting similarities in the cell biology of secretory machinery used by immune cells and melanocytes. Some genetic defects affecting melanocyte secretion have no effect on CTL killing indicating that some components may differ between the two cell types. Griscelli syndrome is characterized by albinism and loss of CTL activity. The defective gene encodes a small GTP binding protein Rab 27a (Menasche et al., 2000). A similar loss of Rab 27a expression has been identified in ashen mice. CTLs from ashen mice cannot kill their targets through the granule pathway. Although they have normal number of granules with normal morphology a clear defect in secretion leads to the phenotypic defect in killing. Live cell imaging revealed that the granules polarize to the IS but do not reach the IS and

therefore the secretory process is not completed. Notably in CTLs missing the geranyl geranyl transferase required for Rab prenylation, lytic granules fail to polarize which suggests that other Rab proteins are involved in earlier phases of granule trafficking.

Chediak-Hegashi Syndrome (CHS) is another rare autosomal recessive disorder that is characterized by partial albinism and NK and CTL killing defect (Ward et al., 2000a). The most prominent morphological feature is that the lysosomes are big in size but are not secreted. The gene that is mutated in this disorder is CHS or LYST (Barbosa et al., 1996). The early biogenesis of the granules is normal in patients with CHS and both perforin and granzymes are sorted correctly to the lysosome (Baetz et al., 1995). However later during development the granules fuse to form giant organelles. This is because there is a defect in the membrane fusion or fission which occur during the biogenesis of the organelles (Stinchcombe et al., 2000). The enlarged organelles in CTLs can polarize and dock normally at the immunological synapse. CHS interacts with the SNARE complex (Tchernev et al., 2002) consistent with a role in membrane fusion or fission.

Familial Hemophagocytic lymphohistiocytosis (FHL) represents another set of genetic disorders associated with secretory defects in CTLs but not melanocytes (Voskoboinik et al., 2006). It is a devastating disease where lymphocytes (especially CD8 T cells) proliferate uncontrollably and infiltrate into tissues. The production of excessive cytokines and infiltration into tissues leads to massive tissue necrosis and organ failure (Henter et al., 1998). Without bone marrow transplant, FHL is usually fatal till the first year of life. Killer lymphocytes from patients with FHL are deficient in their ability to deliver perforin to the IS. FHL type 2 is caused by a loss of perforin itself. Several mutations in perforin cause FHL (Arico et al., 2002; Feldmann et al., 2002; Goransdotter Ericson et al., 2001; Suga et al., 2002). FHL type 3 results from mutations in the gene encoding Munc13-4 (Feldmann et al., 2003). In CTLs derived from patients with FHL type 3, lytic granules dock at the IS but fail to fuse. FHL type 4 which is clinically similar to but less severe than FHL type 3 is caused by defects in Syntaxin11, a SNARE protein (Albayrak et al., 2009; zur Stadt et al., 2005). This deficiency impairs granule exocytosis without affecting polarization which suggests an important function downstream of the recruitment of granules to the IS. FHL type 5

is reported to be due to mutations in Munc18-2 that impair its binding with Syntaxin11 giving further insight into the molecular mechanism of CTL mediated killing (Cote et al., 2009; zur Stadt et al., 2009).

The perforin mediated cell death pathway is also important for immune homeostasis as is revealed in FHL where the function of CTL killing another CTL is lost. It has been reported that CTLs acquire membrane from dying target cells and this leads to the acquisition of MHC class I molecule on the surface of CTLs which are in turn recognized by other CTLs and thus an immunological synapse is formed between two CTLs which results in lytic granule polarization and death. This explains the uncontrollable proliferation of CTLs when perforin gene is not functional. When homeostasis is impaired CTLs begin to proliferate more in order to kill more efficiently.

1.9 Syntaxin7

Since SNAREs are important for the specificity of vesicular transport we were interested in the role of SNARE proteins in CTL mediated cytotoxicity. The functions of some SNARE proteins have been identified in other immune cell types such as mast cells, macrophages and natural killer cells (Murray et al., 2005b). Most SNAREs are membrane proteins that have a C terminal hydrophobic tail through which they bind to their respective membrane (Fasshauer, 2003).

Syntaxin7 is a widely expressed SNARE protein containing 261 amino acids (aa). The region of Syntaxin7 that is most related all other syntaxins lies between residues 157 and 235, just before its C terminal domain (Wong et al., 1998). It shares the highest homology to Syntaxin1. The C terminal hydrophobic domain is important as it serves as a membrane anchor. In addition it contains di-leucine based motifs that are sorting signals and therefore necessary for their intracellular localization and trafficking via distinct transport pathways (Kasai and Akagawa, 2001). The N terminal region of Syntaxin7 has a three helical bundle consisting of Ha, Hb and Hc regions that precede the N terminal domain. The Habc 3-helical bundle in some Qa SNAREs such as Syntaxin1 and Syntaxin7 can fold back to interact with the C terminal SNARE motif to generate a closed conformation (Antonin et al., 2002a; Fasshauer,

2003; Misura et al., 2000). The SNARE motif in the closed conformation cannot bind to other SNAREs and therefore the closed conformation downregulates the capability of Syntaxin to form SNARE complexes. The regulation of neuronal Syntaxin1 by the N-terminus has been well studied. Munc18-1, a member of the Sec1/Munc18 (SM) family, binds tightly to the closed conformation of Syntaxin1 and stabilizes it (Dulubova et al., 1999). Munc18-1 may serve as a negative regulator preventing Syntaxin1 from forming SNARE complexes, although it has an essential role in exocytosis. The N terminal region of Syntaxin7 also decreases the rate of SNARE complex assembly 7-fold, thus playing a regulatory role (Antonin et al., 2002a).

Syntaxin7 is known to be an endosomal SNARE. However, the localization of Syntaxin7 has been controversial. It was first identified to be a member of the early endosome compartment (Nakamura et al., 2000; Prekeris et al., 1999; Wong et al., 1998). However later studies have shown that it is also localized to late endosomes (Collins et al., 2002; Mullock et al., 2000; Ward et al., 2000b) and lysosomes (Wang et al., 1997). Syntaxin7 has also shown to be the only endosomal SNARE apart from Syntaxin8 to be constantly recycling between the endosomes and the plasma membrane (Prekeris et al., 1999). Syntaxin7 forms a SNARE complex with Vti1b, Syntaxin8 and VAMP7 (other endosomal SNAREs) and the structure of this complex has been crystallized (Antonin et al., 2002b), revealing conserved structural features with the neuronal SNARE complex.

In macrophages, Syntaxin7 has been shown to be important for phagocytosis and TNF secretion (Murray et al., 2005a). It has been found to be enriched in the secretory lysosomes of NK cells implicating a role in exocytosis (Casey et al., 2007). The importance of the endosomal trafficking pathway was especially highlighted in a study where HIV1 infected lymphocytes have a severe defect in intracellular trafficking and signaling (Thoulouze et al., 2006). Further studies in the importance of the endocytic pathway and endosomal proteins might give new insight in to the function of CTLs.

1.10 Aim of the work

Immune responses are dependent on effective cell-cell communication. One effective way of communication is by forming necessary contacts between the cells. Cytotoxic T lymphocytes function in killing infected cells in the body and to do so they must first come in contact with the target cell. Once contact is formed, cytotoxic components that are stored in lytic granules are released at the contact zone. This constitutes the granule mediated pathway of target cell killing. Although there are other pathways used by CTLs for killing target cells the granule mediated pathway is the most important (Trapani and Smyth, 2002). Most of the molecular players important for the fusion of lytic granules have not been identified. The function of some of the molecules such as Rab27a (Menasche et al., 2000; Stinchcombe et al., 2001a) and Munc13-4 has been studied in lytic granule fusion (Feldmann et al., 2003). However, the SNARE proteins (essential mediators of almost all fusion events) that are important for CTL function and lytic granule release still remain unknown. Mutations in the SNARE protein Syntaxin11 lead to defective degranulation in lymphocytes (Albayrak et al., 2009; Arneson et al., 2007; zur Stadt et al., 2005). This ascertains the importance of SNAREs in CTL function. The identification of specific SNARE proteins involved in lytic granule release is necessary for understanding the underlying molecular mechanism behind the killing process.

The work presented here is aimed to study the importance of SNAREs for the fusion of lytic granules at the IS. Syntaxin7, a Qa SNARE protein is a key component of NK cell secretory lysosomes (Casey et al., 2007) whose precise function in CTL mediated killing if at all is unknown. We present our results on the detailed functional analysis of Syntaxin7 in CTLs, a work that was performed to better understand CTL effector function.

2. Materials and Methods

2.1 Materials

2.1.1 Reagents

Agarose	Roth
Aprotinin	Sigma
BSA (Bovine Serum Albumin)	Sigma
Chloroform	Roth
Diethylpyrocarbonate (DEPC)	Sigma
Dithiothreitol (DTT)	Sigma
ECL reagent	GE Healthcare
EDTA (Ethylenediaminetetraacetate)	Sigma
Ethanol	Roth
Ethidium Bromide	Invitrogen
FCS (Fetal Calf Serum)	Invitrogen
Ficol	GIBCO
Formaldehyde (16%)	Polysciences
Glucose	Merck
Glycine	Roth
HBSS (Hanks balanced salt solution)	PAA
N-(2-Hydroxyethyl)-1 piperazine-N'-(ethanesulfonic acid) (HEPES)	Sigma
H_20	Sigma
Nitrocellulose membrane	Roth
PBS (Phosphate Buffered Saline)	GIBCO
Propanol	Roth
Skimmed milk powder	Naturaflor
TritonX100	Roth
TRIzol® reagent	Invitrogen

All other chemicals if not specified otherwise were from Sigma.

2.1.1.1 Antibodies

Alexa^{488} anti-CD3	Biolegend
Alexa^{647}anti-CD3	Bioscience
CD3	Euroclone
CD28	BD
EEA1	BD
GranzymeA	Biolegend
Perforin	Biolegend
Rab7	AbCam
SNAP-23	Synaptic System
Syntaxin3	Synaptic System
Syntaxin4	Synaptic Systems
Syntaxin7	Synaptic System
Syntaxin7	Osenses
Syntaxin10	BD
Syntaxin17	BD
Talin	AbCam
VAMP3	Synaptic System
VAMP4	Synaptic System

2.1.2 Plasmids

Perforin-mCherry

Perforin was amplified from human cDNA with primers 5' TAT ATA AGA TCT CCA CCA TGG CAG CCC GTC TGC TCC and 5' TAT ATA TAC CGG TGG CCA CAC GGC CCC ACT CCG G to add BglII and Age1 restriction sites.. The mCherry construct was obtained as a gift from Roger Tsien. After AgeI and BglII restriction digestion, perforin was ligated to mCherry to yield a C-terminal tagged perforin.-mCherry.

Syntaxin7-mTFP1 and Syntaxin7ΔC-mTFP1

Syntaxin7 was amplified from human cDNA with primers 5' TAT ATA AGA TCT CCA CCA TGT CTT ACA CTC CAG GAG TTG and 5' TAT ATA ACC GGT GGG TGG

TTC AAT CCC CAT ATG ATG to add BglII and BsH TI restriction sites.The mTFP construct was obtained from Allele Biotech, San Diego. After BglII and BshTI restriction digestion, Syntaxin7 was ligated to mTFP to yield a C-terminal tagged Syntaxin7. For the dominant negative mutation of Syntaxin7 (Syntaxin7ΔC) only the first 200 amino acids without its transmembrane was amplified from human cDNA with primers 5' TAT ATA AGA TCT CCA CCA TGT CTT ACA CTC CAG GAG TTG and 5' TAT ATA ACC GGT GGC AGG GTT TTT CTG GAT TTG CG to add Bgl2 and BsH T1. The mTFP construct (obtained from Allele Biotech, San Diego). After Bgl2 and BshT1 restriction digestion, Syntaxin7ΔC was ligated to mTFP at the C-terminus.

2.1.3 Media and Solutions

2.1.3.1 Solutions for CTL preparation

- Ficol
- HBSS
- Erythrocyte lysis buffer:
  155 mM NH_4 Cl
  10 mM $KHCO_3$
  0,1 mM EDTA
  pH 7.3
- Buffer 1: PBS (GIBCO) supplemented with 0.5% BSA
- Buffer 2: RPMI medium (Invitrogen) with 0.1% FCS
- AIMV medium supplemented with 10% FCS (Invitrogen)

2.1.3.2 Solutions for CTL fixation and Immunostaining

- Polyornithine (0.1mg/ml)
- PBS (GIBCO)
- 4% PFA in PBS (Diluted from 10%PFA in PBS stock solution)
- PBS in 0.1M Glycine
- PBS 2% BSA 0.1% Triton
- PBS 0.1% Triton

- Mounting medium

    Mowiol 4-88

    Glycerol

    ddH_20

    0.2MTris-Buffer (pH8.5)

2.1.3.3 Solution for TIRFM experiments

- 10 mM extracellular Calcium solution

    140 mM NaCl

    4.5 mM KCl

    5 mM HEPES

    2 mM $MgCl_2$

    10 mM $CaCl_2$

    pH: 7.4 and Osmolarity: 300-310 mOsm.

2.2 Methods

2.2.1 Peripheral blood mononuclearcells (PBMCs) Isolation

The starting material for the isolation of PBMCs was Leukocyte reduction chambers (LRCs) containing whole blood obtained from the clinic of the department of Haematology and Transfusion medicine, University of Saarland. All the steps for the isolation were carried out at room temperature (RT). 15-17 ml Ficoll was added before the isolation in special leukocyte separation tubes and spun at 1000 g for 30 seconds. Shown in figure 5 is a picture of the cone shaped LRC containing blood from a healthy donor. The hose was cut at the two points marked 1 and 2 in figure 5a with sterile scissors cleaned with 70% ethanol. First, the lower hose was cut and made to point to the inside of the leukocyte separation tube as shown in figure 5. Then the top tube was cut to allow the flow of blood through the cone. A 20 ml syringe containing HBSS was used to rinse the blood flowing through the cone into the LRC as shown in figure 5b.

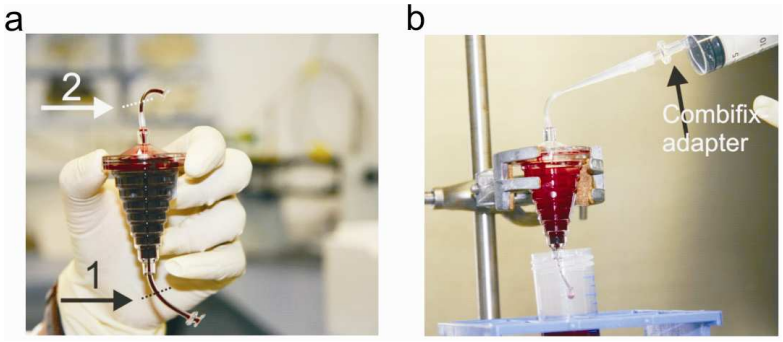

Figure 5. Isolation of PBMCs.
Depicted are the cone shaped leukocyte reduction chambers used for isolation. See text for details.

The leukocyte separation tubes were then carefully mixed and spun at 450 g for 30 min at RT without any brake or special settings. The leukocyte containing ring that was obtained after centrifugation was removed very carefully with a 5 ml pipette and transferred to a 50 ml falcon tube and then filled with HBSS. This was followed by centrifugation at 250 g for 15 min at RT. The red pellet obtained after centrifugation was resuspended in 1-3 ml erythrocyte lysis buffer (see Reagents for details) for the

erythrocyte lysis process. After 60-120 s, 50 ml HBSS was added to the pellet to stop the lysis process and the tubes were spun at 200 g for 10 min.

The white pellet obtained was devoid of erythrocytes and was resuspended in 20 ml cold PBS/0.5%BSA. A small amount of the cells was taken to make a 1:10 dilution in PBS. Trypan blue staining was used for checking the viability of the purified PBMCs before using them for isolating naïve CD8T cells or for stimulation with Staphylococcus enterotoxin A.

2.2.2 Negative isolation of naïve CD8 T cells

Naïve CD8+ T cells were negatively isolated from PBMCs with a CD8 negative isolation kit (Invitrogen). The percentage of CD8+ T cells obtained by this method from PBMCs was usually 10-12% and so the intial number of PBMCs for all the isolations varied according to the number of CD8+ T cells required. PBMCs were resuspended at a concentration of 100 million/ml in a Ca^{2+} and Mg^{2+} free phosphate buffer (supplemented with 0.1% BSA and 2 mM EDTA), as the starting material for the negative isolation. Heat inactivated FCS and the antibody mix that was provided by the kit were mixed in a 1:1 ratio and added to the PBMCs for incubation at 4°C by gentle rotation for 20 minutes. The volume of the antibody mix was recommended as 20 µl by the manufacturer for the starting material of 100 million PBMCs. For isolating from more PBMCs the volumes of all the reagents were scaled accordingly for every step of the isolation. The cells were then washed by adding 2 ml of the isolation buffer and centrifuged at 300 g for 8 min at 4°C. The pellet was resuspended in 800 µl of the isolation buffer and then 200 µl of the pre-washed depletion dynabeads (supplied in the kit) was added. For prewashing, 200 µl of the depletion dynabeads were transferred to a fresh tube and the same volume of isolation buffer, or at least 1 ml, was added and mixed. The tube was kept in a magnet for 1 minute and then the supernatant was discarded. The beads were then resuspended in 200 µl of the isolation buffer. Subsequently, the cells and beads were mixed and incubated for 15 min at 18-25°C by gentle tilting and rotation. The tube was then placed in the magnet for 2 min. The supernatant contained the negatively isolated naïve CD8T cells and was transferred to a new tube. The cells were then stained with trypan blue for checking the viability of the purified CD8T cells. Centrifugation was done at 200 g for

5 minutes at RT and the pellet was resuspended in fresh and pre-warmed AIMV medium (Invitrogen) containing 10% FCS at a volume of 3 million/ml.

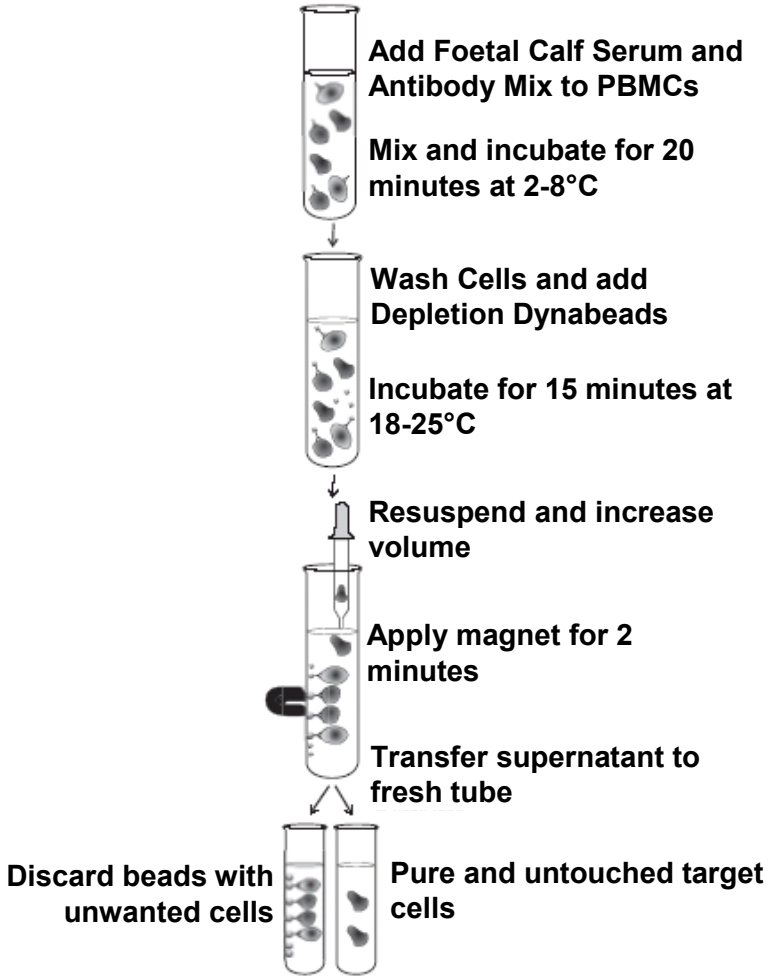

Figure 6. Schematic outline of the negative isolation of naive CD8 T cells.
The isolation of naïve CD8 T cells from PBMCs using dynabeads (See text for details).

2.2.3 Generation of effector Cytotoxic T lympocytes (CTLs)

2.2.3.1 Stimulation by CD3/CD28 coated beads

Naïve CD8T cells from the negative isolation were stimulated with CD3/CD28 T cell expander beads (Invitrogen) at a 1:1 ratio. The appropriate volume of expander beads was transferred to a fresh tube and equal volume of Buffer 1 (PBS supplemented with 0.1% BSA, pH 7.4) was added to the tube. The tube was then placed in a magnet for 1 minute and the supernatant was discarded. The beads were resuspended in the same volume of Buffer 1 that was initially taken for washing. The

beads were then added to the cells and plated at a concentration of 2 million/ml for 3 days.

2.2.3.2 Stimulation by superantigen A

PBMCs from healthy donors were stimulated with 5 µg/ml of Staphylococcus enterotoxin A (SEA) at a density of 100 million/ml, at 37°C for one hour. After that, stimulated PBMCs were diluted to a density of 20 million/ml in AIMV medium (Invitrogen) supplemented with 10% FCS and 100 U/ml of recombinant human IL-2. After 5 days, SEA-specific CTLs were positively isolated with Dynabeads (Invitrogen). The PBMCs were at a concentration of 10 million/ml before starting the isolation. The appropriate volume of Dynabeads as recommended by the manufacturer was added to the cells and incubated by gentle rotation at around 4°C. The beads were coated with anti-CD8 antibody and so only the cells that are CD8 positive would bind to the beads. The cells and beads were then placed in a magnet for about two minutes and then the bead bound cells were washed with the buffer1 that is Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) supplemented with 0.1% BSA and 2mM EDTA. This step was repeated three times and then the cells that were bound to the beads were incubated in buffer 2 (RPMI medium supplemented with 0.1% FCS) for 45 minutes at RT. This step was to ensure that the positively isolated CTLs were detached from the beads. After this detachment, step the cells were placed in a magnet for two minutes and the supernatant containing the CTLs was transferred to a fresh tube. The remaining beads were washed four times to ensure that all the cells that were detached from the beads were obtained. The cells were stained with trypan blue to check the viability of the positively isolated CTLs. The cells were then spun at 200 g for five minutes at RT and the pellet containing cells were resuspended in AIMV medium supplemented with 10% FCS and 100 U/ml of recombinant human IL-2 (Biosource). The cells were used up to day three after isolation for experiments.

2.2.4 Reverse Transcriptase PCR

2.2.4.1 Preparation of probes and RNA isolation

15 million naïve CD8 T cells from one donor that were freshly isolated were spun at 300 g for five minutes at RT and then the pellet was resuspended in 800 µl TRIzol®

Reagent (Invitrogen, #15596018). The sample was then frozen at -80°C. The remaining cells from the same donor were then stimulated with anti-CD3 and anti-CD28 coated beads as mentioned above for cell stimulation. 10 million CD8 T cells after one day of stimulation and 5 million each of CD8 T cells after 3 and 5 days of stimulation were all treated the same way as naïve CD8 T cells. RNA was then isolated from the thawed probes in TRIzol® Reagent. The tubes were spun at 12000 g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and then incubated at RT for 5 minutes. 200 µl of chloroform was added and then the tubes were shaken vigorously for 15 minutes and incubated at RT for 2 or 3 minutes. The tubes were spun again at 12000 g for 5 minutes at 4°C. After this centrifugation, the aqueous phase that was obtained was transferred very carefully to a new tube and 1 µl glycogen was added (this was done only for unstimulated cells to get more optimum amounts of RNA). 500 µl of isopropanol was then added for precipitation and incubated at RT for 10 minutes. The tubes were then spun at 12000 g for 10 minutes at 4°C. The supernatant was then removed and 1 ml 75% ethanol prepared in diethyl-pyrocarbonate (DEPC) water was added. The tubes were spun at 7500 g for 5 minutes at 4°C. The supernatant was removed and the pellet was air dried at RT. Care was taken not to over dry the pellet as that would affect the solubility of the RNA. When the color of the pellet changed from white to transparent, 20 µl of DEPC treated water was added to dissolve the pellet. The concentration of the RNA was measured by UV spectrometry at 260 nm and a small amount of the RNA was also loaded on an agarose gel to check the stability.

2.2.4.2 cDNA preparation and PCR amplification

cDNA obtained after reverse transcription was used for subsequent PCR reactions. All the PCR reactions were normalized based on the amount of cDNA and carried out for all the templates that were unstimulated and stimulated. Primers were designed for human Syntaxin7 gene and were obtained from Gottingen. UbiquitinC was used as a house keeping gene and granzyme B was the positive control.

2.2.5 Preparation of lysates from CTL for western blots and analysis

2.2.5.1 CTL Preparation

All preparative steps were performed at 0-4°C using pre-chilled solutions. Human CTLs were homogenized via syringe five times by hand in a lysis buffer containing 1 mM EDTA, 1 mM DTT, 50 mM Tris-Cl (pH 7.4), 1% TX-100, 1 mM Deoxycholate, 100 mM NaCl and protease inhibitors. Lysates were rotated for thirty minutes at 4°C and insoluble material and cell debris was removed by low speed centrifugation. All extracts contained the protease inhibitors pepstatin A (1 μM), benzamidine (100 μM), leupeptin (1 μM), aprotinin (0.3 μM), phenylmethanesulfonyl fluoride (25 mM), trypsin inhibitor (20 μg/ml), PefBloc SC (1 mM).

2.2.5.2 Western Blot Analysis

Proteins were separated by SDS-PAGE using 4-12% Bis-tris gels. Proteins were then transferred to nitrocellulose (0.2 μm pore diameter). Blots were blocked by incubation with 5% skim milk powder in 20 mM Tris, 0.15 M NaCl (pH 7.4) (TBS) for two hours or overnight. Blots were incubated with affinity-purified antibodies in 5% skim milk powder (SM-TBS) with anti-Syntaxin7 (1:4000 in SM-TBS), anti-GAPDH (1:1000 in SM-TBS). The blots were washed with TBS containing 0.05% Tween-20 (TBST) (five changes), incubated for 1 hour with horseradish peroxidase donkey anti-rabbit (Amersham), diluted 1:40000 or horseradish peroxidase-labeled anti-mouse, diluted 1:10000 in SM-TBST, washed 5-6 times with TBST and developed with ECL reagent (Pierce).

2.2.5.3 Quantitative Analysis

The blots obtained after developing were scanned to generate tif images. Images were analyzed using Adobe CS4 photoshop software. For measurements of pixel density, we multiplied the mean fluorescence value by the pixel value for each band. Normalized data was obtained by dividing the relative intensity over our standard as the common point of comparison according to standard analysis procedures. Measurements were then analyzed using Sigmaplot and Excel.

2.2.6 Electroporation of CTL

Electroporation was done using nucleofector kit supplied by Amaxa/Lonza Biosystems. For electroporating CTLs that were activated with CD3/CD28 beads for three days, the beads were first removed by the magnet. SEA stimulated CTLs after positive isolation were used within 1-3 days of their preparation. The cells were then spun at 100 g for 5 minutes to remove the medium and then washed once with PBS supplemented with 0.5% BSA 5 million CTLs cells were used for one transfection. The maximum amount of DNA used for transfection was 1.5 µg. The program T-023 recommended by the manufacturer for electroporating CTLs cells was used for all the transfections. The cells were washed 6 hours after transfection by a low speed centrifugation at 100 g for 5 minutes and then resuspended in fresh AIMV medium with 50 U/ml IL-2 (Biosource).

2.2.7 Small interfering RNA (siRNA) treatment and Real Time PCR

All siRNA were modified by Qiagen as described by Mantei et al. 2008. SEA-specific CTL were transfected with modified siRNA designed to silence human Syntaxin7 (SI02631307) using nucleofector kit (Lonza) according to the manufacturer's instructions. A modified non-silencing siRNA (#1022076, Qiagen) was used as control. Fresh AIMV medium that was supplemented with recombinant IL-2 (50 U/ml) was given to the cells 12 hours after transfection. The cells were then kept in culture for additional 24 hours before use. For Quantitative RealTime-PCR (qRT-PCR), total RNA was isolated using TRIzol® Reagent (Invitrogen, #15596018) including 1 µl Glycogen (5 µg/µl, Invitrogen, #10814-010) according to the manufacturer's protocol. Templates were prepared from 1.5 x 10^6 SEA-specific CTL and 0.8 µg total RNA was reverse transcribed into cDNA by SuperScript™ II reverse trancriptase (Invitrogen, #18064-014) including 1 µl RNaseOut, (Invitrogen, #10777-019) and 1 µl oligo dT Primer (0.5 µg/µl, Invitrogen, #18418-012) following the manufacturer's instruction. qRT-PCR was carried out in a MX3000 instrument from Stratagene. 1 µl cDNA and 300 nM of each primer were set into PCR reactions (25 µl) using Quanti Tect SYBR green kit (Qiagen, #204145). PCR conditions were: initial denaturation, 15 min, 94 °C; 45 cycles: denaturation, 30 s, 94 °C; annealing, 45 s, 58 °C; elongation, 30 s, 72

°C and finally a dissociation curve cycle (60 s, 95 °C; 30 s 55 °C; 30 s 95 °C). Primers were designed using Primer3 program available at http://frodo.wi.mit.edu/. PCR fragments were confirmed by sequencing (MWG).

2.2.8 Cytotoxicity assay

The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used to detect target lysis. CTL were plated in 96-well plates in AIMV medium (5% FCS) with 1×10^4 SEA-pulsed Raji target cells at various effector/target ratios. CTL and target cells were incubated at 37°C for 4 h and then Lactate dehydrogenase activity in the supernatant was measured. To do this the cells were spun down at 200 g for 4 min. Then 50 µl of the supernatant was taken from each well and incubated with the reaction substrate for 30 min at room temperature. The absorbance was measured at 490 nm with the GENios Pro plate reader (TECAN). Cytotoxity was calculated with the following equation: % Cytotoxicity = (Experimental – Effector Spontaneous – Target Spontaneous) / (Target Maximum – Target Spontaneous) × 100. All cytotoxicity assays were done in triplicates.

2.2.9 CTL fixation and immunofluorescence

2.2.9.1 Conjugation of CTL to target cells

To incubate the SEA CTLs with target cells, Raji cells that were used as cognate target cells were pulsed with 10 µg/ml of SEA at 37°C for 30 min. The stimulation of Raji cells was done in 96 well plates with maximum 1 million cells resuspended in 100 µl AIMV medium. The CTL and SEA-pulsed Raji cells were washed once with AIMV and resuspended at a concentration of 2×10^7 cells/ml. CTLs were mixed with target cells at a 1:1 ratio and left in suspension for 5 min at 37°C. The cell suspension was then diluted to a concentration of 4×10^6 cells/ml with AIMV medium and plated onto poly-ornithine coated 12 mm glass coverslips and incubated at 37°C for 5, 15 and 30 min. Cells were resuspended in a volume of 50 µl for one coverslip.

2.2.9.2 Pre-incubation of CTLs to label recycling TCR

In order to label the recycling TCRs, CTLs were first pre-incubated with alexa 647 anti CD3 Ab at a concentration of 20×10^6 cells/ml. First the required number of CTLs were washed once with AIMV medium and then resuspended in a volume of 50 µl. To this 1 µl of 1 mg/ml alexa 647 anti CD3 Ab was added and plated in one well of a 96 well plate. The maximum number of cells per well was 0.5 million CTLs. After the 30 minute incubation, the CTLs were washed once with AIMV medium and resuspended at the concentration as mentioned above for the incubation of CTLs and Raji cells.

2.2.9.3 CTL fixation and immunostaining

CTLs were fixed with ice cold 4% PFA in PBS (GIBCO) that was diluted from a 10% stock. The fixation was done for 20 minutes at RT in the dark. The cells were then washed with PBS containing 0.1 M Glycine for 3 minutes. This was done to ensure the removal of excess PFA. The cells were washed with PBS for five minutes. The cells were then permeabilized before staining with primary and secondary antibodies with PBS with 0.1% Triton for 20 minutes at RT. Blocking was also done at RT with PBS containing 0.1% Triton and 2% BSA. All the primary antibodies and secondary antibodies were diluted in the blocking buffer. The primary antibody incubation was done either at RT for 90 minutes or at 4°C overnight and the secondary antibody incubation was done at RT for 45 minutes. The antibody incubation was followed by extensive washing for 5 minutes at least three times with PBS containing 0.1% Triton. After the secondary antibody incubation, the cells were ready to be mounted. The last wash before mounting was always done for 5 minutes in PBS. Pre cleaned glass slides were used for mounting. 3 µl mounting medium was used for one coverslip and the cells were removed from PBS and dipped once in distilled water and carefully mounted using clean forceps. The mounted glass slides were kept in the dark at 4°C and carefully stored till they were used for imaging.

2.2.10 Total internal reflection fluorescence microscopy (TIRFM)

2.2.10.1 Setup

The TIRFM setup used for visualizing the accumulation and release of Perforin-mCherry consisted of an inverted Olympus IX70 microscope with the following components: a solid-state laser 85 YCA emitting at 561 nm (Melles Griot, Carlsbad, CA, USA), a Micromax 512 BFT camera (Princeton instruments Inc., Trenton NJ, USA) controlled by Metamorph (Visitron, Puchcheim, Germany), a TILL TIRF condenser (T.I.L.L Photonics, Grafeling, Germany) and an Acousto Optical tunable filter (AOTF)-nC (AA optoelectronic, St Remy-les Chevreuses, France), a dual band FITC/Texas red filter set (# 51006, AHF Analysen technik AG, Tübingen, Germany), a dual-view camera splitter (Visitron, Puchheim, Germany) to separate the red and green channels, a Visichrome Monochromator (Visitron, Puchheim, Germany) to acquire images in epifluorescence. A 100X Olympus objective with a N.A of 1.45 was used for all TIRFM experiments. The object size which is represented in one pixel size was 130 nm.

2.2.10.2 Coating glass coverslips with antibody

25°mm glass coverslips that were precleaned with 70% ethanol were used for all TIRFM experiments and dried. The coverslips were then coated with 0.1 mg/ml poly-ornithine for 30 minutes at RT and then removed before coating the antibodies. The anti-CD3 (1 mg/ml) and anti-CD28 (1 mg/ml) antibodies that were used for coating were diluted in PBS to a final concentration of 30 µg/ml and 90 µg/ ml, respectively. The maximum volume of antibody solution that was used for coating one coverslip was 50 µl. The solution was added to the center of the coverslip and then incubated for 2:30 hours in a 37°C humidified tissue culture incubator. The solution was aspirated after the incubation period and then the coverslips were either used immediately for experiments or left overnight in PBS at 4°C. Coverslips stored in PBS at 4°C could be kept for a maximum period of one week.

2.2.10.3 Experiment protocol

Perforin-mCherry

Human CTLs over expressing perforin-mCherry were washed and resuspended in 50 µl AIMV medium and allowed to settle for 3-4 min on anti-CD3 anti-CD28 coated coverslips. The cells were then perfused with extracellular solution containing 10 mM calcium in order to induce secretion. Cells were imaged for 30 min by TIRFM at 561 nm. The cells were recorded simultaneously in epifluorescence at 561 nm excitation wavelength. Following each TIRF image, three images were acquired as epifluorescence stacks (z = 0.5 µm), the first being at the same plane as that of TIRFM. The epifluorescence images were taken to distinguish between secretion and movement of the vesicle away from the plasma membrane inside the cell. The acquisition speed was 1 Hz and the exposure time was 100 ms. All experiments were performed at RT.

Syntaxin7-TFP

For experiments with Syntaxin7 full length and dominant negative TFP fusion constructs, CTLs were made to co-express perforin-mCherry with either the full length Syntaxin7 protein or its mutant form without the transmembrane domain. The cells were again allowed to settle for 3-4 minutes on CD3/CD28 coated coverslips. For the experiments CTLs expressing either the full length or dominant negative Syntaxin7-TFP were chosen and imaged in TIRF at 561 nm. Since the granules never moved 1 µm above the TIRF plane and for faster imaging, only two images were acquired in epifluorescence at 488 nm and 561 nm, one at the same plane as that of TIRFM and the second at 0.75 µm above the TIRF plane. The acquisition speed was 1 Hz and the exposure time was 100 ms. All experiments were performed at RT.

2.2.10.4 Analysis of movies

2.2.10.4.1 Analysis of accumulation

The analysis of lytic granule accumulation was performed with Metamorph version 6.3. The background was subtracted from the raw data of TIRF stacks and then a threshold value was set for every cell such that only the pixels above a given fluorescence intensity that mark the lytic granules were selected. The value of the

pixels defined as the threshold area was obtained for every frame of the time lapse movie for each individual cell. Due to difficulties in tracking every single vesicle for all the cells, a ratio was made between the threshold area and number of vesicles for a minimum of five frames for every cell. The area threshold was then divided by this ratio for all the time frames to get the number of vesicles for each frame. The average of the number of vesicles was then plotted against the time.

2.2.10.4.2 Analysis of secretion

Metamorph v6.3 was used to analyze the secretion of lytic granules at the IS. Every slice of the TIRF movie after background was checked carefully by eye for secretion. The time interval between each slice of the TIRF movie was one second and within that one second three images in epifluorescence were taken at different planes. One second of one experiment therefore generated four time lapse movies one being at the TIRF plane and another epifluorescence image at the same plane. The other two images were in epifluorescence but at two planes higher than the first plane by a step size of 500 nm. When a vesicle that was present in one slice disappeared in the next slice, a region of interest was made circling that particular vesicle just before it seemed to have been secreted. The same region of interest was copied and pasted on to the three epifluorescence images. If the vesicle had been secreted it would not appear in any of the subsequent epifluorescence images. However if the vesicle had moved back then it would appear in either one of the two planes higher than the TIRF plane one second later.

2.2.11 Confocal and epifluorescence deconvolution microscopy

2.2.11.1 Laser Scanning Confocal Microscope 710

The fixed and stained samples were visualized using a standard laser scanning confocal microscope LSM 710. A 40X oil objective with a N.A. of 1.3 was used for image acquisition. An averaging of 4 with a chip size of 512 x 512 was used with the airy unit (A.U) set to 1 for all experiments. Laser light of wavelengths 488 nm, 561 nm and 647 nm were used for excitation. The laser power that was used was 1-2% of maximal power to avoid bleaching. Serial confocal z-sections were taken at 0.4 µm intervals for whole cell analysis. ImageJ v1.37 software was used to generate

merged images and projections of confocal sections and to quantify the CD3 fluorescence at the IS and at the whole plasma membrane of the cells.

2.2.11.2 Time-lapse imaging and epifluorescence deconvolution microscopy

Time lapse imaging was performed with a Zeiss Cell Observer HS system with a 63X alpha Plan-Fluar Objective (N.A. 1.45) and an AxioCam MRm Rev. 3 with the exposure time of 200–500 ms. A Colibri lamb (LED based) was the light source for excitation and two single bandpass filter sets were used: GFP (38HE, Zeiss) and ET-Cy5 (Chroma).

Human CTLs overexpressing either Syntaxin7-TFP or Syntaxin7ΔC-TFP were pre-incubated with Alexa^{647}-labeled anti-CD3 mAb at 37°C for 30 min to label TCR. Cells were kept on the microscope stage in chambers filled with AIMV culture medium. The entire stage of the microscope was covered by a climate chamber. In this chamber, cells had the same conditions as in a cell culture incubator (37°C, 100% humidity, 5% CO_2). For a single experiment 5 to 15 stage positions were repetitively monitored every minute for a total of 90 minutes. Data were collected and analysed with Zeiss Axiovision software.

For imaging fixed cells, the above mentioned single bandpass filtersets were used with the following additional filter: ET-TxRed (Chroma). Images were acquired with no binning and a z-stepsize of 0.2 μm. Constrained iterative deconvolution was performed using a point spread function calculated with the z-stack acquisition from 170 nm yellow-green, orange or deep-red fluorescent beads (PS-Speck, Invitrogen) using 30-40 iterations.

2.2.12. Structured Illumination Microscopy

Structured illumination microscopy (SIM) is a recently developed high resolution technique (Gustafsson et al., 2008) which allows decrease of the diffraction barrier by the factor of 2-2.5 (resolution of about 100 nm). The setup used was at that time a non-commercially available prototype of a SIM system from Zeiss.

The principle of SIM is as follows: Each plane of a sample is illuminated with light which beforehand had passed an optical grade (and so the name structured illumination). The illumination is turned 3 times around the optical axes by a discrete angle and moved 5 times along a line in the focus plane. As a result for each plane

we get a set of 15 single pictures which are then used by a computer algorithm to calculate the high resolution picture. The high resolution information itself is hidden in the strew light in the pattern illuminated raw data sets of pictures. As a result one gets a high resolution picture with a z resolution of 250 nm and a xy resolution of about 100 nm. Images at the SIM were taken with a 63X oil objective with a N.A. of 1.40. Images were acquired with excitation light of 488, 561 and 635 nm wavelengths and then processed for SIM to obtain higher resolutions. Z stacks of 200 nm step size were used to scan the whole cell. Zen 2009 software was used for acquiring the images and for processing for high resolution.

2.2.13 Colocalization analysis

Image J v3.3 with the plugin Just Another Colocalization Plugin (JACOB) was used for all analysis (Bolte and Cordelieres, 2006). The colocalization plugin was based on Pearson's coefficient correlation factor 'r'. The fluorescence values of one pixel from one channel were plotted against the corresponding fluorescence values from the other channel. The slope of this curve would determine the association rate of two fluorophores and the Pearson's correlation coefficient determined the probability of this association. The value of the Pearson's coefficient factor can range from 1 to -1 with 1 for positive correlation of association and -1 for negative correlation of association and 0 for no correlation. The background was corrected for the raw image stacks from each of the channels and then a threshold was set to eliminate the noise. The two channels were then analyzed for two different measures – the Pearson's correlation coefficient and the Van Steensel's correlation coefficient.

3. Results

3.1 Syntaxin7 is expressed in CTLs, upregulated upon activation and is preferentially localised at the IS

Syntaxin7 has been shown previously to be enriched in the secretory lysosomes of NK cells and has been implicated to function in the fusion of lysosomes with the plasma membrane (Casey et al., 2007). The secretory machinery of NK cells and CTLs has been shown to be very similar (Lowin et al., 1995). Since we were interested in identifying the potential SNAREs involved in the fusion of lysosomes in CTLs, we thought Syntaxin7 would be a promising candidate. To determine whether Syntaxin7 is expressed in CTLs we first performed RT-PCR on CTLs at different times after stimulation (Fig. 7a). We used two different methods of stimulation (see methods section) – one by CD3/CD28 coated beads and the other by Staphylococcus enterotoxin A (SEA). PCR reactions were normalized to the amount of cDNA. The expression of Syntaxin7 was tested in naïve cells, in CTLs that had been stimulated for 1, 3 and 5 days after CD3/CD28 coated bead stimulation and in CTLs that were stimulated for three days with SEA. There was an upregulation of Syntaxin7 expression in both methods of stimulation in comparison to naïve cells. Western blots further confirmed the presence and upregulation of Syntaxin7 in CTLs (Fig. 7b). The expression of Syntaxin7 was comparable between CTLs that were stimulated for three days with beads and SEA. The killing capacity of CTLs when tested was also maximum at these particular time points after stimulation and therefore hinted towards the importance or requirement of Syntaxin7 for the same (data not shown).

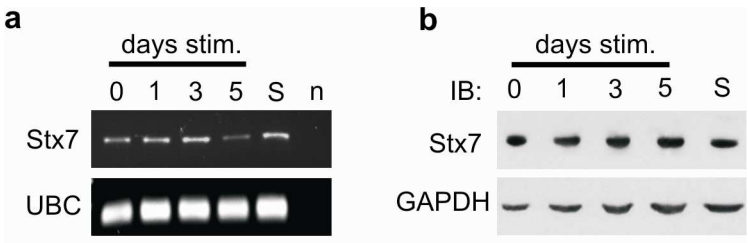

Figure 7. Syntaxin7 is upregulated upon activation of CD8 T cells
(a) Expression of Syntaxin7 by reverse transcriptase PCR in CD8 T cells at the different times after stimulation that are indicated on top with anti-CD3/anti-CD28 coated beads and SEA. UbiquitinC (Lyubchenko et al., 2001) was used as the internal control. n is the negative control. (b) Western blots showing the expression of Syntaxin7 in naïve and activated CTLs. Glyceraldehyde phosphate deydrogenase (GAPDH) was used as the loading control.

Killing of target cells occurs by polarized secretion of lytic granules at the IS. If Syntaxin7 plays a role in the killing function of CTLs, it should then preferentially accumulate at the IS in CTLs. In order to check this we performed immunocytochemistry with SEA stimulated CTLs and SEA pulsed Raji cells (target cells) (Fischer et al., 1989) that had been in contact for 30 minutes (allowing the formation of a mature IS) and stained for Syntaxin7 using a commercially available antibody (See Methods for details). Confocal microscopy on these cell conjugates showed that in contrast to CTLs that had no contact with target cells (resting CTLs), Syntaxin7 preferentially accumulated at the IS in CTLs within 30 minutes of its formation (Fig. 8a). To confirm the results obtained with the Syntaxin7 antibody we overexpressed CTLs with a full length Syntaxin7-TFP fusion construct (Stx7 FL-TFP). We saw a preferential localization of Stx7 FL-TFP at the IS (Fig. 8b, upper panel), similar to that seen with endogenous Syntaxin7. When the transmembrane region of Syntaxin7 was deleted and this fusion construct was overexpressed in CTLs (Stx7 ΔC-TFP), the staining was not vesicular but cytoplasmic as was also shown previously (Kasai and Akagawa, 2001). There was no accumulation of Stx7 ΔC-TFP at the IS (Fig. 2b, lower panel). These results indicated that Syntaxin7 is preferentially localized to the IS in CTLs.

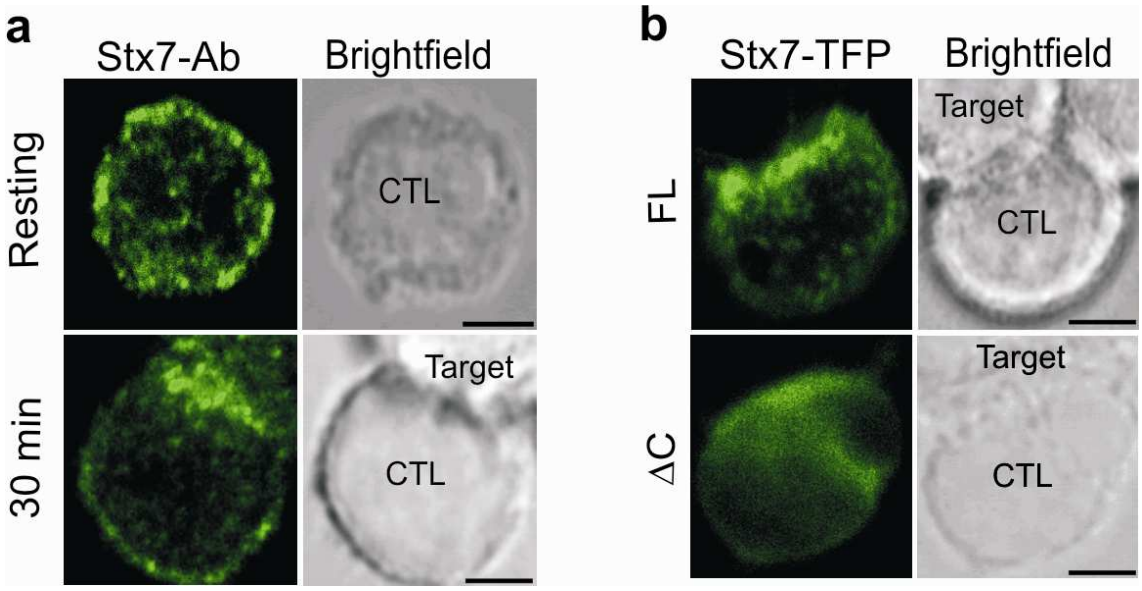

Figure 8. CTLs in contact with target cells show an accumulation of Syntaxin7 at the IS.
(a) Representative images showing the endogenous staining of Syntaxin7 in resting CTLs (upper panel) and in CTLs that were incubated with target cells for 30 minutes (lower panel). (b) CTLs expressing full length Syntaxin7 protein fused to TFP (upper panel) in contact with a target cell for 30 minutes showed an accumulation of Syntaxin7 at the IS. When the trans-

membrane region of Syntaxin7 was deleted (lower panel) no accumulation at the IS was observed. Scale bars are 3 µm.

In order to check the specificity of the Syntaxin7 antibody we co-stained Syntaxin7-TFP cells with Syntaxin7 antibody. Using structured illumination microscopy (SIM), we improved the resolution of fluorescence microscopes by a factor of 2 in all directions (Gustafsson et al., 2008). We then imaged serial sections along the whole cells and used 0.2 µm tetraspeck™ microspheres (Invitrogen) to correct for any chromatic aberration. We saw a robust overlap between Stx7 FL-TFP and the Syntaxin7 antibody (Fig. 9), showing antibody specificity for staining Syntaxin7.

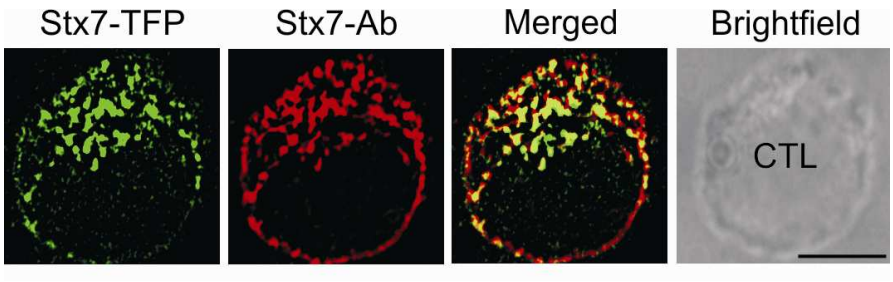

Figure 9. Syntaxin7-TFP transfected CTLs showed a strong overlap with Syntaxin7 antibody.
CTLs transfected with Syntaxin7-TFP (green) were stained with Syntaxin7 antibody (red). Images were taken by SIM to obtain higher resolution. There was a large overlap between Syntaxin7-TFP and Syntaxin7 antibody. Scale bar is 5 µm.

3.2 Syntaxin7 is required for CTL mediated killing and perforin release

3.2.1 Population killing assay

The preferential accumulation of Syntaxin7 at the IS led us to investigate the requirement of Syntaxin7 for the killing function of CTLs. To do this we used a modified siRNA (Mantei et al., 2008) that was specific for Syntaxin7 to downregulate its expression in SEA stimulated CTLs. A nonsilencing siRNA was used as the control. 36 hours after transfection of CTLs with Syntaxin7 and control siRNAs, we saw a significant reduction in the relative expression of Syntaxin7 in Syntaxin7 siRNA transfected CTLs. Quantitative real Time PCR (qRT-PCR) from two donors showed an 80% reduction in Syntaxin7 mRNA expression (Fig. 10a). Two different house keeping genes, the TATA box binding protein (TBP) and an RNA polymerase were used as controls. Data from four different donors showed a 60% reduction in

Syntaxin7 protein expression (Fig. 10b+c). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the loading control. Thus, after having verified the reliability of the modified siRNA in downregulating Syntaxin7 expression in CTLs we performed a killing assay to verify if there was any change in the killing capacity of CTLs when Syntaxin7 expression was downregulated.

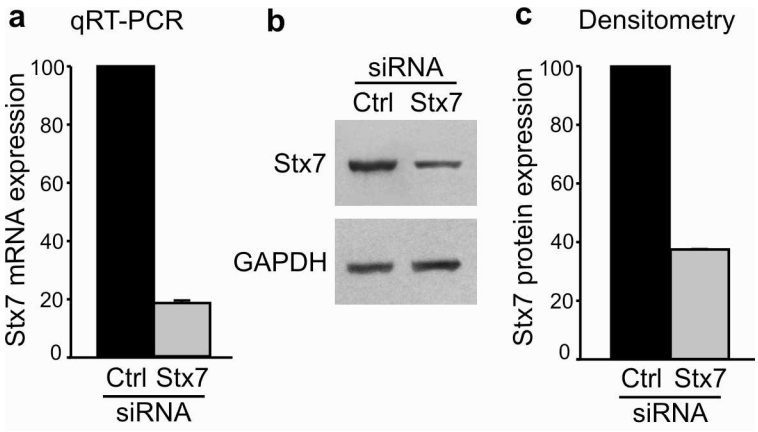

Figure 10. Syntaxin7 expression was downregulated using modified siRNA in primary human CTLs.
Downregulation of Syntaxin7 at the (a) mRNA level by qRTPCR and (b) protein level by Western blot in control (Ctrl) and Syntaxin7 (Stx7) siRNA treated CTLs. (c) Quantification of b from 4 independent experiments. Expression of Syntaxin7 was quantified by normalizing to the loading control (GAPDH).

A population based killing assay with varying ratios of effector to target cells showed a significant reduction in the killing capability of Syntaxin7 siRNA transfected CTLs in contrast to Control siRNA transfected CTLs (Fig. 11). CTLs and SEA pulsed Raji cells were incubated together for 4 hours at 37°C. The activity of lactate dehydrogenase (LDH) in the supernatant was used as the measure for killing (see 2.2.8). The reduction in the killing efficiency of target cells was independent of the varying effector to target cell ratios that were used.

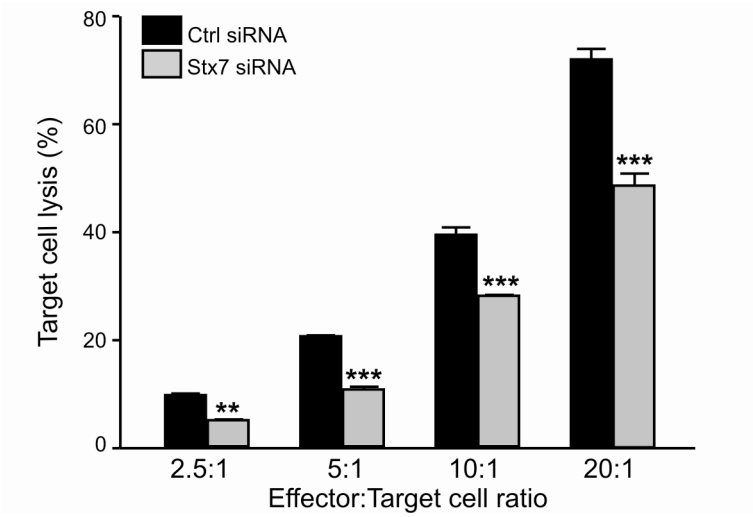

Figure 11. Reduced CTL mediated cytotoxicity when endogenous Syntaxin7 expression is downregulated.
CTL-mediated cytotoxicity was impaired when Syntaxin7 was downregulated. SEA-specific CTLs and SEA-pulsed Raji cells were co-cultured at the indicated effector:target cell ratios for 4 hours. Each condition was done in triplicate. One representative experiment out of three independent experiments is shown. Error bars show standard deviation.

In order to check whether the reduction in the killing capacity of Syntaxin7 siRNA transfected CTLs was because they had less lytic granule content we performed two sets of control experiments. In the first experiment we checked whether there was any difference in the total expression of the lytic granule marker perforin in Syntaxin7 siRNA cells when compared to control siRNA cells. The expression was quantified and normalized to GAPDH which was used as the loading control. There was no significant difference in the total expression of perforin (Fig. 12a+b). Secondly, to verify whether the numbers of perforin granules per cell were different or not we stained resting CTLs that were transfected with control and Syntaxin7 siRNA with anti-perforin antibody and counted the number of perforin vesicles per cell. We found that there was no significant difference in the number of perforin vesicles between control and Syntaxin7 siRNA transfected CTLs (Fig. 12c). The average number of vesicles counted were 32.7 ± 2.54 and 33.8 ± 2.6 in control and Syntaxin7 siRNA transfected cells (Fig. 12d).

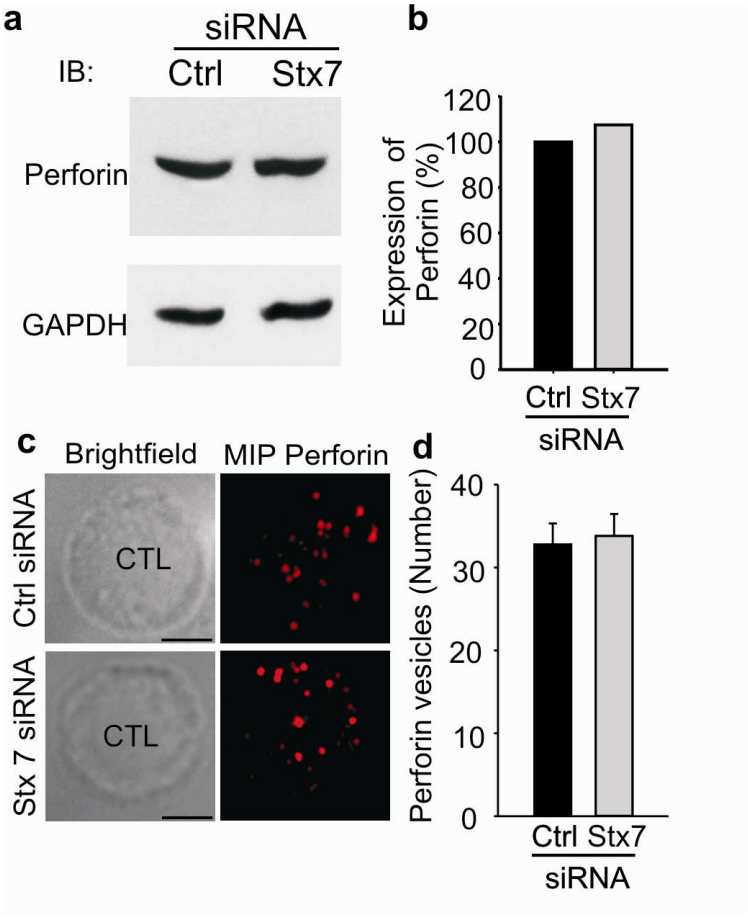

Figure 12. Control and siRNA transfected CTLs have no change in endogenous perforin expression.
(a) Lysates of Control and siRNA transfected CTLs were blotted for perforin and GAPDH.(b) Quantification of the total expression of perforin normalized to GAPDH which was used as the loading control (c) Images showing the total number of perforin loaded vesicles in control and siRNA transfected CTLs (d) Quantification of (c). There was no significant difference in the total number of perforin vesicles per cell. Scale bars are 3 μm. Error bars show SEM.

3.2.2 TIRFM

The killing of target cells requires the fusion of lytic granules with the plasma membrane at the IS. We therefore analyzed granule movement and fusion by total internal reflection fluorescence microscopy (TIRFM). This technique has been well established to study synaptic transmission from neurons and neuroendocrine cells with high spatial resolution (Zenisek et al; Chow Nature, Nofal et al.), but has not been used before to study the secretion of lytic granules. The early events of IS formation in T cells have been studied using TIRFM. Glass coverslips coated with with either membrane bilayers containing laterally mobile anti-CD3 and ICAM1 molecules (Kaizuka et al., 2007) or with anti-CD3/anti-CD28 molecules have been used to effectively to form functional synapses with T cells (Quintana et al., 2007). In order to test the applicability of studying secretion of lytic granules by TIRFM we

transfected bead stimulated CTLs with the lytic granule marker perforin fused to mCherry and allowed the cells to form functional synapses with anti-CD3/anti-CD28 coated coverslips. Shortly after the addition of CTLs on the glass coverslips, the appearance of lytic granules at the cell/coverslip interface could be observed. The number of lytic granules approaching the IS increased over time (Fig. 13a). The number of vesicles approaching the IS could be reliably quantified (Fig. 13b; see also Methods section 2.2.10.4.1).

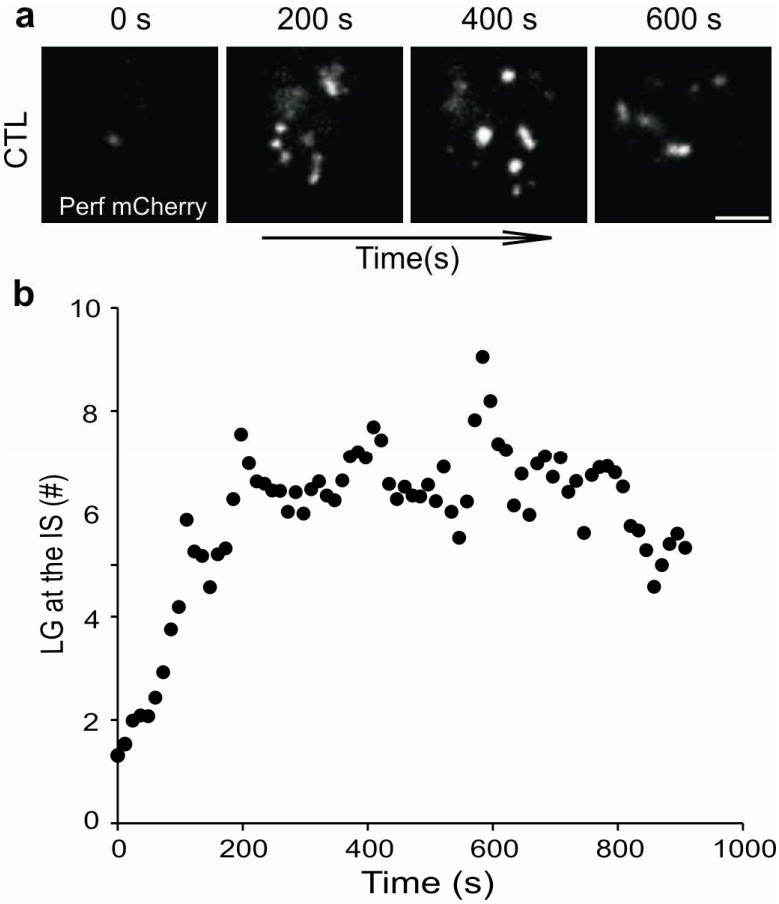

Figure 13. Visualization of lytic granule accumulation at the IS by TIRFM.
CTLs were transfected with perforin mCherry to label lytic granules and imaged 24 hours later. The cells were allowed to settle on anti CD3 coated coverslips to form a synapse and imaged immediately for 30 minutes. Scale bar is 3µm. (a) Typical accumulation of perforin mCherry at the TIRF plane after minutes of addition to glass coverslips. (b) Quantification of the number of lytic granules approaching the IS from 21 cells. LG: lytic granule.

Furthermore, some of the lytic granules that accumulated over time at the IS quite regularly fused with the plasma membrane (Fig. 14). Fusion of lytic granules could be distinguished from their movement back into the cell (See Methods section 2.2.10.4.2 for details).

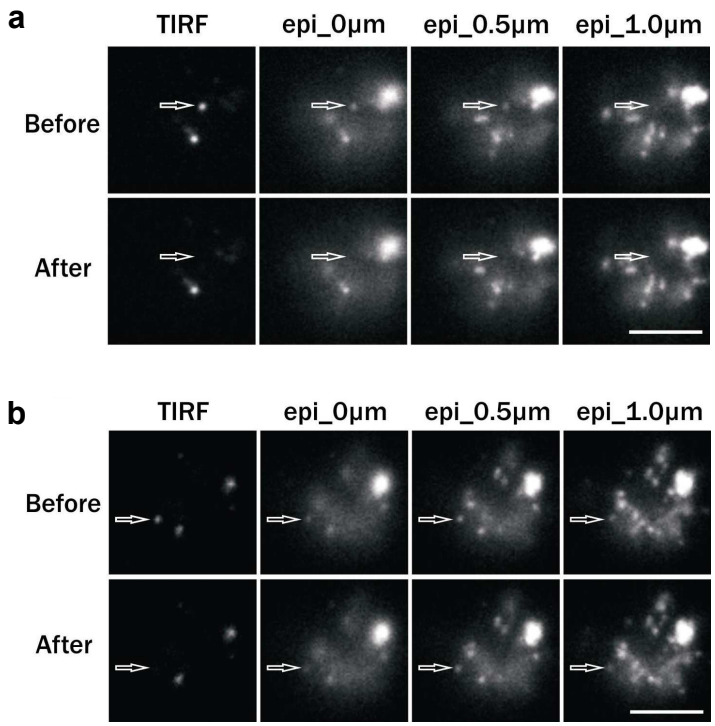

Figure 14. Secretion of lytic granules at the IS can be distinguished from movement. To distinguish between secretion of vesicles and their movement away from the plasma membrane images were acquired simultaneously in TIRF and epifluorescence. (a) TIRF and epifluorescence images taken at planes 0, 0.5 and 1.0 µm above the TIRF plane, before and after secretion or movement. The lytic granule (marked by an arrow), which was secreted, was neither present in the TIRF image nor in the epifluorescence stack images. (b) A lytic granule that moved away from the TIRF plane. It had disappeared in the TIRF image (lower panel, After), but was still visible in the epifluorescence images. Scale bars are 3 µm.

Quantification of fusion from 24 cells showed that the average number of vesicles released was 2 ± 0.13. The time of the first fusion event was 405.75 ± 37.66 s. The low number of fusion events could be because CTLs do not need many lytic granules for killing one target cell (Lyubchenko et al., 2001).

Having established the validity of the method we then performed similar experiments with CTLs coexpressing either Stx7 FL-TFP or Stx7 ΔC-TFP in addition to perforin-mCherry. As shown in the exemplary traces in Figure 15a, expression of the dominant-negative Stx7 ΔC-TFP led to a large reduction in the number of lytic granules appearing in the TIRFM field compared to cells expressing the full length Stx7 FL-TFP. Quantification of the data from 11 and 14 cells revealed that after 15 min of IS formation Syntaxin7 expressing cells had about five times more lytic granules at the IS than cells expressing the dominant-negative mutant (Fig. 15b).

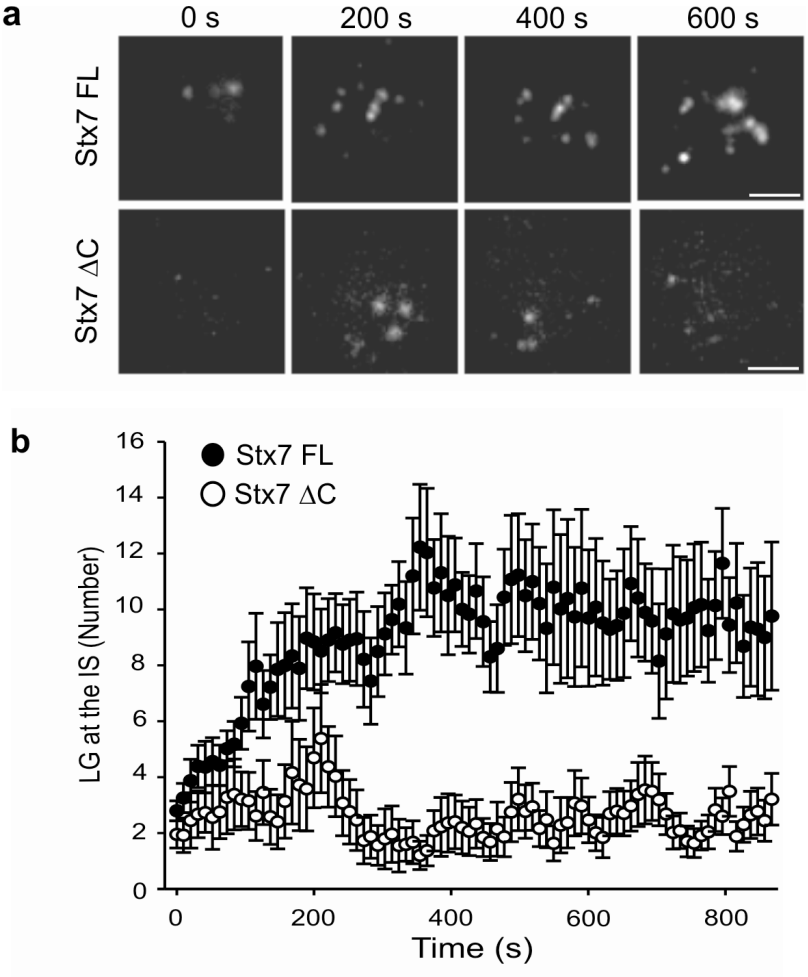

Figure 15. Reduced accumulation of lytic granules was seen in TIRFM.
(a) TIRFM images of CTLs expressing perforin mCherry in Stx7-TFP cells (upper panel) or Stx7 ΔC-TFP cells (lower panel). The extracellular solution contained 10 mM Ca^{2+} in order to induce secretion in the cells. Cells were allowed to settle for 2-3 minutes on antiCD3/antiCD28 coated coverslips and then imaged for 15 minutes. Stx7 FL-TFP cells (upper panel) showed an increase in perforin accumulation overtime in contrast to Stx7 ΔC-TFP cells (lower panel). (b) Quantification of (a) as the number of lytic granules approaching the IS over time from 11 Stx7 FL-TFP cells and 14 Stx7 ΔC-TFP cells from at least three independent donors. Scale bars are 3 µm.

Also, the cells expressing Stx7 FL-TFP cells secreted perforin at the IS indicating that the fusion construct did not block the function of CTLs. The average number of perforin vesicles released was 1.6±0.24 which was similar to the cells overexpressing only perforin mCherry. This indicated that the overexpression of Syntaxin7 in CTLs did not interfere with the function of CTLs.

In order to confirm our observation of a significant reduction in perforin accumulation at the IS in Stx7 ΔC-TFP cells in comparison to that seen in Stx7 FL-TFP cells, we fixed and stained transfected CTLs that were in prior contact with target cells for 30 minutes for perforin.

Confocal microscopy imaging of serial sections of the whole cell also showed a a relative reduction in the accumulation of perforin at the IS (Fig. 16). This was comparable to the results obtained from TIRFM. These data allow us to conclude that Syntaxin7 function is required for the correct transport of lytic granules to the IS.

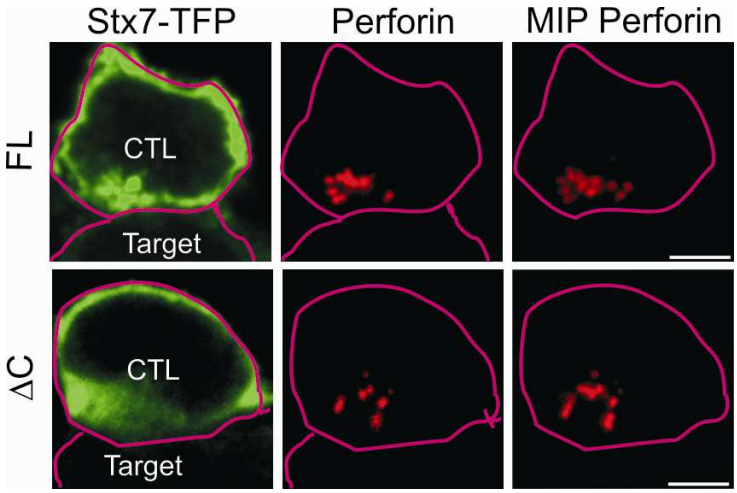

Figure 16. Confocal microscopy shows a reduction in perforin accumulation at the IS. Shown are representative confocal images and maximum intensity projections (MIP) of CTLs transfected with either Stx7 FL-TFP (upper panel) or Stx7 ΔC-TFP (lower panel) and stained with perforin antibody. A qualitative difference in the reduction of perforin accumulation was seen in cells overexpressing Stx7 ΔC-TFP on comparison with Stx7 FL-TFP expressing CTLs. Scale bars are 3 µm.

After confirming by two independent methods the importance of Syntaxin7 for the normal accumulation of perforin at the IS in CTLs we asked the next important question concerning the localization of Syntaxin7. In order to investigate whether Syntaxin7 and perforin were colocalized we used SIM microscopy to scan serial sections along the whole cell. We found that there was very little colocalization between Syntaxin7 and perforin (Fig 17).

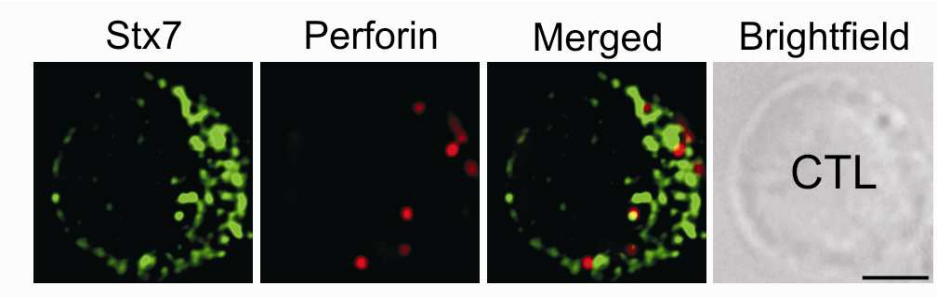

Figure 17. Colocalization of Syntaxin7 and Perforin in activated CTLs. Resting CTLs were fixed and stained with Syntaxin7 and perforin antibodies. Colocalization analysis was done using Jacob plugin from ImageJ. Scale bar is 3 µm.

The amount of colocalization between Syntaxin7 and perforin was quantified using Pearson's coefficient (r) from 15 cells (See also methods section 2.2.13). SEA stimulated CTLs that had no prior contact with target cells were used for the analysis which yielded a Pearson's coefficient value of 0.05 ± 0.02. This indicated that Syntaxin7 and perforin were not colocalized. We therefore conclude that the reduction in the killing capacity of CTLs that had impaired or reduced Syntaxin7 expression cannot be due to a defect in the trafficking/sorting of perforin.

3.3 Defective TCR accumulation when Syntaxin7 function is blocked

Lytic granule polarization and release is one of the later steps occurring after a mature IS is formed. Since Syntaxin7 and perforin were found to be present in two different vesicle populations we decided to examine the function of Syntaxin7 in the early trafficking events necessary for IS formation. TCR signaling at the CTL-target cell contact zone is one of the earliest signaling events that initiate the formation of a mature IS. Accumulation of TCRs at the IS to form the cSMAC marks the formation of a mature IS. We therefore wanted to check whether the effect of impaired Syntaxin7 function on the accumulation of perforin at the IS in CTLs was because of a defect in the formation of the cSMAC. A fluorescently labeled anti-CD3 antibody was used to label recycling TCRs. CTLs transfected with siRNA for human Syntaxin7 showed a dramatic loss of CD3 accumulation at the IS in comparison to cells that were transfected with control siRNA control (Fig. 18a). In an attempt to quantify the observed reduction we divided the CTL into three equal parts and measured the percentage of CD3 fluorescence in the third facing the target cell (containing the IS). If the CD3 fluorescence was distributed randomly throughout the cell, we would expect a 28.9% value in that area (dotted line in Fig. 18b). In cells transfected with control siRNA, 30 minutes after CTL-target cell contact 61.4 ± 3.3% of CD3 fluorescence was present in the region near the IS. In contrast, only 43.0 ± 2.7% of CD3 fluorescence occurred in the third facing the target cells in cells transfected with siRNA against Syntaxin7 (Fig. 18b; $p < 0.001$). A qualitatively and quantitatively similar block of CD3 accumulation was observed in CTLs expressing Stx7 ΔC-TFP cells (40.9 ± 1.6%) when compared to CTLs expressing Stx7 FL-TFP (56.5 ± 1.3%; $p < 0.001$; Fig. 18c+d). Thus, our two independent methods of interfering with Syntaxin7 function reveal that Syntaxin7 is needed for the stable formation of the cSMAC at the IS.

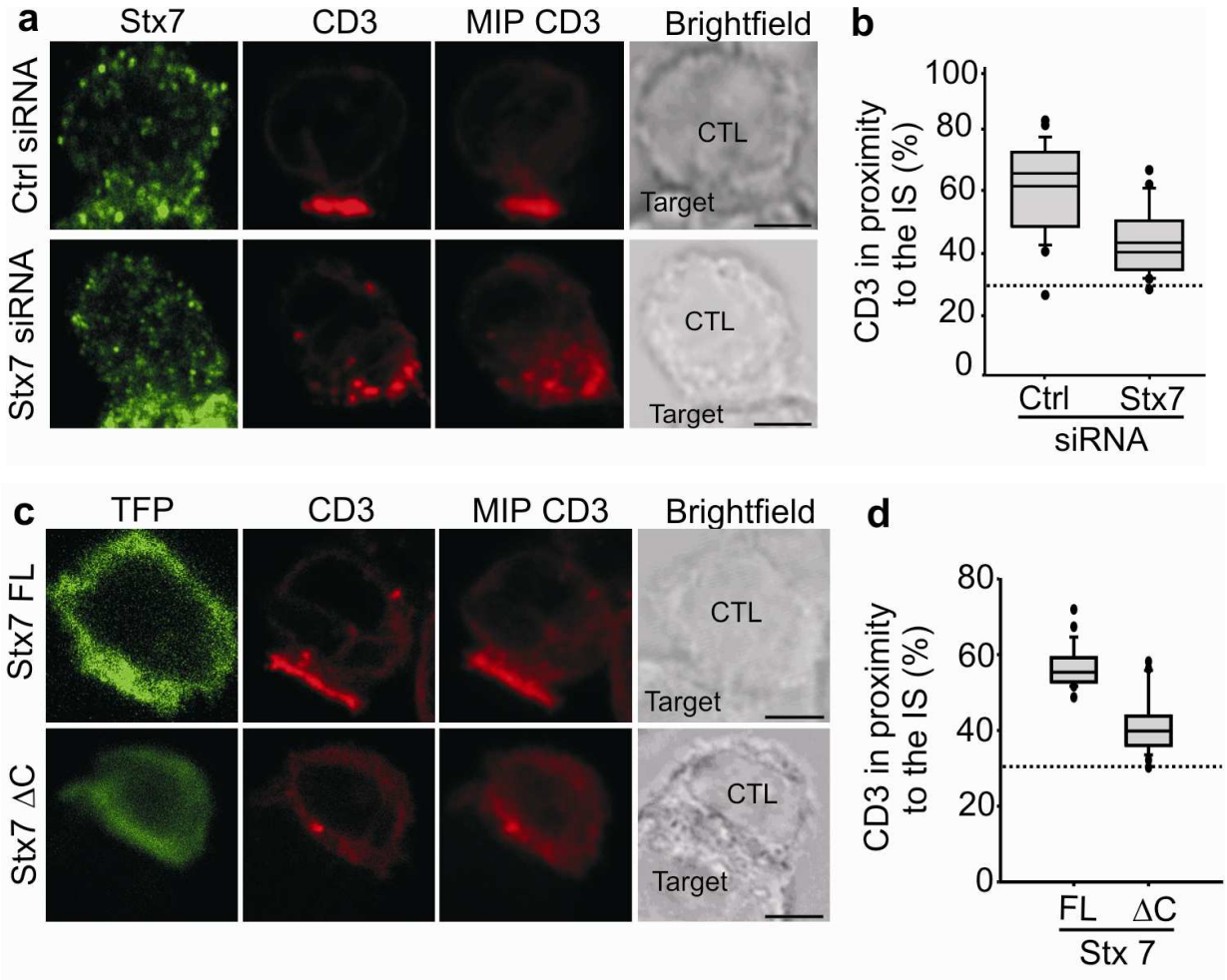

Figure 18. Reduced accumulation of CD3 in CTLs when Syntaxin7 function is blocked. CD3 staining to mark the formation of a mature IS after 30 minutes of CTL-target cell contact in (a) Ctrl siRNA and Syntaxin7 siRNA cells and (c) Stx7FL-TFP and Stx7ΔC-TFP cells. (b) and (d) are the quantification of CD3 accumulation in (a) and (c) respectively. Scale bars are 3 µm.

In principle, this observation could be due to either a reduced transport of intracellular organelles containing recycling TCR towards the IS (Das et al., 2004) or to a reduced lateral mobility of membrane bound TCR towards the IS membrane (Favier et al., 2001). In order to distinguish between these possibilities we performed live imaging in both Stx7 ΔC-TFP and Stx7 FL-TFP transfected CTLs. In order to exclude the possibility of labeling newly synthesized TCR, we pre-incubated CTLs with a fluorescent conjugated anti-CD3 antibody (see also section 2.2.9.2). As shown in Figure 19, we observed no accumulation of CD3 at any time of observation in Stx7 ΔC-TFP transfected CTLs, thereby indicating that the transport of recycling TCR to the IS is blocked in these cells resulting in defective cSMAC formation.

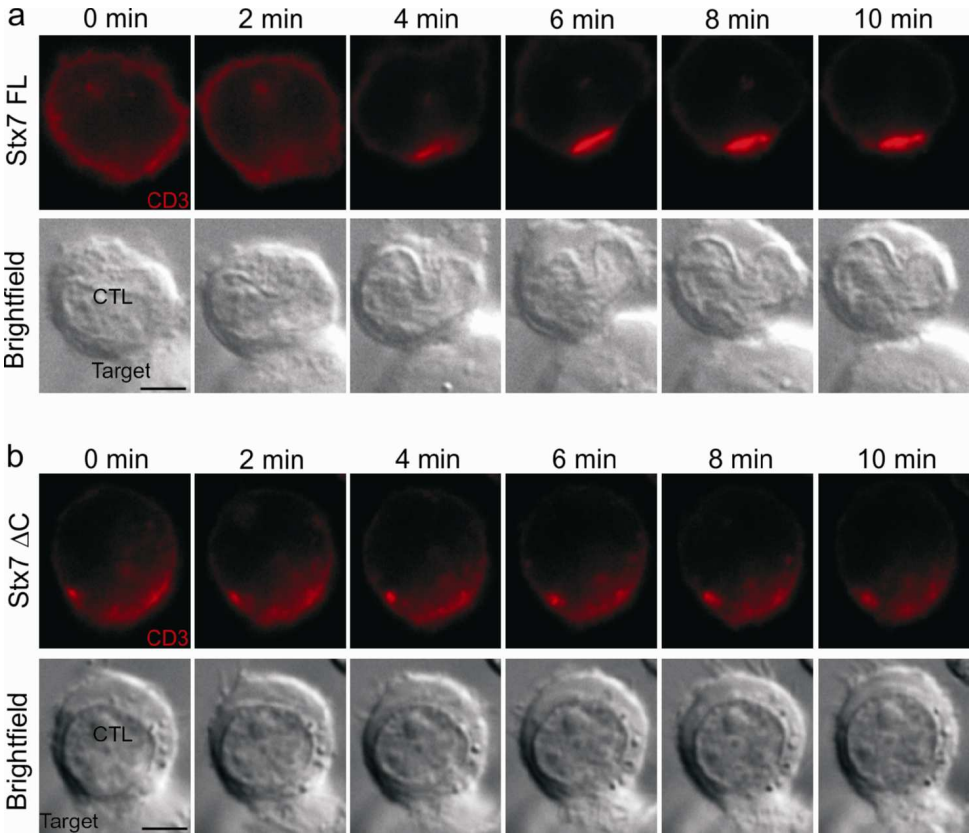

Figure19. Time lapse imaging of CD3 polarisation towards the IS.
Live cell imaging showing the dynamics of CD3 polarisation towards the IS after CTL-target cell contact in (a) Stx7 FL-TFP and (b) Stx7 ΔC-TFP cells. The cells are a representative of a total of 6 (Stx7 FL-TFP) and 17 (Stx7 ΔC-TFP) cells. Cells were taken from three independent donors. Scale bars are 3 μm.

It was not feasible to quantify the accumulation of recycling TCRs from the time lapse imaging experiments. The cells were scanned only along three planes to avoid bleaching during the movie. We were interested in analyzing the accumulation of recycling TCR at different time points. To do this, we performed immunocytochemistry on fixed CTL-target cell conjugates that were transfected with either Stx7 ΔC-TFP or with Stx7 FL-TFP. We pre-incubated CTLs with Alexa-647 conjugated CD3 for 30 minutes and then washed the cells before incubating them with target cells for 5, 15 and 30 minutes before fixation (Fig. 20). The accumulation of CD3 was later quantified by first making a projection of all the stacks of the cell. The cell was then divided into three equal parts. The third facing the target cell was defined as the immunological synapse and the CD3 fluorescence in this region was measured over the fluorescence along the entire region of the cell.

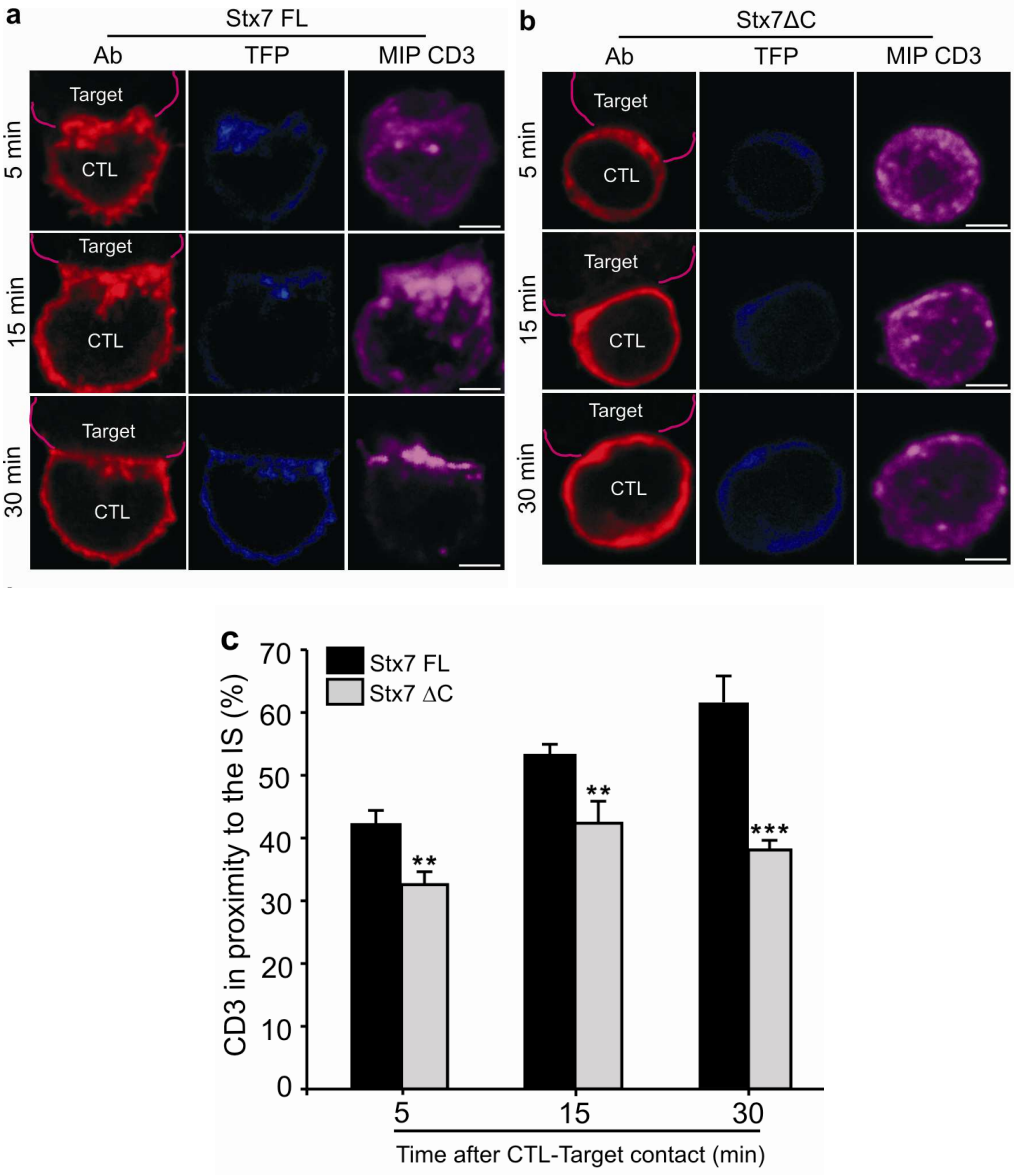

Figure 20. CD3 localization at different times after CTL-Target cell contact.
(a, b)TFP tagged with either Syntaxin7 protein lacking its transmembrane region (Stx7 ΔC-TFP) or full length Syntaxin7 (Stx7 FL-TFP) were expressed in CTLs. 24 hours after transfection CTLs were pre-incubated for 30 minutes with Alexa647–labeled anti-CD3 mAb to label recycling TCR and then washed. CTLs were then incubated with target cells for 5, 15 and 30 minutes (Panels A, B and C respectively; shown above). (c) Accumulation of CD3 was quantified by dividing the fluorescence of CD3 in proximity to the IS (defined as one third the distance into the cell from the immunological synapse) by the total fluorescence in the cell. CTLs expressing Stx7 ΔC-TFP showed no CD3 accumulation over time in contrast to Stx7 FL-TFP expressing CTLs. Scale bar is 5 μm.

After 5 minutes of CTL-Target cell contact we observed internalization of TCR in both the Stx7 FL-TFP and Stx7 ΔC-TFP cells. The CD3 fluorescence in the third facing the target cell was 42.13 ± 2.25%, 53.17 ± 1.76% and 61.41 ± 4.19% in Stx7 FL-TFP at and 32.63 ± 2.05%, 42.41 ± 3.48% and 38.13 ± 1.15% in Stx7 ΔC-TFP cells at 5, 15

and 30 minutes after CTL-target cell contact respectively. The dramatic reduction in the accumulation of recycling TCR seen at 30 minutes restates the importance of Syntaxin7 for a fully formed cSMAC. In order to check whether the CTLs with an impaired Syntaxin7 function could even form a stable contact with their target cells we used Talin as marker for the pSMAC. We observed that Talin accumulated normally in both control and Syntaxin7 siRNA treated cells indicating that Syntaxin7 functions specifically in the transport of recycling TCR towards the IS (Fig. 21).

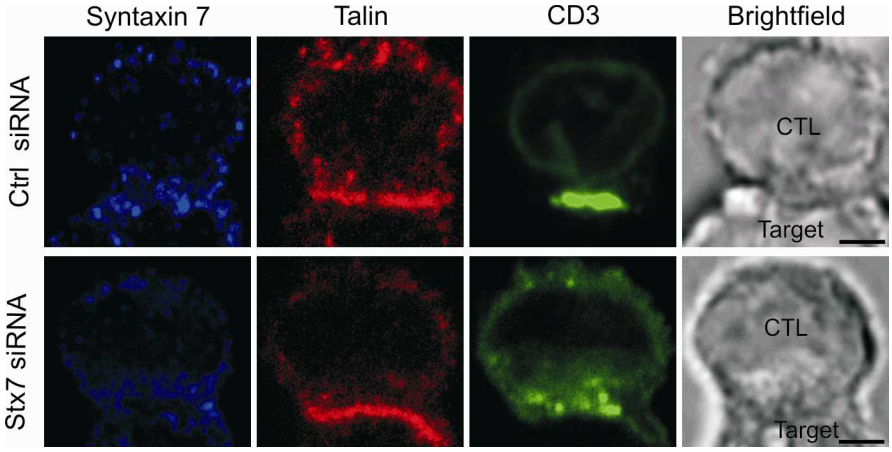

Figure 21. Accumulation of Talin at the IS after 30 minutes of CTL-target cell contact. Representative cells from three independent experiments showing the normal accumulation of Talin, but defective accumulation of CD3 in Syntaxin7 siRNA treated cells. In contrast, control siRNA cells show a normal accumulation of Talin and CD3. Scale bars are 3 µm.

3.4 Defective TCR recycling in CTLs where Syntaxin7 function is blocked

The live cell imaging experiments confirmed that the internalization of TCR was not affected but only their polarization towards the synapse was blocked. we thought that the trafficking step of the TCR either from the early to late/recycling endosome or from the late endosome to lysosome was blocked. In order to discern which specific step was blocked by Syntaxin7, we checked the localization of T cell receptors first with the early endosome marker early endosome autoantigen 1 (EEA1) (Barysch et al., 2009). Interestingly, we found that there was an increase in the colocalization between EEA1 and TCR. This suggests that when Syntaxin7 was down regulated, the TCR recycling pathway is blocked at the early endosome (Fig. 22a). Pearson's coefficient and Van Steensels CCF were used as the measure for colocalization (Fig. 22b+c). The difference in the Pearson's coefficient calculated for 17 Syntaxin7 siRNA treated cells was 0.2 ± 0.03, while that for control siRNA transfected cells was only 0.09±0.01 ($p<0.001$).

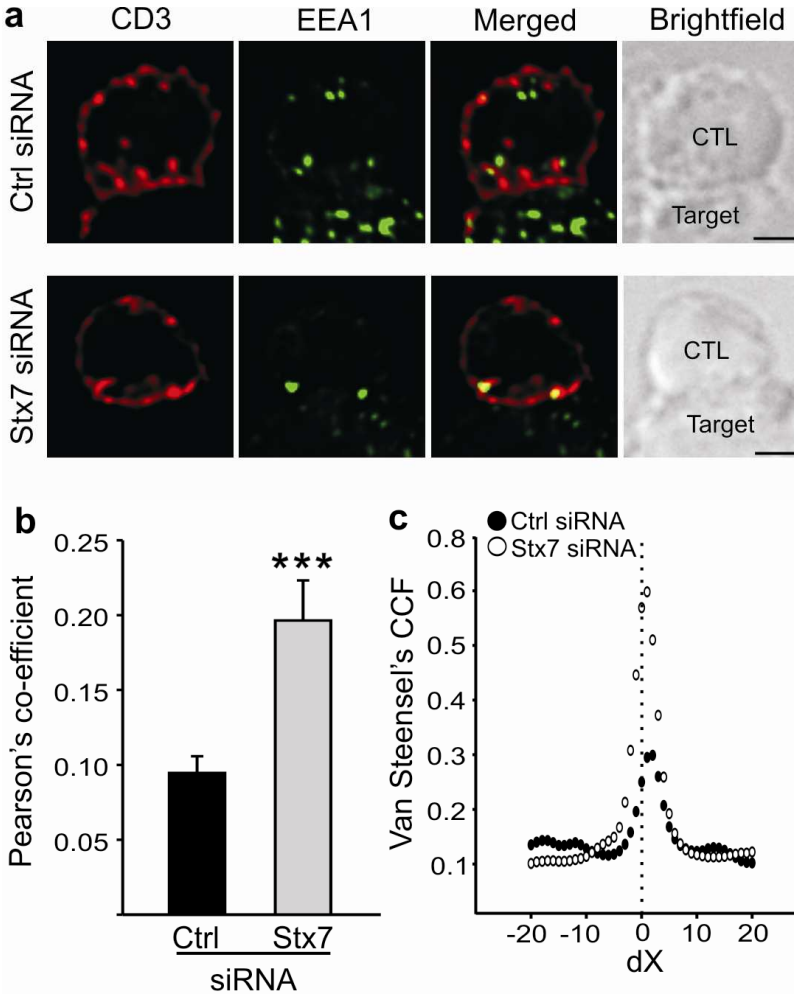

Figure 22. Defective TCR recycling in Syntaxin7 downregulated cells
(a) Representative images showing the colocalization of early endosomes with TCR in control siRNA and Syntaxin7 siRNA treated cells. EEA1 was used as the marker for early endosomes TCR was labeled using CD3-647 antibody. A significant increase in the colocalization of EEA1 and TCR was seen in Syntaxin7 siRNA treated cells when compared to control siRNA treated cells. Scale bars are 3 µm. (b) Pearsons's co-efficient was calculated as a measure for colocalization. 22 cells from control conditions and 17 cells from Syntaxin7 siRNA conditions were used for analysis. (c) One representative cell was taken from each of the above treated conditions to show the Van Steensel's CCF as another measure for colocalization.

Since the previous results indicated that the function of Syntaxin7 was to ensure the correct trafficking of TCR from early to late endosomes, we wanted to investigate the mechanism of its function. The important question we asked was to which compartment Syntaxin7 was localized.

Therefore, we analyzed the colocalization of Syntaxin7 with the early and late endosome markers EEA1 and Rab7 respectively in CTLs using SIM. We used the value of the Pearson's coefficient (r) as a measure for colocalization.

We found there was no significant colocalization between Syntaxin7 with EEA1 (r = 0.04 ± 0.01; Fig. 23a). There was partial colocalization with the late endosome marker Rab7 (r = 0.13 ± 0.02; Fig. 23b). The partial colocalization with Rab7 might be due to the possibility that Syntaxin7 is only localized to the TCR containing late endosomes and not the entire population of late endosomes in CTLs as there are several sub-populations of late endosomes in cells. Additionally, the partial colocalization with TCRs could also be because not all the TCRs in the cells are present in the late endosomes.

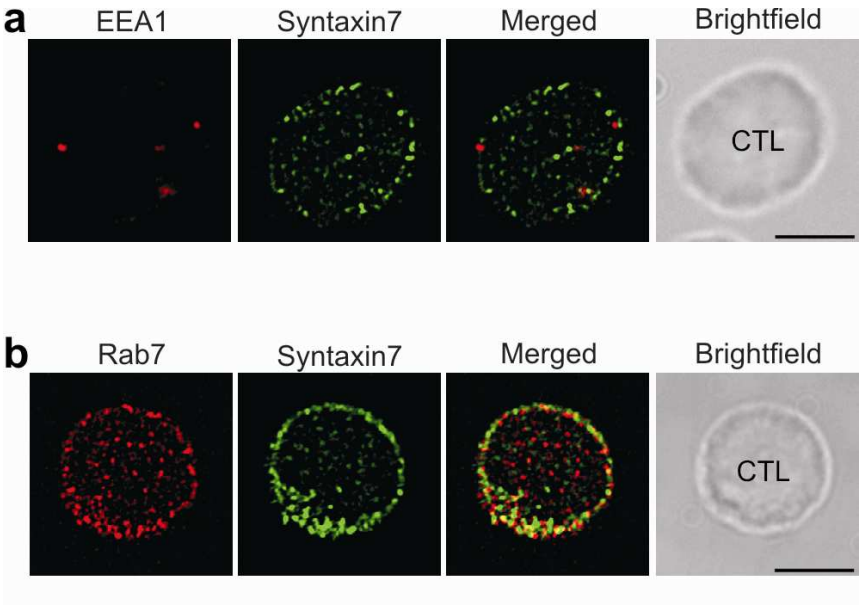

Figure 23. Syntaxin7 is preferentially localized to the late endosomes in CTLs.
Colocalization of Syntaxin7 with the (a) early endosome marker EEA1 and (b) late endosome marker Rab7 in resting CTLs. Shown are representative images that were acquired using Structured illumination microscopy to obtain an improved resolution in comparison to the acquisitions using the conventional laser scanning confocal microscopy. Scale bars are 5 µm.

Syntaxin7 is a Qa SNARE and therefore is the SNARE present on the target membrane of fusion. Since there was more colocalisation between TCR and EEA1 in the cells that were downregulated with Syntaxin7, it implies that Syntaxin7 is the Qa SNARE residing on the late endosomes and is required for fusion between the early and late endosomes.

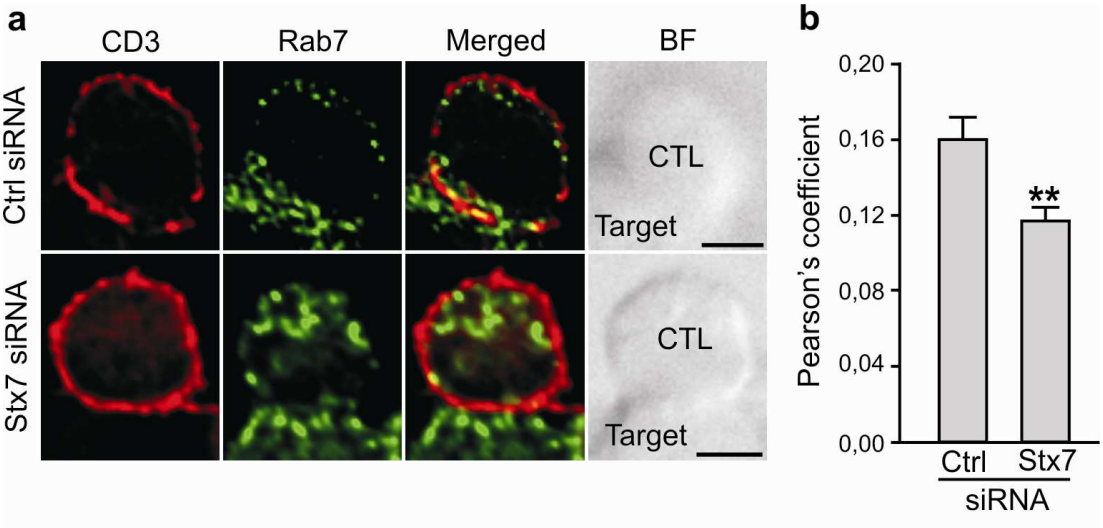

Figure 24. Less colocalization between CD3 and Rab7 in Syntaxin7 siRNA transfected cells
(a) Representative images showing the colocalization of CD3 and Rab7 in control and Syntaxin7 siRNA transfected CTLs. CTLs were incubated with SEA plused Raji cells for 30 minutes before the fixation and subsequent staining. (b) Colocalization analysis for Pearson's coefficient that was done using Jacob plugin in the Image J software. Scale bars are 3 μm.

If the TCR compartments were stuck in the early endosome compartment in the absence of functional Syntaxin7 then we would assume there should be less TCR colocalizing at the late endosome. This was indeed the case when we compared the colocalization between TCR and Rab7 in Syntaxin7 siRNA and control siRNA transfected CTLs (Fig. 24a). A significant reduction in the Pearson's correlation coefficient (Fig. 24b) further indicates that the increase in colocalization between the TCR and EEA1 was specifically due to the incomplete trafficking of TCRs from the early to late endosomes.

3.5 Expression of SNARES in CTLs under naïve and stimulated conditions

The importance of SNAREs in immune cells has been highlighted in Stow et al. (2006). The specific roles of few of the SNARE proteins have been identified in some of the immune cells. The importance of Syntaxin7 in CTLs that is needed for the killing of cognate target cells directed us towards investigating the importance of other SNAREs in CTLs. Several mutations have been identified in Syntaxin11, a Qa SNARE that cause defective NK cell degranulation and cytotoxicity. The outcome of this phenotype leads to the devastating FHL (see also section1.8). Recent studies from a VAMP8 knock out mouse by Loo et al. (2009) have shown that it is the R-

SNARE that is present on lytic granules and is required for their fusion with the plasma membrane. We tested the expression of all the SNAREs with the idea of determining their expression profile upon CTL activation. We used CD3/CD28 bead stimulated cells and compared the expression of all the SNAREs first at the mRNA level by RT-PCR. We saw that some of the SNAREs were not expressed in CTLs. Out of the remaining SNAREs some were upregulated upon activation and some showed no change in expression. The mRNA expression pattern of some of the tested SNAREs is shown in figure 25. Syntaxin11 was expressed in CTLs and was upregulated upon stimulation along with several other SNAREs such as Syntaxin3, Syntaxin4, Syntaxin6 and Syntaxin17. Some SNAREs like VAMP3 and VAMP4 showed no change in expression. The presence and upregulation of only certain SNAREs might implicate their importance in CTLs.

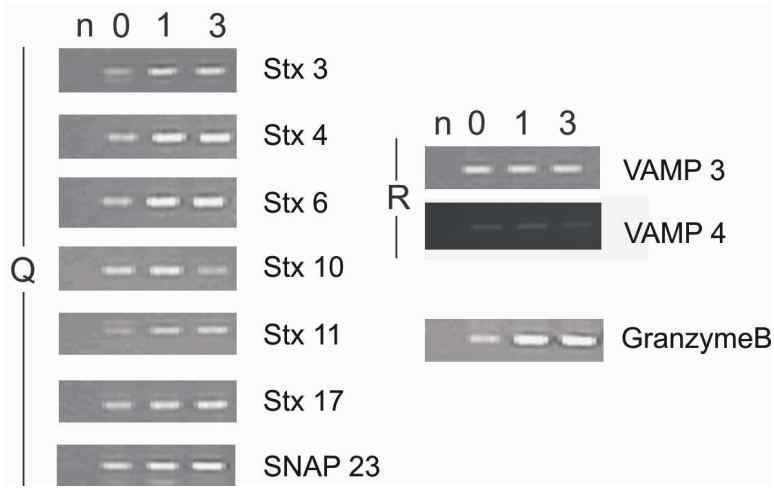

Figure 25. mRNA profile of some SNARE proteins with varying regulation upon activation.
RT-PCR on naïve (indictated as 0) and CD3/CD28 bead stimulated CTLs at day 1 and 3 after stimulation, n is the negative control. Shown are the expression profiles of some of the SNAREs tested.

We next wanted to test whether the SNARES that were either upregulated or that showed no change in their expression upon activation had any role in the release of lytic granules. We systematically tested the localization of various SNAREs in CTLs at the IS by immunocytochemistry as that could give a hint about its potential role in CTL effector function. Perforin was used to mark the IS. CTLs and target cells were incubated for 30 minutes to induce IS formation. The cell pairs were then fixed and stained for various SNARE proteins (Fig. 26). Confocal images of serial sections along the whole cell were taken to analyze the localization of various SNAREs at the

IS. Interestingly not all the SNAREs that were upregulated upon stimulation from the RT-PCR results accumulated at the IS in CTLs. Some among the various SNAREs tested with different patterns of localization at the IS are shown in Figure 26. Syntaxin3, 4 and 6 in addition to VAMP3 and 4 accumulated at the IS. Syntaxin 10, 17 and SNAP-23 did not accumulate at the IS.

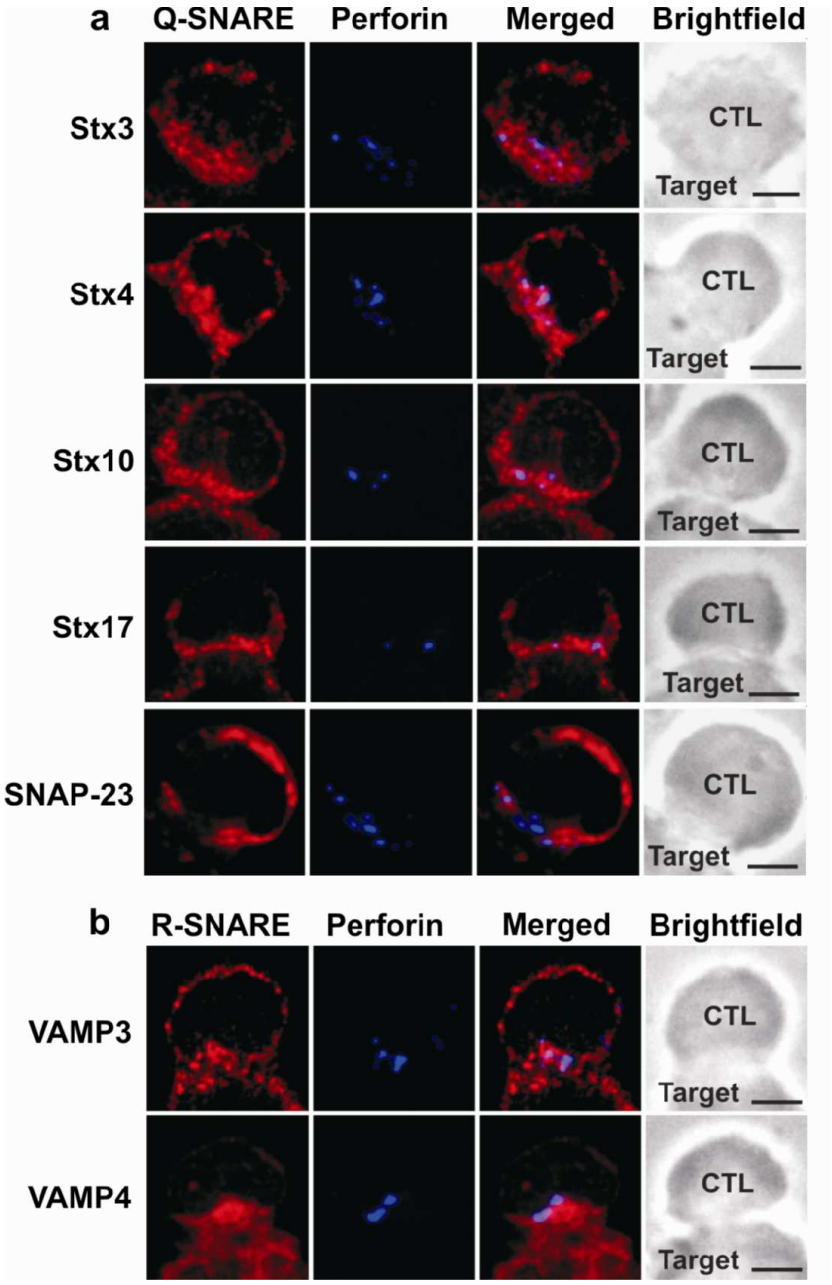

Figure 26. Accumulation of some of the Q and R SNAREs at the IS along with perforin
Shown are confocal images of the localization of selected Q (a) and R (b) SNAREs among the various that were tested. CTLs and target cells were incubated for 30 minutes. before fixation and subsequent immunostaining. Perforin was used as the lytic granule marker. Scale bars are 3 µm.

These results indicated that several SNAREs show promising localization at the IS with perforin implicating their possible role in lytic granule release at the IS. Detailed functional analyses of SNARE proteins could hint towards further understanding of the still mysterious killing process of CTLs.

4. Discussion

4.1 Are SNAREs indispensable for immune function?

Extensive studies have been done in the recent past showing the presence of several SNARE proteins in various cells of the immune system. There are many processes such as the release of molecules that mediate inflammation or other immune responses and the delivery of receptors to and from the cell surface that mediate signaling, which all need SNARE dependent fusion reactions (Lacy, 2005; Logan et al., 2003). In macrophages for example, the presence and upregulation of particular SNAREs such as Syntaxin4, 6 and Vti1b have been reported. More importantly, the protein levels were found to be correlating with the requirements for trafficking and were rate limiting for the secretion of TNF from activated macrophages (Murray et al., 2005a; Murray et al., 2005b; Pagan et al., 2003).

Platelets have fast release of granules content by direct fusion with the plasma membrane or slow release of granule contents through surface connected invaginations. The distinct location of specific Q SNAREs in these cell types determines the fate of the granule fusion (White, 1970). The Q SNARE Syntaxin4 alone or the Q-SNARE complex of Syntaxin2 and SNAP-23 regulate specific and distinct granule secretion events within the cell (Chen et al., 2000a; Chen et al., 2000b; Flaumenhaft et al., 1999; Lemons et al., 2000). The study by Huse et al. (2006) on activated T helper cells also show that there are a specific set of SNARE proteins that mediate secretion at the synapse that are different from the ones that mediate multi-directional cytokine secretion. In mast cells, the translocation of two SNAREs VAMP7 and Syntaxin3 containing granules to the plasma membrane has been consistent with their role in membrane fusion (Hibi et al., 2000). It was also shown that specific inhibition of VAMP7 blocks release of granules from neutrophils (Logan et al., 2006; Mollinedo et al., 2006). The regulation of SNAREs also plays an important role in immune function as shown by Hepp et al. (2009), where the phosphorylation of SNAP-23 is needed for activated mast cells to degranulate. The N terminal domains of SNARE proteins by interaction with Sec/Munc-like proteins also regulate SNARE complex formation as seen in the case of Syntaxin4 in mast cells. When Syntaxin4 dissociates from Munc18c, it allows Q-SNARE complex assembly formation for cytokine release and phagocytosis (Kay et al., 2006).

4.2 What do we know about SNAREs and CTLs??

However, the most compelling evidence about the absolute requirement for SNAREs in immune cells is seen in the form of genetic defects in lymphocyte mediated killing. The importance of the Qa SNARE Syntaxin11 in lymphocytes was identified by the defect in granule mediated killing that was linked to specific mutations in its gene (Albayrak et al., 2009; zur Stadt et al., 2005). Impaired binding of Munc18 to Syntaxin11 which results in the decreased stability of both proteins leads to a reduction in the function of CTLs and NK cells (Cote et al., 2009; zur Stadt et al., 2009).These lines of work in favour of the specificity of SNAREs in mediating various fusion events in the cells. Defects in SNARE regulating proteins such as Rab27a (Menasche et al., 2000; Stinchcombe et al., 2001a) and Munc13-4 (Feldmann et al., 2003) also have severe effects that lead to a complete defect in granule secretion. The protein LYST, mutations in which cause the genetic syndrome CHS, was shown to interact with the SNARE complex. This might reveal further mechanistic insights into the underlying cause of the severe genetic syndrome that results in defective lytic granule secretion in lymphocytes (Bossi and Griffiths, 2005; Tchernev et al., 2002).

Our results show for the first time that there is a differential regulation of SNAREs upon activation of naïve CD8T cells (Fig. 25). This is in agreement with studies on other immune cells which show also specific upregulation of certain SNAREs upon cell activation (Murray et al., 2005b). The SNARE proteins barring Syntaxin11 that are critically required for the function of CTLs have not been identified (Hong, 2005; Stow et al., 2006). Our data provide the first clue as to which SNAREs could play important roles in the activation or cytolytic function of CTLs. The dramatic upregulation of Syntaxin11 upon CTL activation is consistent with its function in lymphocyte degranulation (Arneson et al., 2007). The preferential localization of some SNAREs at the IS such as Syntaxin3, 4 and 10 (Fig. 26) could implicate their possible role in mediating secretion of specific components at the IS (Hibi et al., 2000). Taken together these results imply that SNAREs are important for CTL function and that identifying the specific SNAREs important for different steps in CTL function might give new insights into the function of CTL.

4.3 Syntaxin7 function in CTLs

In this study we show that the Qa SNARE protein Syntaxin7 is critically required for the cytolytic function of CTLs. Some aspects of the function of Syntaxin7 in the innate immune system has been studied earlier. Syntaxin7 was shown to mediate the secretion of TNF from macrophages (Murray et al., 2005a). It was also reported to function in the formation of phagosomes in macrophages (Collins et al., 2002). Using two independent methods of stimulation, we determined the expression profile of Syntaxin7 in naïve and stimulated CTLs and found that Syntaxin7 is upregulated upon CTL activation showing a strong expression at the days that CTLs show maximum killing efficiency. Most SNARE proteins that are upregulated upon immune cell activation have been shown to play a crucial role for the function of those activated cells (Murray et al., 2005b; Pagan et al., 2003). While it is not essential that the proteins important for the cytolytic function of T cells should always show an increased expression upon T cell activation (as it was the case for Munc13-4 in Menager et al., 2007), the upregulation of Syntaxin7 clearly implicated that the protein is important for some function of activated T cells. Our results on the localization of Syntaxin7 in resting and activated CTLs clearly indicated a preferential localization of Syntaxin7 at mature synapses. Studies on the Jurkat T cell lines have shown the accumulation of the plasma membrane Q SNAREs Syntaxin4 and SNAP-23 at the IS with a possible role for mediating the fusion of recycling TCRs with the plasma membrane (Das et al., 2004). We show that Syntaxin7 is needed for CTL mediated killing of target cells. Perforin, a marker for secretory lysosomes was shown to be one of the key players for CTL granule mediated target cell killing. A previous study in NK cells showed the preferential enrichment of Syntaxin7 in the lysosome fractions (Casey et al., 2007). However, our results using SIM showed that Syntaxin7 and perforin almost exclude each other in terms of colocalization. NK cells and CTLs both share similar pathways for target cell killing (Kagi et al., 1994) though NK cells require no pre-activation for killing as they belong to the innate immune system. This could account for the differences in the requirement of trafficking proteins in CTLs and NK cells for their respective functions.

We discuss in two parts about the two major findings from our studies on Syntaxin7 in CTLs in this chapter.

4.3.1 Perforin accumulation and TIRF microscopy

The first major surprise from our study was the significant effect of the lack of functional Syntaxin7 on the accumulation of perforin at the IS though they seemed to present on two different vesicle populations.

In order to check whether Syntaxin7 is influencing the final fusion event of perforin vesicles, we proposed to use TIRFM. TIRFM is a high resolution technique that allows exclusive visualization of biological processes within 200 nm from the plasma membrane (Holz and Axelrod, 2008; Nofal et al., 2007). TIRFM has been used to study secretion from neurons and chromaffin cells (Zenisek et al., 2000). The use of membrane bilayers with CD3 and ICAM antibodies have been used to form a functional IS with T cells to enable studies using TIRFM (Dustin, 2008; Ilani et al., 2009). TCR microcluster formation and the Actin dynamics have been studied using TIRF microscopy (Dustin, 2008). Mitochondrial movement to the IS and calcium signaling have been studied using TIRF microscopy with the IS formed between CTLs and coated glass coverlips with CD3 and other co-stimulatory molecules (Quintana et al., 2009; Quintana et al., 2007). When we employed TIRF microscopy for the first time to study secretion of lytic granules at the IS we observed a gradual accumulation of lytic granules at the IS over time. The accumulation of perforin at the synapse was rapid and occurred within minutes of CTL adhering to antibody coating. This was expected of synapses formed from CTLs as they are known to form rapid and short synapses leading to target cell destruction (Bossi et al., 2002; Isaaz et al., 1995). The rapid accumulation led to a phase where there was no further significant increase in the accumulation of lytic granules. CTLs do synthesize new perforin granules after TCR stimulation (Isaaz et al., 1995). The limitation to the number of granules that turn out to be fusion competent for killing at the IS is probably a safety mechanism for CTL function. CTLs probably to ensure efficient killing and to always have a supply of perforin have devised a method where only limited granules are targeted to the IS which could explain the sustained phase in perforin accumulation that was reliably seen after the initial rise in accumulation after 5 minutes of CTLs adhering to the glass coverslips. Not all the lytic granules in the CTL even approach the IS as seen in our results where simultaneous epifluorescence imaging shows the presence of lytic granules in the epifluorescence images taken above the TIRF plane persisting overtime. Studies using labeled granules of CTLs have shown that very few granules need to be released to destroy a target. Lyubchenko et al. (2009) have

shown that in TALL-104 cells (CTL cell lines) only 15% of lytic granules in a cell are released at the IS. We show that approximately only 20% of the vesicles that approach the IS fuse with the glass coverslip.

The experiments with Stx7 FL-TFP show a similar pattern of perforin accumulation at the TIRF plane over time. In Stx7 ΔC-TFP there is an almost complete block of perforin accumulation and fusion at the IS. The results were confirmed in a qualitative manner by confocal microscopy with fixed CTL-target cell conjugates. The TIRFM method can therefore be used reliably to study the events occurring at the CTL secretory synapse prior to the secretion of lytic granules and can probably be used to quantify pre secretion events such as docking and priming.

4.3.2 TCR trafficking and Syntaxin7

One of the other major findings in this study is the function of Syntaxin7 in the trafficking of recycling TCRs to the IS and the importance of this pathway for CTL effector function. We report that in the absence of functional Syntaxin7, recycling TCRs cannot get past the early endosomes and remain trapped there. There have been reports of fast recycling where the early endosomes bypass recycling endosomes and fuse with the plasma membrane (Sheff et al., 1999). One explanation for our result could have been that Syntaxin7 is localized to the early endosome and needed for the fusion of early endosomes with the plasma membrane constituting a role in the fast recycling. Evidently, the first report on Syntaxin7 showed it to be colocalizing with transferrin receptors marking the recycling compartment of the early endosomes (Wong et al., 1998). This could explain the findings from Prekeris et al. (1999) where Syntaxin7 constantly cycles through the plasma membrane. However, in that scenario the SNARE hypothesis would predict Syntaxin7 to be localized on the plasma membrane, since as a Qa SNARE it would reside on the target membrane of the two fusing compartments. Other authors also claimed a localization of Syntaxin7 to the early endosome (Nakamura et al., 2000). In contrast, our results show that Syntaxin7 is partially colocalized to the late endosome fraction in CTLs. Our findings are in agreement with studies by Wang and coworkers who have shown Syntaxin7 to be localized to both late endosome and lysosome compartments (Wang et al., 1997). Studies using electron microscopy have also shown that Syntaxin7 was localized to the multivesicular bodies along with Rab7 (Mullock et al., 2000). In NK cells the secretory lysosomes and not the early

endosomes or late endosomes were reported to have an enriched fraction of Syntaxin7. This implied that the TCR trafficking from the early to late endosomes required Syntaxin7. This was in agreement with the work reported by Prekeris et al. (1999) which showed that Syntaxin7 was mediating the fusion of early and late endosome compartments. Since in this scenario the Syntaxin7 would reside on the correct target membrane, we consider a localization of Syntaxin7 in the membrane of late endosomes more likely.

Studies using truncated Syntaxin7 with deletion of the transmembrane domain have shown to block the function of Syntaxin7 leading to cytoplasmic staining (Kasai and Akagawa, 2001; Nakamura et al., 2000). We report here that the over-expression of truncated Syntaxin7 leads to a clear phenotype, the loss of TCR accumulation at the IS. The use of tetanus toxin which specifically inactivates VAMP3 on Jurkat cells shows a defect in recycling TCR accumulation (Das et al., 2004), indicating that the accumulation of recycling TCR at the IS is SNARE-dependent. In our study the defect in recycling TCR accumulation leading to the defect in killing clearly implicates the importance of this process in CTL. It also goes to show that all three processes of TCR transport to the IS, lateral diffusion of TCR to the IS (Favier et al., 2001), actin mediated transport and the trafficking mediated by SNAREs are required for the cluster of TCR accumulation (Das et al., 2004) and do not compensate for the loss of the other.

Both siRNA and dominant negative approach show an increase in colocalization between EEA1 and TCR in Syntaxin7 downregulated cells. This was specifically supported by a significant decrease in the reduction of colocalization between Rab7 and TCR in the Syntaxin7 downregulated cells. Since the accumulation of recycling TCR contributes to the formation of a cSMAC, in the next part we discuss about the role of the cSMAC in CTL effector function.

The requirement and function of the cSMAC has been under debate. Naïve T cells have been shown not to be dependent on the formation of a cSMAC for their activation (O'Keefe et al., 2004). Studies have also shown that a mature synapse is not required for the effector function of CTLs (Purbhoo et al., 2004) and that CTLs can kill target cells without the formation of a complete cSMAC. Killing of target cells was shown to occur in the presence of low concentrations of antigen presentation. Strong antigen signals ensure complete signaling and the formation of SMACs. However it was shown recently that low TCR signaling can ensure the polarization of

MTOC to the IS but only in response to strong TCR signals can the efficient transport of lytic granules to the IS take place (Jenkins et al., 2009). Since recycling TCR do contribute to the formation of TCR accumulaton at the IS (Das et al., 2004), granule polarization therefore requires the normal accumulation of of recycling TCRs as is also shown by our results.

Trafficking of TCR is probably also very important in CTLs for the termination of signals after ensuring the killing of target cells. It has been shown that at the cSMAC TCR is internalized and degraded (Liu et al., 2000). The trafficking of TCR from the early to late endosome could be the decisive factor in determining the fate of TCR to the plasma membrane or to the lysosome for degradation. Previous studies have shown that early endosomes are the compartments where the decision of recycling or degradation takes place (Rink et al., 2005; Sheff et al., 1999). Late endosomes mature from early endosomes and from the late endosomes components are recylyed back though the trans golgi network to reach the recycling endosomes (Maxfield and McGraw, 2004). This decision is probably regulated by the function of Syntaxin7 that mediates the fusion between the early and late endosome which is crucial in determining the persistence of TCR signaling required for full effector potential of CTLs.

Another finding from our results is the transient localization of Syntaxin7 at the plasma membrane which is probably because of a necessity to have a second step of TCR regulation. A previous study on Syntaxin7 proposed that it is not only colocalized to both late endosomes and lysosomes but also functionally active on both membranes (Ward et al., 2000b). The yeast Vam3p was also shown to be mediating homotypic fusion of yeast vacuoles. This fusion reaction is NSF dependent and requires the vSNARE Nyv1p to be both present and functionally active on both partners. This model was used to explain that Syntaxin7 may be active on both late endosome and lysosome compartments and catalyzes fusion events from a number of different pathways that converge at late endosome/lysosome compartments. Our results show that Syntaxin7 is not only present in late endosomes but also at the plasma membrane. One reason for the strong plasma membrane localization of Syntaxin7 could be that it is functional in both membranes. SNAP-23 was proposed to be important for the recycling of TCRs in the Jurkat cells. However, in our results, we did not see any accumulation of SNAP-23 (Qbc SNARE) in primary human T cells. One possible explanation could be that primary cells have different trafficking

partners. Syntaxin7 (Qa SNARE) probably also functions at the plasma membrane as the target SNARE for the fusion of TCR granules. Syntaxin8 (Qc SNARE), a known endosomal SNARE, is also supposed to be localized to the plasma membrane (Prekeris et al., 1999) and is a known interacting partner for Syntaxin7 (Antonin et al., 2002b). VAMP3 (R SNARE) which is the vSNARE on the recycling TCR (Das et al., 2004) has been shown to interact with Syntaxin7 (Murray et al., 2005a) and therefore Syntaxin7 and Syntaxin8 along with another Qb SNARE (probably Vti1a or Vti1b) could interact with VAMP3 in controlling the fusion of recyling TCR. One could envision that CTLs have a two step mechanism to regulate the signaling of TCR at the IS. One step would be at the level of the early/late endosomes and, if for some reason this is bypassed, then the other would be at the plasma membrane. Syntaxin7 is the only endosomal SNARE that adopts an open and closed conformation for regulation (Antonin et al., 2002a). Normally this regulation is supposed to be only for plasma membrane SNAREs (Dulubova et al., 1999). This could be one possible explanation for how Syntaxin7 regulates the trafficking of TCR at the IS.

Syntaxin7 therefore regulates the accumulation of TCR at the IS at two levels. CTLs are very efficient killers and sustained TCR signaling at the IS might result in CTL-target cell remaining in contact even after target cell killing. Therefore termination of signal after target cell killing is very important for CTLs to move to kill the next target for a quick and efficient effector response. One important question remains to be studied: Which molecule is binding to be the closed form of Syntaxin7 to ensure its regulation? An attractive candidate would be Munc18 that is known to mediate this function at the active zone of CNS neurons.

Our results on the function of Syntaxin7 in CTLs establish that SNAREs are important for recycling of TCR to the membrane (Das et al., 2004). We show in addition that this specific process is critically required for the killing function of CTLs.

4.4 Outlook

The importance of Syntaxin7 in CTL function leads to several open questions about the specific function of other SNAREs and SNARE related proteins for CTL effector function. A lot is left to be discovered in the field about the importance of SNARE trafficking. Recently it was shown that VAMP8 is important for lytic granule release (Loo et al., 2009). If this is indeed the case then it could be the v-SNARE on the lytic granules that fuses with the t-SNARES Stx4 and SNAP-23 which are both known

interacting partners for VAMP8 (Paumet et al., 2000; Polgar et al., 2002; Puri and Roche, 2006). Syntaxin4 and SNAP-23 are both localized to the plasma membrane. The explanation for SNAP-23 not accumulating at the IS but being important for a fusion event at the IS could be that there are so few lytic granules that are released at the IS for killing one target cell that there is no necessity for SNAP-23 to accumulate in the plasma membrane. It could be that SNAP-23 is specific only for the release of lytic granules. Syntaxin4 the other Q-SNARE accumulates very well at the IS. This could be because it might be regulating some other fusion event at the IS as well such as the release of cytokines.

What is also not known is the importance of other regulators for the release of lytic granules. Neuronal synapses are well studied. There are several additional molecules apart from SNAREs such as Munc18, Synaptotagmin, and CAPS that are well known to be playing an important role in the fusion of neuronal vesicles (Rettig and Neher, 2002; Walent et al., 1992). What are these molecules good for in mediating the function of CTLs? Impaired binding of Syntaxin11 to Munc18 causes FHL-5. The mechanism or the reasons for the mutations in Syntaxin11 leading to FHL are not known. Is it blocking the final fusion event or some very important pathway in between? It is known to be localized to the TGN. One might not be able to rule out the possibility that the mutations in Syntaxin11 that lead to FHL might be because of blocking the sorting of molecules that are important for the cytolytic function of T cells. It would also be very interesting to know what role Munc18 is indeed playing in this whole process.

A recent study showed the function of Synaptotagmin in the release of lytic granules leaving us to believe that there is a lot of similarity in the basic mechanism of lytic granule and neurotransmitter release (Fowler et al., 2007).

Another protein that is involved in the secretion of dense core vesicles in neuroendocrine cells is the Ca^{2+}-dependent Activator Protein for secretion (CAPS). Studies have shown the presence of CAPS in non neuronal tissues (Sadakata et al., 2007). Interestingly both the thymus and spleen as important sites for T cell maturation and activation show the expression of CAPS. CAPS protein has a Munc-13 homology domain (MHD) that functions as a Syntaxin interacting domain. Will CAPS have a function to play by binding to Syntaxin11 or some other SNARE in CTLs? The function of CAPS in CTL effector function if any might give new insights into the molecular mechanisms of the release of lytic granules.

5. Summary

The function of cytotoxic T lymphocytes (CTLs) is to kill infected cells in the body. In order to kill their targets they must first come in contact with them to form an immunological synapse (IS). It is at the IS that the lytic granules containing cytotoxic components such as perforin and granzymes fuse with the plasma membrane of the CTL, an event that is necessary to bring about apoptosis of the specific target cell. SNARE proteins (soluble NSF-attachment receptor proteins) are well known as mediators of fusion events in cells. The specific SNARE proteins that are important for the fusion of lytic granules have not been identified. Here we analyzed the function of a SNARE protein, Syntaxin7, in CTL function using two independent approaches to interfere with its function – overexpression of a Syntaxin7 dominant negative construct and siRNA.

We show that Syntaxin7 is critically required for the accumulation and fusion of lytic granules at the IS. Surprisingly we found no colocalization of Syntaxin7 with the lytic granule maker perforin. Our results therefore established that the reduction in lytic granule fusion at the IS was not because of a defect in the trafficking of perforin itself. Further studies showed that there was a defective accumulation of T cell receptors (TCRs) at the IS indicating that the formation of a complete cSMAC required the function of Syntaxin7. We observed a significant increase in colocalization between EEA 1 (early endosome autoantigen 1), a marker for early endosomes and TCR upon interference with Syntaxin7 function. High resolution nanoscopy showed that Syntaxin7 and Rab7, a marker for late endosomes were partially colocalized. We show that Syntaxin7 is required for the trafficking of recycling TCR and that in the absence of functional Syntaxin7 the TCRs are trapped in the early endosomes. This results in incomplete recycling and defective accumulation of TCRs at the IS. Our results provide evidence that the accumulation of recycling TCRs at the IS is a necessity or a pre-requisite for CTL effector function. We also ascertain the specific function of Syntaxin7 in the recycling pathway of TCRs in CTLs.

6. Literature

Albayrak, M., Kaya, Z., Yilmaz-Keskin, E., Stadt, U.Z., Kocak, U., and Gursel, T. (2009). Fatal Epstein-Barr virus infection in a case of familial hemophagocytic lymphohistiocytosis with syntaxin-11 mutation. The Turkish journal of pediatrics *51*, 371-374.

Andersen, M.H., Schrama, D., Thor Straten, P., and Becker, J.C. (2006). Cytotoxic T cells. The Journal of investigative dermatology *126*, 32-41.

Aniento, F., Gu, F., Parton, R.G., and Gruenberg, J. (1996). An endosomal beta COP is involved in the pH-dependent formation of transport vesicles destined for late endosomes. The Journal of cell biology *133*, 29-41.

Antonin, W., Dulubova, I., Arac, D., Pabst, S., Plitzner, J., Rizo, J., and Jahn, R. (2002a). The N-terminal domains of syntaxin 7 and vti1b form three-helix bundles that differ in their ability to regulate SNARE complex assembly. The Journal of biological chemistry *277*, 36449-36456.

Antonin, W., Fasshauer, D., Becker, S., Jahn, R., and Schneider, T.R. (2002b). Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. Nature structural biology *9*, 107-111.

Arico, M., Allen, M., Brusa, S., Clementi, R., Pende, D., Maccario, R., Moretta, L., and Danesino, C. (2002). Haemophagocytic lymphohistiocytosis: proposal of a diagnostic algorithm based on perforin expression. British journal of haematology *119*, 180-188.

Arneson, L.N., Brickshawana, A., Segovis, C.M., Schoon, R.A., Dick, C.J., and Leibson, P.J. (2007). Cutting edge: syntaxin 11 regulates lymphocyte-mediated secretion and cytotoxicity. J Immunol *179*, 3397-3401.

Baetz, K., Isaaz, S., and Griffiths, G.M. (1995). Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. J Immunol *154*, 6122-6131.

Balaji, K.N., Schaschke, N., Machleidt, W., Catalfamo, M., and Henkart, P.A. (2002). Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. The Journal of experimental medicine *196*, 493-503.

Barbosa, M.D., Nguyen, Q.A., Tchernev, V.T., Ashley, J.A., Detter, J.C., Blaydes, S.M., Brandt, S.J., Chotai, D., Hodgman, C., Solari, R.C*., et al.* (1996). Identification

of the homologous beige and Chediak-Higashi syndrome genes. Nature *382*, 262-265.

Barysch, S.V., Aggarwal, S., Jahn, R., and Rizzoli, S.O. (2009). Sorting in early endosomes reveals connections to docking- and fusion-associated factors. Proceedings of the National Academy of Sciences of the United States of America *106*, 9697-9702.

Bennett, M.K., Calakos, N., and Scheller, R.H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science (New York, NY *257*, 255-259.

Beresford, P.J., Zhang, D., Oh, D.Y., Fan, Z., Greer, E.L., Russo, M.L., Jaju, M., and Lieberman, J. (2001). Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. The Journal of biological chemistry *276*, 43285-43293.

Bolte, S., and Cordelieres, F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. Journal of microscopy *224*, 213-232.

Bossi, G., and Griffiths, G.M. (2005). CTL secretory lysosomes: biogenesis and secretion of a harmful organelle. Seminars in immunology *17*, 87-94.

Bossi, G., Trambas, C., Booth, S., Clark, R., Stinchcombe, J., and Griffiths, G.M. (2002). The secretory synapse: the secrets of a serial killer. Immunological reviews *189*, 152-160.

Bousso, P. (2008). T-cell activation by dendritic cells in the lymph node: lessons from the movies. Nature reviews.

Bright, N.A., Gratian, M.J., and Luzio, J.P. (2005). Endocytic delivery to lysosomes mediated by concurrent fusion and kissing events in living cells. Curr Biol *15*, 360-365.

Bright, N.A., Reaves, B.J., Mullock, B.M., and Luzio, J.P. (1997). Dense core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. Journal of cell science *110 (Pt 17)*, 2027-2040.

Bromley, S.K., and Dustin, M.L. (2002). Stimulation of naive T-cell adhesion and immunological synapse formation by chemokine-dependent and -independent mechanisms. Immunology *106*, 289-298.

Burkhardt, J.K., Hester, S., Lapham, C.K., and Argon, Y. (1990). The lytic granules of natural killer cells are dual-function organelles combining secretory and pre-lysosomal compartments. The Journal of cell biology *111*, 2327-2340.

Casey, T.M., Meade, J.L., and Hewitt, E.W. (2007). Organelle proteomics: identification of the exocytic machinery associated with the natural killer cell secretory lysosome. Mol Cell Proteomics *6*, 767-780.

Chattopadhyay, P.K., Betts, M.R., Price, D.A., Gostick, E., Horton, H., Roederer, M., and De Rosa, S.C. (2009). The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. Journal of leukocyte biology *85*, 88-97.

Chen, D., Bernstein, A.M., Lemons, P.P., and Whiteheart, S.W. (2000a). Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 in dense core granule release. Blood *95*, 921-929.

Chen, D., Lemons, P.P., Schraw, T., and Whiteheart, S.W. (2000b). Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 and 4 in lysosome release. Blood *96*, 1782-1788.

Chen, Y.A., and Scheller, R.H. (2001). SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol *2*, 98-106.

Collins, R.F., Schreiber, A.D., Grinstein, S., and Trimble, W.S. (2002). Syntaxins 13 and 7 function at distinct steps during phagocytosis. J Immunol *169*, 3250-3256.

Costello, P.S., Gallagher, M., and Cantrell, D.A. (2002). Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. Nature immunology *3*, 1082-1089.

Cote, M., Menager, M.M., Burgess, A., Mahlaoui, N., Picard, C., Schaffner, C., Al-Manjomi, F., Al-Harbi, M., Alangari, A., Le Deist, F.*, et al.* (2009). Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. The Journal of clinical investigation *119*, 3765-3773.

Das, V., Nal, B., Dujeancourt, A., Thoulouze, M.I., Galli, T., Roux, P., Dautry-Varsat, A., and Alcover, A. (2004). Activation-induced polarized recycling targets T cell antigen receptors to the immunological synapse; involvement of SNARE complexes. Immunity *20*, 577-588.

Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T.C., and Rizo, J. (1999). A conformational switch in syntaxin during exocytosis: role of munc18. The EMBO journal *18*, 4372-4382.

Dustin, M.L. (2008). T-cell activation through immunological synapses and kinapses. Immunological reviews *221*, 77-89.

Dustin, M.L., Bromley, S.K., Kan, Z., Peterson, D.A., and Unanue, E.R. (1997). Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. Proceedings of the National Academy of Sciences of the United States of America *94*, 3909-3913.

Ebnet, K., Hausmann, M., Lehmann-Grube, F., Mullbacher, A., Kopf, M., Lamers, M., and Simon, M.M. (1995). Granzyme A-deficient mice retain potent cell-mediated cytotoxicity. The EMBO journal *14*, 4230-4239.

Eisenbarth, S.C., and Flavell, R.A. (2009). Innate instruction of adaptive immunity revisited: the inflammasome. EMBO molecular medicine *1*, 92-98.

Faroudi, M., Utzny, C., Salio, M., Cerundolo, V., Guiraud, M., Muller, S., and Valitutti, S. (2003). Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. Proceedings of the National Academy of Sciences of the United States of America *100*, 14145-14150.

Fasshauer, D. (2003). Structural insights into the SNARE mechanism. Biochimica et biophysica acta *1641*, 87-97.

Fasshauer, D., Eliason, W.K., Brunger, A.T., and Jahn, R. (1998a). Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. Biochemistry *37*, 10354-10362.

Fasshauer, D., Sutton, R.B., Brunger, A.T., and Jahn, R. (1998b). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proceedings of the National Academy of Sciences of the United States of America *95*, 15781-15786.

Favier, B., Burroughs, N.J., Wedderburn, L., and Valitutti, S. (2001). TCR dynamics on the surface of living T cells. International immunology *13*, 1525-1532.

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.

Feldmann, J., Le Deist, F., Ouachee-Chardin, M., Certain, S., Alexander, S., Quartier, P., Haddad, E., Wulffraat, N., Casanova, J.L., Blanche, S.*, et al.* (2002).

Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. British journal of haematology *117*, 965-972.

Fischer, H., Dohlsten, M., Lindvall, M., Sjogren, H.O., and Carlsson, R. (1989). Binding of staphylococcal enterotoxin A to HLA-DR on B cell lines. J Immunol *142*, 3151-3157.

Flaumenhaft, R., Croce, K., Chen, E., Furie, B., and Furie, B.C. (1999). Proteins of the exocytotic core complex mediate platelet alpha-granule secretion. Roles of vesicle-associated membrane protein, SNAP-23, and syntaxin 4. The Journal of biological chemistry *274*, 2492-2501.

Fowler, K.T., Andrews, N.W., and Huleatt, J.W. (2007). Expression and function of synaptotagmin VII in CTLs. J Immunol *178*, 1498-1504.

Furst, J., Sutton, R.B., Chen, J., Brunger, A.T., and Grigorieff, N. (2003). Electron cryomicroscopy structure of N-ethyl maleimide sensitive factor at 11 A resolution. The EMBO journal *22*, 4365-4374.

Goldschneider, I., Komschlies, K.L., and Greiner, D.L. (1986). Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. The Journal of experimental medicine *163*, 1-17.

Goransdotter Ericson, K., Fadeel, B., Nilsson-Ardnor, S., Soderhall, C., Samuelsson, A., Janka, G., Schneider, M., Gurgey, A., Yalman, N., Revesz, T.*, et al.* (2001). Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. American journal of human genetics *68*, 590-597.

Gowans, J.L., and Knight, E.J. (1964). The Route of Re-Circulation of Lymphocytes in the Rat. Proceedings of the Royal Society of London Series B, Containing papers of a Biological character *159*, 257-282.

Gustafsson, M.G., Shao, L., Carlton, P.M., Wang, C.J., Golubovskaya, I.N., Cande, W.Z., Agard, D.A., and Sedat, J.W. (2008). Three-dimensional resolution doubling in

wide-field fluorescence microscopy by structured illumination. Biophysical journal *94*, 4957-4970.

Hao, M., and Maxfield, F.R. (2000). Characterization of rapid membrane internalization and recycling. The Journal of biological chemistry *275*, 15279-15286.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Sudhof, T.C., and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. The EMBO journal *13*, 5051-5061.

Henter, J.I., Arico, M., Elinder, G., Imashuku, S., and Janka, G. (1998). Familial hemophagocytic lymphohistiocytosis. Primary hemophagocytic lymphohistiocytosis. Hematology/oncology clinics of North America *12*, 417-433.

Hibi, T., Hirashima, N., and Nakanishi, M. (2000). Rat basophilic leukemia cells express syntaxin-3 and VAMP-7 in granule membranes. Biochemical and biophysical research communications *271*, 36-41.

Hirst, C.E., Buzza, M.S., Bird, C.H., Warren, H.S., Cameron, P.U., Zhang, M., Ashton-Rickardt, P.G., and Bird, P.I. (2003). The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. J Immunol *170*, 805-815.

Hohl, T.M., Parlati, F., Wimmer, C., Rothman, J.E., Sollner, T.H., and Engelhardt, H. (1998). Arrangement of subunits in 20 S particles consisting of NSF, SNAPs, and SNARE complexes. Molecular cell *2*, 539-548.

Holz, R.W., and Axelrod, D. (2008). Secretory granule behaviour adjacent to the plasma membrane before and during exocytosis: total internal reflection fluorescence microscopy studies. Acta physiologica (Oxford, England) *192*, 303-307.

Hong, W. (2005). Cytotoxic T lymphocyte exocytosis: bring on the SNAREs! Trends in cell biology *15*, 644-650.

Horejsi, V., Zhang, W., and Schraven, B. (2004). Transmembrane adaptor proteins: organizers of immunoreceptor signalling. Nature reviews *4*, 603-616.

Huppa, J.B., Gleimer, M., Sumen, C., and Davis, M.M. (2003). Continuous T cell receptor signaling required for synapse maintenance and full effector potential. Nature immunology *4*, 749-755.

Hurez, V., Saparov, A., Tousson, A., Fuller, M.J., Kubo, T., Oliver, J., Weaver, B.T., and Weaver, C.T. (2003). Restricted clonal expression of IL-2 by naive T cells reflects differential dynamic interactions with dendritic cells. The Journal of experimental medicine *198*, 123-132.

Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89-95.

Ilani, T., Vasiliver-Shamis, G., Vardhana, S., Bretscher, A., and Dustin, M.L. (2009). T cell antigen receptor signaling and immunological synapse stability require myosin IIA. Nature immunology *10*, 531-539.

Isaaz, S., Baetz, K., Olsen, K., Podack, E., and Griffiths, G.M. (1995). Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. European journal of immunology *25*, 1071-1079.

Jahn, R., Lang, T., and Sudhof, T.C. (2003). Membrane fusion. Cell *112*, 519-533.
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.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31-37.

Kaizuka, Y., Douglass, A.D., Varma, R., Dustin, M.L., and Vale, R.D. (2007). Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. Proceedings of the National Academy of Sciences of the United States of America *104*, 20296-20301.

Kasai, K., and Akagawa, K. (2001). Roles of the cytoplasmic and transmembrane domains of syntaxins in intracellular localization and trafficking. Journal of cell science *114*, 3115-3124.

Kataoka, T., Schroter, M., Hahne, M., Schneider, P., Irmler, M., Thome, M., Froelich, C.J., and Tschopp, J. (1998). FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. J Immunol *161*, 3936-3942.

Kay, J.G., Murray, R.Z., Pagan, J.K., and Stow, J.L. (2006). Cytokine secretion via cholesterol-rich lipid raft-associated SNAREs at the phagocytic cup. The Journal of biological chemistry *281*, 11949-11954.

Kojima, H., Shinohara, N., Hanaoka, S., Someya-Shirota, Y., Takagaki, Y., Ohno, H., Saito, T., Katayama, T., Yagita, H., Okumura, K.*, et al.* (1994). Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. Immunity *1*, 357-364.

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

Kuta, A.E., Reynolds, C.R., and Henkart, P.A. (1989). Mechanism of lysis by large granular lymphocyte granule cytolysin: generation of a stable cytolysin-RBC intermediate. J Immunol *142*, 4378-4384.

Lacy, P. (2005). The role of Rho GTPases and SNAREs in mediator release from granulocytes. Pharmacology & therapeutics *107*, 358-376.

Lee, K.H., Holdorf, A.D., Dustin, M.L., Chan, A.C., Allen, P.M., and Shaw, A.S. (2002). T cell receptor signaling precedes immunological synapse formation. Science (New York, NY *295*, 1539-1542.

Lemons, P.P., Chen, D., and Whiteheart, S.W. (2000). Molecular mechanisms of platelet exocytosis: requirements for alpha-granule release. Biochemical and biophysical research communications *267*, 875-880.

Lieberman, J. (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nature reviews *3*, 361-370.

Liu, H., Rhodes, M., Wiest, D.L., and Vignali, D.A. (2000). On the dynamics of TCR:CD3 complex cell surface expression and downmodulation. Immunity *13*, 665-675.

Logan, M.R., Lacy, P., Odemuyiwa, S.O., Steward, M., Davoine, F., Kita, H., and Moqbel, R. (2006). A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils. Allergy *61*, 777-784.

Logan, M.R., Odemuyiwa, S.O., and Moqbel, R. (2003). Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion. The Journal of allergy and clinical immunology *111*, 923-932; quiz 933.

Loo, L.S., Hwang, L.A., Ong, Y.M., Tay, H.S., Wang, C.C., and Hong, W. (2009). A role for endobrevin/VAMP8 in CTL lytic granule exocytosis. European journal of immunology *39*, 3520-3528.

Lowin, B., Beermann, F., Schmidt, A., and Tschopp, J. (1994a). A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity. Proceedings of the National Academy of Sciences of the United States of America *91*, 11571-11575.

Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. (1994b). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature *370*, 650-652.

Lowin, B., Peitsch, M.C., and Tschopp, J. (1995). Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. Current topics in microbiology and immunology *198*, 1-24.

Lyubchenko, T.A., Wurth, G.A., and Zweifach, A. (2001). Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. Immunity *15*, 847-859.

Mantei, A., Rutz, S., Janke, M., Kirchhoff, D., Jung, U., Patzel, V., Vogel, U., Rudel, T., Andreou, I., Weber, M.*, et al.* (2008). siRNA stabilization prolongs gene knockdown in primary T lymphocytes. European journal of immunology *38*, 2616-2625.

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Faure, J., Blanc, N.S., Matile, S., Dubochet, J., Sadoul, R.*, et al.* (2004). Role of LBPA and Alix in multivesicular liposome formation and endosome organization. Science (New York, NY *303*, 531-534.

Maxfield, F.R., and McGraw, T.E. (2004). Endocytic recycling. Nat Rev Mol Cell Biol *5*, 121-132.

Menasche, G., Pastural, E., Feldmann, J., Certain, S., Ersoy, F., Dupuis, S., Wulffraat, N., Bianchi, D., Fischer, A., Le Deist, F.*, et al.* (2000). Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nature genetics *25*, 173-176.

Misura, K.M., Scheller, R.H., and Weis, W.I. (2000). Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature *404*, 355-362.

Mollinedo, F., Calafat, J., Janssen, H., Martin-Martin, B., Canchado, J., Nabokina, S.M., and Gajate, C. (2006). Combinatorial SNARE complexes modulate the secretion of cytoplasmic granules in human neutrophils. J Immunol *177*, 2831-2841.

Mullock, B.M., Bright, N.A., Fearon, C.W., Gray, S.R., and Luzio, J.P. (1998). Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. The Journal of cell biology *140*, 591-601.

Mullock, B.M., Smith, C.W., Ihrke, G., Bright, N.A., Lindsay, M., Parkinson, E.J., Brooks, D.A., Parton, R.G., James, D.E., Luzio, J.P.*, et al.* (2000). Syntaxin 7 is localized to late endosome compartments, associates with Vamp 8, and Is required for late endosome-lysosome fusion. Molecular biology of the cell *11*, 3137-3153.

Murray, R.Z., Kay, J.G., Sangermani, D.G., and Stow, J.L. (2005a). A role for the phagosome in cytokine secretion. Science (New York, NY *310*, 1492-1495.

Murray, R.Z., Wylie, F.G., Khromykh, T., Hume, D.A., and Stow, J.L. (2005b). Syntaxin 6 and Vti1b form a novel SNARE complex, which is up-regulated in activated macrophages to facilitate exocytosis of tumor necrosis Factor-alpha. The Journal of biological chemistry *280*, 10478-10483.

Nagata, S. (1996). Fas-mediated apoptosis. Advances in experimental medicine and biology *406*, 119-124.

Nakajima, H., Park, H.L., and Henkart, P.A. (1995). Synergistic roles of granzymes A and B in mediating target cell death by rat basophilic leukemia mast cell tumors also expressing cytolysin/perforin. The Journal of experimental medicine *181*, 1037-1046.

Nakamura, N., Yamamoto, A., Wada, Y., and Futai, M. (2000). Syntaxin 7 mediates endocytic trafficking to late endosomes. The Journal of biological chemistry *275*, 6523-6529.

Nofal, S., Becherer, U., Hof, D., Matti, U., and Rettig, J. (2007). Primed vesicles can be distinguished from docked vesicles by analyzing their mobility. J Neurosci *27*, 1386-1395.

Norcross, M.A. (1984). A synaptic basis for T-lymphocyte activation. Annales d'immunologie *135D*, 113-134.

O'Keefe, J.P., Blaine, K., Alegre, M.L., and Gajewski, T.F. (2004). Formation of a central supramolecular activation cluster is not required for activation of naive CD8+

T cells. Proceedings of the National Academy of Sciences of the United States of America *101*, 9351-9356.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., and Wilson, M.C. (1989). The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. The Journal of cell biology *109*, 3039-3052.

Pagan, J.K., Wylie, F.G., Joseph, S., Widberg, C., Bryant, N.J., James, D.E., and Stow, J.L. (2003). The t-SNARE syntaxin 4 is regulated during macrophage activation to function in membrane traffic and cytokine secretion. Curr Biol *13*, 156-160.

Pannetier, C., Even, J., and Kourilsky, P. (1995). T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunology today *16*, 176-181.

Paul, W.E., Brown, M., Hornbeck, P., Mizuguchi, J., Ohara, J., Rabin, E., Snapper, C., and Tsang, W. (1987). Regulation of B-lymphocyte activation, proliferation, and differentiation. Annals of the New York Academy of Sciences *505*, 82-89.

Paumet, F., Le Mao, J., Martin, S., Galli, T., David, B., Blank, U., and Roa, M. (2000). Soluble NSF attachment protein receptors (SNAREs) in RBL-2H3 mast cells: functional role of syntaxin 4 in exocytosis and identification of a vesicle-associated membrane protein 8-containing secretory compartment. J Immunol *164*, 5850-5857.

Peters, P.J., Borst, J., Oorschot, V., Fukuda, M., Krahenbuhl, O., Tschopp, J., Slot, J.W., and Geuze, H.J. (1991). Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. The Journal of experimental medicine *173*, 1099-1109.

Poirier, M.A., Hao, J.C., Malkus, P.N., Chan, C., Moore, M.F., King, D.S., and Bennett, M.K. (1998). Protease resistance of syntaxin.SNAP-25.VAMP complexes. Implications for assembly and structure. The Journal of biological chemistry *273*, 11370-11377.

Polgar, J., Chung, S.H., and Reed, G.L. (2002). Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. Blood *100*, 1081-1083.

Potter, T.A., Grebe, K., Freiberg, B., and Kupfer, A. (2001). Formation of supramolecular activation clusters on fresh ex vivo CD8+ T cells after engagement of the T cell antigen receptor and CD8 by antigen-presenting cells. Proceedings of the National Academy of Sciences of the United States of America *98*, 12624-12629.

Prekeris, R., Yang, B., Oorschot, V., Klumperman, J., and Scheller, R.H. (1999). Differential roles of syntaxin 7 and syntaxin 8 in endosomal trafficking. Molecular biology of the cell *10*, 3891-3908.

Purbhoo, M.A., Irvine, D.J., Huppa, J.B., and Davis, M.M. (2004). T cell killing does not require the formation of a stable mature immunological synapse. Nature immunology *5*, 524-530.

Puri, N., and Roche, P.A. (2006). Ternary SNARE complexes are enriched in lipid rafts during mast cell exocytosis. Traffic (Copenhagen, Denmark) *7*, 1482-1494.

Quintana, A., Kummerow, C., Junker, C., Becherer, U., and Hoth, M. (2009). Morphological changes of T cells following formation of the immunological synapse modulate intracellular calcium signals. Cell calcium *45*, 109-122.

Quintana, A., Schwindling, C., Wenning, A.S., Becherer, U., Rettig, J., Schwarz, E.C., and Hoth, M. (2007). T cell activation requires mitochondrial translocation to the immunological synapse. Proceedings of the National Academy of Sciences of the United States of America *104*, 14418-14423.

Rettig, J., and Neher, E. (2002). Emerging roles of presynaptic proteins in Ca++-triggered exocytosis. Science (New York, NY *298*, 781-785.

Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. Cell *122*, 735-749.

Robertson, L.K., Mireau, L.R., and Ostergaard, H.L. (2005). A role for phosphatidylinositol 3-kinase in TCR-stimulated ERK activation leading to paxillin phosphorylation and CTL degranulation. J Immunol *175*, 8138-8145.

Russell, M.R., Nickerson, D.P., and Odorizzi, G. (2006). Molecular mechanisms of late endosome morphology, identity and sorting. Current opinion in cell biology *18*, 422-428.

Sadakata, T., Washida, M., Morita, N., and Furuichi, T. (2007). Tissue distribution of Ca2+-dependent activator protein for secretion family members CAPS1 and CAPS2 in mice. J Histochem Cytochem *55*, 301-311.

Sauer, H., Pratsch, L., Tschopp, J., Bhakdi, S., and Peters, R. (1991). Functional size of complement and perforin pores compared by confocal laser scanning microscopy and fluorescence microphotolysis. Biochimica et biophysica acta *1063*, 137-146.

Seaman, M.N. (2004). Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. The Journal of cell biology *165*, 111-122.

Sheff, D.R., Daro, E.A., Hull, M., and Mellman, I. (1999). The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. The Journal of cell biology *145*, 123-139.

Shinkai, Y., Takio, K., and Okumura, K. (1988). Homology of perforin to the ninth component of complement (C9). Nature *334*, 525-527.

Shiver, J.W., and Henkart, P.A. (1991). A noncytotoxic mast cell tumor line exhibits potent IgE-dependent cytotoxicity after transfection with the cytolysin/perforin gene. Cell *64*, 1175-1181.

Smyth, M.J., Thia, K.Y., Street, S.E., MacGregor, D., Godfrey, D.I., and Trapani, J.A. (2000). Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. The Journal of experimental medicine *192*, 755-760.

Sollner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993a). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell *75*, 409-418.

Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993b). SNAP receptors implicated in vesicle targeting and fusion. Nature *362*, 318-324.

Stinchcombe, J.C., Barral, D.C., Mules, E.H., Booth, S., Hume, A.N., Machesky, L.M., Seabra, M.C., and Griffiths, G.M. (2001a). Rab27a is required for regulated secretion in cytotoxic T lymphocytes. The Journal of cell biology *152*, 825-834.

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

Stinchcombe, J.C., Page, L.J., and Griffiths, G.M. (2000). Secretory lysosome biogenesis in cytotoxic T lymphocytes from normal and Chediak Higashi syndrome patients. Traffic (Copenhagen, Denmark) *1*, 435-444.

Stow, J.L., Manderson, A.P., and Murray, R.Z. (2006). SNAREing immunity: the role of SNAREs in the immune system. Nature reviews *6*, 919-929.

Stradal, T.E., Rottner, K., Disanza, A., Confalonieri, S., Innocenti, M., and Scita, G. (2004). Regulation of actin dynamics by WASP and WAVE family proteins. Trends in cell biology *14*, 303-311.

Suga, N., Takada, H., Nomura, A., Ohga, S., Ishii, E., Ihara, K., Ohshima, K., and Hara, T. (2002). Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. British journal of haematology *116*, 346-349.

Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature *395*, 347-353.

Tchernev, V.T., Mansfield, T.A., Giot, L., Kumar, A.M., Nandabalan, K., Li, Y., Mishra, V.S., Detter, J.C., Rothberg, J.M., Wallace, M.R.*, et al.* (2002). The Chediak-Higashi protein interacts with SNARE complex and signal transduction proteins. Molecular medicine (Cambridge, Mass *8*, 56-64.

Thoulouze, M.I., Sol-Foulon, N., Blanchet, F., Dautry-Varsat, A., Schwartz, O., and Alcover, A. (2006). Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse. Immunity *24*, 547-561.

Trambas, C.M., and Griffiths, G.M. (2003). Delivering the kiss of death. Nature immunology *4*, 399-403.

Trapani, J.A., and Smyth, M.J. (2002). Functional significance of the perforin/granzyme cell death pathway. Nature reviews *2*, 735-747.

Trimble, W.S., Cowan, D.M., and Scheller, R.H. (1988). VAMP-1: a synaptic vesicle-associated integral membrane protein. Proceedings of the National Academy of Sciences of the United States of America *85*, 4538-4542.

Tschopp, J., Masson, D., and Stanley, K.K. (1986). Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. Nature *322*, 831-834.

Tugores, A., Alonso, M.A., Sanchez-Madrid, F., and de Landazuri, M.O. (1992). Human T cell activation through the activation-inducer molecule/CD69 enhances the activity of transcription factor AP-1. J Immunol *148*, 2300-2306.

van den Broek, M.E., Kagi, D., Ossendorp, F., Toes, R., Vamvakas, S., Lutz, W.K., Melief, C.J., Zinkernagel, R.M., and Hengartner, H. (1996). Decreased tumor

surveillance in perforin-deficient mice. The Journal of experimental medicine *184*, 1781-1790.

Van Parijs, L., Peterson, D.A., and Abbas, A.K. (1998). The Fas/Fas ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens. Immunity *8*, 265-274.

Villalba, M., Bi, K., Rodriguez, F., Tanaka, Y., Schoenberger, S., and Altman, A. (2001). Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells. The Journal of cell biology *155*, 331-338.

Voskoboinik, I., Smyth, M.J., and Trapani, J.A. (2006). Perforin-mediated target-cell death and immune homeostasis. Nature reviews *6*, 940-952.

Walent, J.H., Porter, B.W., and Martin, T.F. (1992). A novel 145 kd brain cytosolic protein reconstitutes Ca(2+)-regulated secretion in permeable neuroendocrine cells. Cell *70*, 765-775.

Wang, H., Frelin, L., and Pevsner, J. (1997). Human syntaxin 7: a Pep12p/Vps6p homologue implicated in vesicle trafficking to lysosomes. Gene *199*, 39-48.

Ward, D.M., Griffiths, G.M., Stinchcombe, J.C., and Kaplan, J. (2000a). Analysis of the lysosomal storage disease Chediak-Higashi syndrome. Traffic (Copenhagen, Denmark) *1*, 816-822.

Ward, D.M., Pevsner, J., Scullion, M.A., Vaughn, M., and Kaplan, J. (2000b). Syntaxin 7 and VAMP-7 are soluble N-ethylmaleimide-sensitive factor attachment protein receptors required for late endosome-lysosome and homotypic lysosome fusion in alveolar macrophages. Molecular biology of the cell *11*, 2327-2333.

White, J.G. (1970). A search for the platelet secretory pathway using electron dense tracers. The American journal of pathology *58*, 31-49.

Wong, S.H., Xu, Y., Zhang, T., and Hong, W. (1998). Syntaxin 7, a novel syntaxin member associated with the early endosomal compartment. The Journal of biological chemistry *273*, 375-380.

Zal, T., Zal, M.A., and Gascoigne, N.R. (2002). Inhibition of T cell receptor-coreceptor interactions by antagonist ligands visualized by live FRET imaging of the T-hybridoma immunological synapse. Immunity *16*, 521-534.

Zenisek, D., Steyer, J.A., and Almers, W. (2000). Transport, capture and exocytosis of single synaptic vesicles at active zones. Nature *406*, 849-854.

zur Stadt, U., Rohr, J., Seifert, W., Koch, F., Grieve, S., Pagel, J., Strauss, J., Kasper, B., Nurnberg, G., Becker, C.*, et al.* (2009). Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. American journal of human genetics *85*, 482-492.

zur Stadt, U., Schmidt, S., Kasper, B., Beutel, K., Diler, A.S., Henter, J.I., Kabisch, H., Schneppenheim, R., Nurnberg, P., Janka, G.*, et al.* (2005). Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. Human molecular genetics *14*, 827-834.

Curriculum Vitae

Personal information

Name: Varsha Pattu

Date of birth: 20th July, 1982

Place of birth: Chennai, India.

Marital Status: Single

Education

2005	Master degree in Molecular Biology at University of Madras, India.
2003	Bachelor degree in Biochemistry at University of Madras, India.
2000	Secondary education
1998	Primary school

Research work

July 2005 – April2009	Research assistant at the National Centre for Biological Sciences (NCBS), Bangalore, India.
July 2006 - present	Research in the department of Prof.Dr. Jens Rettig in Physiologisches Institut, Geb 59 Universität des Saarlandes, Homburg, Germany.

Publications

Syntaxin7 is required for lytic granule release from cytotoxic T lymphocytes

Varsha Pattu*, Bin Qu*, Misty Marshall, Christian Junker, Ulf Matti, Eva M. Schwarz, Elmar Krause, Markus Hoth, and Jens Rettig. (in preparation)

CTL function requires Vti1b-dependent interaction between lytic granules and endocytosed TCR compartments

Bin Qu*, Varsha Pattu*, Christian Junker, Eva C. Schwarz, Misty Marshall, Ute Becherer, Ulf Matti, Heiko Rieger, Jens Rettig, Markus Hoth (in preparation)

*** - Equal contribution**