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Functions of Syntaxin 8 in human cytotoxic T lymphocytes

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To my beloved parents and teachers

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ABBREVIATIONS

Ab	Antibody
APC	Antigen presenting cell
AP1	Adaptor Protein 1
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHX	Cycloheximide
CHO	Chinese hamster ovary cells
CMA	Conconamycin A
CRAC current	Calcium release-activated calcium current
CRISPRs	Cluster Regularly Interspaced Short Palindromic Repeats
CTLA4	Cytotoxic T-Lymphocyte antigen 4
Ctrl-	Control
cSMAC	Central supra-molecular activation complex
DG	Diacylglycerol
dSMAC	Distal supra-molecular activation complex
DISC	Death Inducing Signalling Complex
EEA1	Early Endosome Autoantigen 1
ER	Endoplasmic reticulum
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FHL	Familial hemophagocytic lymphohistiocytosis
GTPase	Gaunosine triphoshatase
HBSS	Hank's balanced salt solution
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid,
IP3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activating motif
IS	Immunological synapse
JACoP	Just Another Colocalization plugin

LAMP	Lysosomal associated membrane protein
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LRC	Leucocyte reduction chamber
MHC	Major histocompatibility complex
MTOC	Microtubule organization centre
NK	Natural killer cells
NKT	Natural killer T cells
NRK	Normal rat kidney epithelial
NRL	Normal rat liver
NSF	N-ethylamide sensitive factor
PBMCs	Pheripheral blood mononuclear cells
pSMAC	Pheripheral supra-molecular activation complex
RT	Room temperature
SDS	Sodium dodecyl sulphate
SE	Staphylococcal enterotoxins
si-RNA	small interfering RNA
SNAP	Soluble NSF Attachment Protein
SNARE	Soluble N-ethylmaleimide sensitive factor Attachment protein Receptors
Stx	Syntaxin
TCR	T cell receptor
TGN	Trans Golgi network
Tc	T cytotoxic cell
Th	T helper cell
Treg	T regulatory cell
VAMP	Vesicle associated membrane protein

ZUSAMMENFASSUNG

Zytotoxische T-Lymphozyten (CTL) sind die $CD8^+$ T-Effektor-Lymphozyten des Immunsystems, welche Zellen, die entweder mit Pathogenen infiziert sind, Tumorzellen darstellen oder als Nicht-selbst erkannt wurden, identifizieren und töten, ohne benachbarte gesunde Zellen zu schädigen. Dies wird durch die spezifische Freisetzung zytotoxischer Moleküle an der immunologischen Synapse (IS), einer engen Kontaktstelle zwischen CTL und Zielzelle, erreicht. Soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE)-Komplexe und SNARE-Komplex-verwandte Proteine, die an dem Signalweg des Vesikeltransports beteiligt sind, wurden auch eine Beteiligung beim Sortieren und beim Transport der lytischen Granula spezifisch zur IS hin zugesprochen.

Die Untersuchung des Expressionsmusters verschiedener SNARE-Proteine in primären humanen CTL hat gezeigt, dass Syntaxin 8 sowohl in naiven als auch in aktivierten $CD8^+$ T-Zellen sehr hoch exprimiert ist. Zusätzlich war Syntaxin 8 eines der untersuchten SNARE-Proteine, das zu einen hohen Anteil mit lytischen Granula in CTL kolokalisiert war [1]. Aktuell werden Qa-, Qb- und R-SNARE, aber bisher kein Qc-SNARE, mit Funktionen von CTL in Zusammenhang gebracht. Daher wird in dieser Arbeit der Fokus auf Syntaxin 8 liegen, da Syntaxin 8, ein Qc-SNARE, an der IS akkumuliert und teilweise mit lytischen Granula kolokalisiert, was für eine möglicherweise wichtige Rolle in der Funktion von CTL sprechen kann. Somit haben wir uns das Ziel gesetzt, die Bedeutung von Syntaxin 8 bei der Zytotoxizität humaner CTL zu untersuchen. Unsere Untersuchungen weisen darauf hin, dass Syntaxin 8 am Transportweg der Endosomen beteiligt ist, was durch die Kolokalisation mit frühen Endosomen (teilweise), späten, Recycling- und lysomalen Kompartimenten, vor allen

Dingen aber mit späten und Recycling-Endosomen, unterstützt wird. Einhergehend mit einer effizienten Herunterregulation von Syntaxin 8 haben wir eine reduzierte Zytotoxizität in CTL beobachtet, die mit Hilfe eines Zytotoxizitätsassays in Echtzeit gemessen wurde. Weiterhin konnten wir mittels eines ELISA für Perforin zeigen, dass die verminderte Zytotoxozität durch Syntaxin 8 Herunterregulation nicht durch eine verminderte Exozytose (von Perforin) bedingt wird, sondern vorangegangene Prozesse, die zu einer verspäteten Freisetzung von Perforin oder lytischer Granula führen können, betroffen sind. Diese vorangegangenen Prozesse können zum einen die Generierung zytotoxischer Moleküle sein, zum anderen können sie aber auch das Sortieren und den Transport zytotoxischer Moleküle in zur Freisetzung bereite lytische Granula hin zur IS der CTL betreffen. Die Behandlung mit Cycloheximid von Kontroll- und Syntaxin 8 herunterregulierten CTL weist darauf hin, dass Mechanismen betroffen sind, die zeitlich nach der Proteinsynthese liegen. Da die Zytotoxizität zu späteren Zeitpunkten in den Syntaxin 8 herunterregulierten Zellen sich unter DMSO leicht erhöht hat (was in den mit Cyclohexamid behandelten Syntaxin 8 herunterregulierten Zellen nicht beobachtet wurde), kann man davon ausgehen, dass die Sortierung und Ausschüttung der zytotoxischen Moleküle an der IS beeinflusst ist, schließt aber nicht die Möglichkeit aus, dass Syntaxin 8 die Generierung der zytotoxischen Moleküle beeinflusst. FACS Analysen haben gezeigt, dass sich der Perforin-Gehalt zwischen Kontroll- und Syntaxin 8 herunterregulierten CTL, die mit Zielzellen in Kontakt gebracht wurden, zu unterschiedlichen Zeitpunkten nicht unterschieden hat. Dennoch haben wir eine generelle Tendenz zu einem erhöhten Perforin-Gehalt in Kontroll-CTL beobachtet. Die Untersuchung von mit Zielzellen konjugierten CTL mittels Immunzytochemie und hochauflösenden bildgebenden Verfahren (mit Hilfe von Auflichtfluoreszenzmikroskopie und

anschließender Dekonvolution) zu unterschiedlichen Zeitpunkten hat gezeigt, dass die Akkumulation des T-Zell-Rezeptors (TZR) und der lytischen Granula nur nach zwei Stunden Konjugation in Syntaxin 8 herunterregulierten Zellen beeinträchtigt war. Syntaxin 8 herunterregulierte Zellen verfügten im Vergleich zu Kontroll-Zellen über größere Tropfen-ähnliche Perforin enthaltende Vesikel, ein Hinweis darauf, dass Perforin schon in der frühen Phase der Sortierung zurückbleibt, wahrscheinlich in Kompartimenten des trans-Golgi-Netzwerkes. Diese Verzögerung beim Sortieren und Verteilen in Syntaxin 8 herunterregulierten Zellen mag der Grund dafür sein, dass lytische Granula zwar die IS erreichen, dort aber sehr langsam freigesetzt werden im Vergleich zu den Kontrollzellen und damit eine verminderte Zytotoxizität hervorrufen. Wenn Syntaxin 8 herunterregulierte CTL im Vergleich zu Kontrollzellen eine verminderte Zytotoxizität zeigen, haben wir zu späteren Zeitpunkten eine langsamere Degradation des TZR in diesen Zellen im Vergleich zu Kontrollzellen gefunden. Aber auch wenn das Recycling des TZR zur Plasmamembran vielleicht langsamer ist, so ist die Bildung der IS in Syntaxin 8 herunterregulierten Zellen nicht vermindert. Aus diesem Grunde machen Syntaxin 8 herunterregulierte CTL normale Kontakte mit Zielzellen, zeigen eine nicht beeinträchtigte Exozytose, aber weisen eine verminderte oder verzögerte Sortierung bzw. einen verminderten oder verzögerten Transport zytotoxischer Moleküle in zur Freisetzung fähige lytische Granula auf. Demnach reguliert Syntaxin 8 die Zytotoxizität von CTL durch den Einfluss auf das Sortieren und Verteilen der zytotoxischen Moleküle durch den Reifungsweg der Endosomen.

1. INTRODUCTION

1.1. Immune system

All living organisms have their own defense mechanisms to survive against foreign invaders. The higher the organism is, in the evolutionary tree, more evolved is its defense mechanism executed by its immune system. Single cell prokaryotes like bacteria have rudimentary defense mechanisms such as secretion of certain enzymes targeting viruses and also CRISPRs (Cluster Regularly Interspaced Short Palindromic Repeats) which have partial palindromic repeats separated by short stretches of DNA called spacers, acquired from extrachromosomal elements like viral genetic materials which are also cleaved by these CRISPRs [2]. Primitive eukaryotes, plants, insects and primitive chordates have slightly evolved immune systems involving phagocytosis, complement system and production of anti-microbial peptides. In mammals the immune system is more extensive and highly evolved consisting of a dynamic network of cells and molecules acting together to identify and eliminate foreign particles, pathogens, cancerous cells or mutated self cells. Immune system in mammals can be classified into innate immunity and adaptive immunity. Innate immunity is an organism's first line of defense. It has broad reactivity and is not specific for a particular pathogen. It includes physical barriers like skin, mucus membrane, tears; components of complement system, cytokines and cells like macrophages, neutrophils, eosinophils, basophils and also natural killer (NK) cells. On encountering an infection, components of the innate immune system initially induce inflammation and vasodilation by release of chemicals like histamine, the components of the complement system, which in turn mobilize the immune cells like

macrophages, NK cells, dendritic cells, neutrophils and other leukocytes to phagocytose the pathogen at the site of the inflammation [3]. Recently the evidences emerged show that NK cells are also part of adaptive immune response as they are known to posses antigen specific immune memory and can respond to secondary infections by the same antigen [4,5]. On the other hand, adaptive immune responses are highly specific, as every antigen is distinguished and selectively eliminated. Another significant feature of adaptive immunity is its immunological memory which facilitates the system to react rapidly to antigens on its second encounter, thus providing lifelong immunity against many antigens. The ability of adaptive immune system to recognize self and non-self cells is essential to eliminate foreign antigens without harming the self cells.

1.2. Cell mediated and humoral immunity

Adaptive immunity consists of cell-mediated and humoral immune responses. B lymphocytess and T lymphocytes are the main components of cell-mediated immunity and soluble immunoglubulins (antibodies) secreted by B lymphocytes majorly consist the humoral immunity [6].

B lymphocytes mature within the bone marrow, express and secrete antibodies which are specific and unique for each antigen. Antibodies are glycoproteins with antigen binding sites which can bind specifically to its corresponding antigenic epitopes. When naïve B cells come in contact with an antigen, they proliferate rapidly and mature into effector cells also called as plasma cells (that rapidly secrete huge amount antibodies which neutralize the antigen) and memory cells. This antigen specific immune memory is preserved in the memory cells which live longer than the naïve cells [7]. Such antibodies are functional components of humoral immunity.

T lymphocytes are another main aspect of cell-mediated immunity. Like B Lymphocytes, T lymphocytes also originate in the bone marrow but they mature in thymus. Unlike B cells, T cells can recognize antigens only when they are processed and presented as antigenic peptides by special receptors called major histocompatibility complex (MHC) molecules expressed on cell membranes. The two major variants of MHC molecules that are expressed in mammals are MHC class I and class II variants. MHC class I is expressed by almost all types of cells in the body and composes of a heavy chain linked to β2 – microglobulin. Whereas MHC class II is expressed by antigen presenting cells and is made up of alpha and beta subunits [8]. The T cell receptors (TCR) expressed by T cells identify and bind to the antigenic peptides bound to the MHC molecules. This stimulates the T cells and causes them to proliferate and mature into effector and memory T cells. T cells can be classified into four main types. T Helper (Th) cells , T Cytotoxic (Tc) cells, Natural killer T (NKT) cells and γδ T cells [9]. Th cells are also called as $CD4^+$ T cells and Tc cells as $CD8^+$ T cells as they have CD4 (Cluster of Differentiation 4) and CD8 co-receptors respectively. A subpopulation of these cells develop into regulatory T cells (Tregs) or suppressor T cells (Ts) which help in the immune cell homeostasis [10-12]. $CD4^+$ and $CD8^+$ cells on coming in contact with the immunogen, mature into effector cells and later into memory T cells [13]. Th ($CD4^+$) cells recognize and bind to antigenic peptides presented on MHC class II molecules. This brings about stimulation of the $CD4^+$ cells causing them to proliferate and stimulate cytokines like IL-2, IL-4, IL-10, INF-γ etc. These cytokines in turn induce the activation and proliferation of B lymphocytes, Tc cells, macrophages and other components of the immune system [14].

Tc cells or $CD8^+$ T cells recognize and interact with antigenic peptides presented on MHC class I molecules via T cell receptors (TCR) [15]. This interaction stimulates and

activates these $CD8^+$ cells to mature into effector T cells and later into memory cells [13,16]. Such activated effector $CD8^+$ T cells are also called as Cytotoxic T Lymphocytes (CTLs), as they kill the target cells via release of cytotoxic molecules. These target cells are the cells which are pathogen infected cells or tumor cells or foreign cells from graft tissues. Memory T cells have longer life span and can be easily activated and converted to effector memory T cells which are cytotoxic and are capable of killing their targets [17].

1.3. Cytotoxic T Lymphocytes (CTLs)

The effector $CD8^+$ T cells or CTLs and also NK cells are known to kill their targets by two major pathways, by secretion of lytic granules containing perforin and granzymes and by expression of FAS ligands [18-21] (Fig. 1). Another mechanism of killing mostly used by activated macrophages, NK cells and $CD4^+$ T cells is through TNF-α mediated cytokine pathway [22,23] but it is not yet clear if it is used as a killing mechanism by CTLs though TNF-α expression increases on activation of $CD8^+$ T cells [23,24]. CTLs form with their cognate target cells a tight junction, called the immunological synapse (IS), where they specifically release their cytotoxic molecules to kill the target cells, without harming the healthy bystander cells. One CTL or NK cell can kill more than one target cell either simultaneously and/or consecutively [25]. The effector $CD8^+$ T cells recognize peptides presented by MHC I molecules on target cells to form conjugates and finally kill the target cells. Endogenous proteins or peptides expressed in virus infected or cancerous cells are cleaved to smaller peptides in organelles called proteosomes. Such peptides are then bound to MHC I molecules assembled in rough (ER) endoplasmic reticulum. These peptide MHC I complexes are transported to the plasma membrane of these altered target cells, which are then

identified by TCR complexes of the $CD8^+$ T cells. $CD8^+$ T cells can be activated in vitro by an artificial stimulation with superantigens derived from Staphylococcus aureus. Such superantigens are called Staphylococcal Enterotoxins A, B, E (SEA, SEB and SEE). These superantigens are not expressed as processed peptides on MHC, but they bind to MHC class II molecule at a region distinct from its antigenic peptide binding site and interact with CD8 or CD4 co-receptors of the T cells [26-28].

1.3.1. T Cell Receptor complex

TCR complex is the functional unit of the T cells responsible for the recognition of the processed antigens leading to the activation of the T cells. They can recognize only processed antigenic peptides expressed on MHC molecules of the target cells. In $CD8^+$ T cells, the TCR and its CD8 co-receptor recognize and bind to the antigen MHC class I complex. Once they recognize and bind to the antigenic complex, another important component of the TCR complex called the CD3 molecules, which have intracellular domains, transduce the signals to other signalling molecules leading to T cell activation. Most T cells have αβTCR where a membrane bound immunoglobulin-like heterodimer TCR is made of disulphide bonded α and β chains. These are the antigen binding units of the TCR complex [29].

Around 5 % of T cells have been shown to express γδTCR which can recognize and bind to non classical MHC molecules or directly to pathogen glycoproteins [30].

The CD3 receptor which is another functional unit of the TCR complex is composed of CD3γε, CD3δε subunits and CD3ζζ chains [31].

When the TCR identifies and binds to the antigen-MHC complex, there is clustering of the CD8 co-receptors which causes the Lck bound to the cytoplasmic tail of the CD8 co-receptor to phosphorylate the immunoreceptor Tyrosine- based activation

motifs (ITAMs) found on the cytoplasmic tails of the CD3 co-receptors [32]. These phosphorylated tyrosine motifs become docking sites for downstream effector molecules such as ZAP-70, a tyrosine kinase, which further phosphorylates SLP-76 and LAT. These phosphorylated SLP-76 and LAT serve as docking elements for numerous cytoplasmic signalling molecules including Grb2, Vav (triggering Ras/MAP (Mitogen Activated Protein) kinase pathway), PLC-γ1 or PI3-kinase [33]. The PLC1 γ derived calcium signalling is crucial for normal T cell functioning. Activated PLC1 γ generates second messengers Inositol trisphosphate (IP3) and Diacylglycerol (DG) by cleaving PIP_2. IP3 then facilitates active depletion of ER Ca^{2+} stores which mediates ORAI mediated CRAC currents causing Ca^{2+} influx into the T cell [34-36]. Ca^{2+} influx and maintenance of elevated levels of $[Ca^{2+}]_i$ is then brought about by the mitochondrial Ca^{2+} uptake [37]. Ca^{2+} is necessary for many T cell functions like T cell activation, cytotoxicity, activation of transcription factors that bring about proliferation of the T cell and also production of cytokines like IL-2 [38].

1.3.2. Immunological Synapse

Activation of the T cells promotes the polarization of the T cell by movement of the microtubule organization centre (MTOC) to the junction where the CTL conjugates with the target cell [39]. This junction is termed as an immunological synapse (IS) (Fig. 1). TCR accumulation and MTOC movement is said to be essential for the formation of a functional IS. The IS has been divided into different domains: the central Supramolecular Activation Complex (cSMAC), peripheral Supramolecular Activation Complex (pSMAC) and the distal Supramolecular Activation Complex (dSMAC) [40]. The cSMAC is the central region containing the TCR MHC-antigen complex, CD28,

CD8 coreceptors along with signalling molecules like Lck, PKCθ. Surrounding this region is the pSMAC which consists of the adhesion molecules, like Lymphocyte Function-associated Antigen 1 (LFA1), and a cytoskeletal protein called talin, which can link the intergrins to actin cytoskeleton [41]. During the formation of the IS, the actin filaments of CTL cytoskeleton rearrange themselves towards the outermost part of the IS called the dSMAC [40]. It has been reported that the lytic granules move along the microtubules after the MTOC polarization, to be secreted at the IS [42,43]. It has also been shown that the strength of the TCR signals effect the lytic granule accumulation but not the MTOC polarization to the IS, meaning that the lytic granule polarization and centrosome (MTOC) polarization are independently regulated with respect to the strength of the TCR signals [44]. It has been further proven that the enrichment, docking and release of the lytic granules at the IS is dependent on its tethering with CD3 endosomes and that the Qb-SNARE protein Vti1b facilitates this tethering process [45]. When the lytic granules are tethered with the CD3 endosomes, their dwell time at the IS increases leading to their release at the IS [45].

1.3.3. Lytic granules, the secretory lysosomes in immune cells

As mentioned earlier, lytic granules, also termed cytotoxic granules, are involved in one of the main mechanisms by which the CTLs and NK cells kill their targets. Lytic granules are modified lysosomes called as secretory lysosomes that undergo regulated secretion in response to some external stimuli like the activated TCR signaling in CTLs. Such a secretion is induced by the clustering of lytic granules around the polarized MTOC [42,43]. It has also been shown that a protein called Vti1b tethers the lytic granules to the TCR to facilitate lytic granule secretion [45].

These are acidic vesicles which contain cytotoxic proteins such as, pore forming protein called perforin, proteoglycans like Chondroitin sulphate A, different varieties of serine proteases called granzymes and different other lysosomal enzymes such as Carboxypeptidase A [46]. The core of the lytic granules have Mannose 6-phosphate receptors, so that the granzyme A and B which have Mannose 6-phosphate residues are targeted and concentrated to the lytic granules [47]. These cytotoxic molecules are stored in their inactive form in these lytic granules by their acidic environment (pH 4.5 – 5.5). Once these molecules are released out of their acidic environment they are converted to their lethal active cytolytic form. Apart from the cytotoxic molecules, lytic granules contain lysosomal transmembrane proteins like CD63, lysosomes associated membrane proteins (Lamp1 and Lamp2) and other soluble proteins like cathepsins [48].

1.3.3.1. Perforin

Perforin is a pore forming glycoprotein present in the lymphocyte lytic granules. The native 70 kDa form of perforin, when synthesised is made up of 555 amino acids, starting with a signal peptide of 21 amino acids that is cleaved off when the native form is transferred from ER to the lytic granules, to become a 534 amino acid mature 60 kDa form [49]. Approximately 280 amino acids present in the centre of the perforin molecule are highly conserved and homologous to those present in complement protein C9. Both have a conserved domain from aminoacid 191 – 211 (called LB) which have been known to form α helix that interact with the lipid bilayer [46]. Another feature common in both perforin and C9 protein is that they both do not have the TPS (Thrombospodin) domain which is present at the amino terminus of all other complement proteins. The absence of this domain is believed to account for their

ability to form circular polymers [50]. The carboxy C2 domain of mature perforin, facilitates the insertion of perforin into the target cell plasma membrane [49].

Once the perfroin reaches the lytic granules, the acidic environment of these granules favours the interaction of active perforin with proteoglycans to render perforin inactive and harmless to the organelle, thus making it possible for lytic granules to store active perforin [51]. When it is exocytosed from the lytic granules at the IS, due to the extracellular neutral pH as well as high calcium (Ca^{2+}) concentrations in the cleft, perforin loses its interaction with the proteoglycan and is released in its active form. High concentration of free Ca^{2+}, a minimum of 200 μM [52] facilitates the activation and also polymerization of perforin to cylindrical polymers which are inserted into the target membrane to form pores [46] to permeabilize target cell membrane and facilitate entry of granzymes into target cells (Fig. 1) [53]. Perforin is delivered directly to the target membrane by fusion of the lytic granule to the target membrane thus limiting the possiblity of it binding to the T cell membrane [46].

1.3.3.2. Granzymes, lytic granule serine proteases

Granular enzymes also called as granzymes are highly conserved serine proteases present in the lytic granules of CTLs and NK cells [46]. They have also been reported to be present without perforin in non lymphoid cells like macrophages, dendritic cells, mast cells, basophils, keratinocytes, sertoli cells, photoreceptors and brain cells where their function is unknown [54]. Five human granzymes (A, B, H, K, M) and ten mouse granzymes (A, B, C, D, E, F, G, K, M and N) have been reported till date [53,54]. All the granzymes are monomeric polypeptide chains of 27 to 55 kDa molecular weights except granzyme A which is a disulphide bonded homodimer of 60 kDa [55]. Of these, grazyme A and B are the most abundantly occurring ones. They are delivered into the

target cells by the pore forming perforin. On entering the target cells they induce apoptosis of the target cell by various mechanisms. They are also said to exhibit non-cytotoxic functions like stimulation of pro-inflammatory cytokines, altering and remodeling the extracellular matrices, cleavage of viral peptides [54].

As any other protein, the synthesis of granzyme protein is followed by its post translational modifications. Before it reaches the lytic granule, in the ER, its signal peptide is cleaved to form an inactive proenzyme which has an N- terminal dipeptide which needs to be cleaved to convert it to an active protease. In the Golgi apparatus mannose 6-phosphate residue tag, a sorting signal, is added to the proenzyme which targets them to the mannose 6-phosphate receptors containing lytic granules [53]. Once it reaches the lytic granule, the N- terminal dipeptide is removed but even then remains as an inactive protease as it is bound along with perforin to proteoglycans and is inactive in the acidic pH of the granule and thus can be stored efficiently in the lytic granule [56].

Perforin is the major carrier to deliver granzymes into the target cells. The most popular model is that granzyme enter target cells through pores generated by perforin. There is also evidence emerging to support the model that endocytosis following pore formation by perforin brings membrane-bound granzymes into the target cells (Fig. 1) [57]. Granzymes use three major mechanisms to induced target cell apoptosis: (1) cleaving different cytosolic (Bid (BH3 interacting domain death agonist) protein) and nuclear (Histones, lamins, PARP1) substrates; (2) by mitochondrial and DNA damage; (3) inducing the caspase pathway by activating caspase 3 and also activating other caspases like caspase 7 and 8 which lead to cleavage and inactivation of Inhibitor of Caspase Activated DNase (ICAD) causing apoptosis [53,54,58].

1.3.4. Fas and Fas ligand pathway

Another important mechanism by which the CTLs and NK cells eliminate their targets is by a receptor ligand pathway called the Fas/Fas ligand pathway. The occurrence of a cytotoxic pathway other than the perforin/granzyme pathway was observed in perforin knock out mouse CTLs and also in perforin deficient CTL cell line [59]. This alternate pathway was confirmed to be the Fas/Fas ligand pathway, as they observed some killing activity in perforin deficient lymphocytes against Fas expressing target cells but this was eliminated when the same perforin deficient lymphocytes were used to kill Fas receptor deficient lpr-mutant cells which have defective Fas genes [18,19]. Thus proving the existance of an alternate cytotoxic pathway induced by Fas/Fas ligand. At the IS, the Fas ligand expressed by the CTLs or the effector cells binds to the Fas receptor on the target cell, triggering the activation of apoptotic pathway leading to target cell death.

Fas ligand (FasL) also called as CD95L, Apo-1L, CD178, TNFSF6 or APT1LG1, is a 231 amino acid type 2 transmembrane protein belonging to the Tumor Necrosis Factor (TNF) family of death receptors [60]. FasL has a TNF homology domain at its distal end, right next to which is the C-terminal receptor binding site that binds to the Fas receptors on the target cells [61]. It has been shown that FASL is localized in the membrane of secretory lysosomes also called as lytic granules, which also contain perforin and granzymes [62]. Upon formation of an IS with a target cell, the CTL releases lytic granules whose lipid bilayer fuses with the target cell plasma membrane exposing the FasL to interact with the Fas receptor [63].

On engagement of FasL to the target cell Fas receptor, Death Inducing Signalling Complex (DISC) is activated leading to further activation of caspase 8 which directly mediates activation of downstream effector caspases -3, -6 and -7. Caspase 8 cleaves

BID to tBID which releases cytochrome C from mitochondria. Cytochrome C leads to the formation of Apoptosome where caspase 9 is activated which cleaves and activates downstream effector caspases causing apoptosis and target cell death [64].

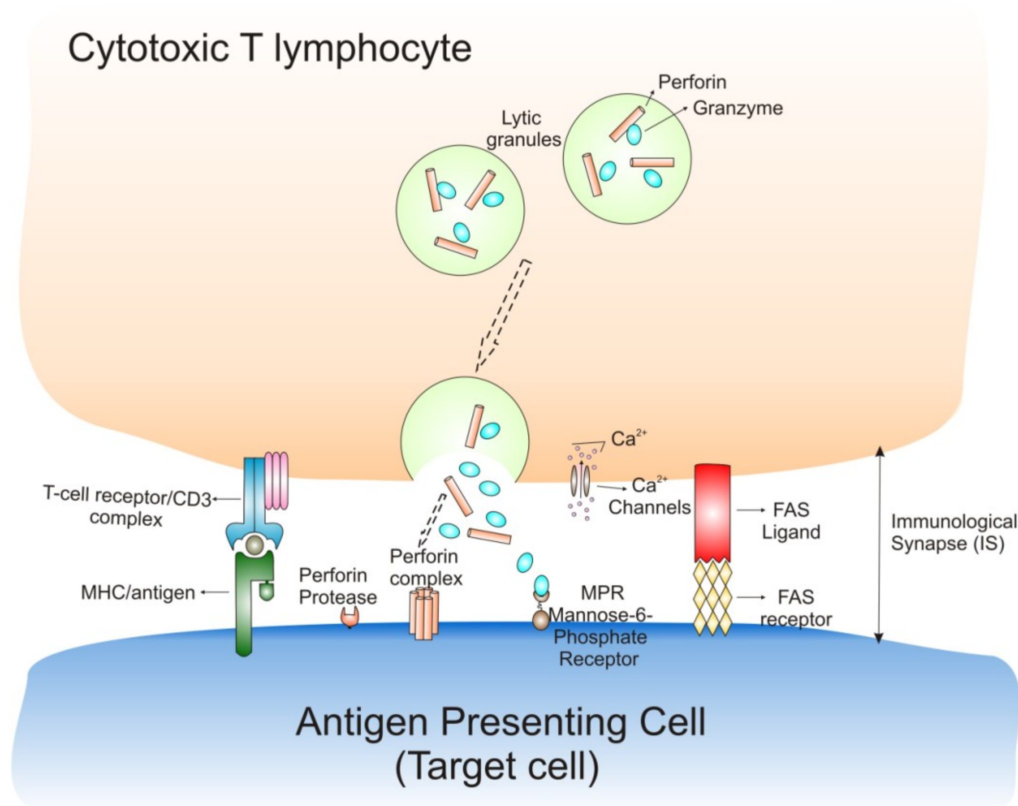

Figure 1: Target cell killing by CTL at the immunological synapse (IS)
On TCR identification and binding of antigen-MHC complex of the antigen presenting cells or target cells, activation of CTLs leads to the killing of target cells at the IS by two main pathways. One is by release of perforin and granzymes from the lytic granules and the other is by the release of the FAS ligand. (Figure modified from Trapani, J.A. & Smyth, M.J., 2002).

1.4. Sorting, delivery and maturation of proteins and vesicles through endosomal pathway

As we already know, after the synthesis of cytotoxic molecules, they are targeted and transported (by mannose 6-phosphate receptor pathway) to the secretory lysomomes (lytic granules) and stored there. These secretory lysosomes also go through a

synthesis and maturation pathway before they are transported to the IS for their targeted secretion. The lytic granule and other lysosomal enzymes and proteins are synthesised in the ER and translocated to the cis-Golgi and then to the trans-Golgi of trans-Golgi Network (TGN). The trans-Golgi is the final port through which the cargo proteins are delivered to vesicles or to the cell surface [65]. From the trans-Golgi, they can follow two different pathways to reach the lysosomes [66]. One is the well studied indirect endocytic pathway, where proteins like lysosomal acid phosphatases are transported to the cell surface [67] in Adaptar Protein 1 (AP1) containing clathrin coated vesicles [68,69]. From here it passes through different compartments of the endocytic pathway strating from early endosomes to late endosomes and then finally to the lysosomes for degradation or recycling back to the cell membrane or to return to the TGN (Fig. 2) [70]. There is also a much faster direct pathway determined by the shorter transit time (half-time 1 to 2 hr) for transfer to the lysosomal compartment [71] compared to the longer transit time (half-time approximately 6 hr) required for the indirect pathway [67]. Lysosomal Associated Membrane Protein (LAMP) 1 is said to be delivered to the lysosome by this direct pathway [71] although in some cell types minority of the LAMP1 molecules have been shown to be delivered through the indirect / long pathway [72]. Other proteins like FasL, Cytotoxic T-Lymphocyte antigen 4 (CTLA4), proteins with mannose 6-phosphate tags also follow the direct route to lysosomes but some proteins like CD63 (LAMP3) can be delivered by both direct and indirect pathways [73,74]. The route taken by the cargo transported through the direct pathway can be described as follows: the cargo is dispatched from the TGN by budding into vesicles or fusion into early endosomal compartments which then fuses with or transforms to late endosomes from which it finally becomes or fuses with lysosomes. Many molecules are essential for the formation or fusion of early

endosomal compartment. Rab 5, a regulatory gaunosine triphosphatase which belongs to the family of small Guanosine TriPhosphatases (GTPase) is a marker for early endosomes and is considered to regulate sorting endosome fusion. Rab 5 has two effectors, Rabaptin-5 and Early Endosome Autoantigen 1 (EEA1) which is an another marker of early endosomes [75,76]. Rab 5 binds to EEA1 which forms a high molecular weight complex with Rabaptin-5 in the presence of N-ethylmaleimide Sensitive Factor (NSF). This complex then interacts with certain SNARE proteins driving the membrane fusion of the vesicles [77] delivering the cargo. Cargo transport from early endosomes to late endosomes can be either through evolution of the early endosomes into late endosomes by the loss of their receptors / markers to gain new receptors or by simple transfer of cargo from the early endosome vesicles to late endosome vesicles. This was answered by the observation of loss of Rab5 marker and gain of Rab7 (a late endosome marker, also a member of the family of GTPase) in the cargo carrying vesicles. Thus confirming that coordinated exchange of Rab5 to Rab7 is the mechanism of cargo progression between early and late endosomes [78]. The final transfer of the cargo from late endosomes to lysosomes can take place in three different ways. Firstly by vesicular transport between two organelles which has not yet been proven. Secondly by 'kiss and run' mechanism, where transient fusion pores are formed when the late endosome comes in contact with the lysosomal vesicle and the cargo is transferred through by repeated transient fusion and fission [79]. The final hypothesis is by direct and complete fusion of late endosomes with lysosomes leading to the formation of a hybrid organelle with subsequent regeneration of lysosomes [80]. This was supported by the possibility to collect late endosome-lysosome hybrid organelles which have characteristics of both late endosomes and lysosomes [81]. It has been shown that the fusion between late endosomes and lysosomes is ATP, cytosol

and temperature dependent and requires the presence of NSF, Soluble NSF Attachment Protein (SNAP), SNARE proteins and Rab-GTPase [82,83].

1.5. SNARE proteins

Membrane fusion which is crucial for the trafficking and secretion of the vesicle cargo is facilitated by the participation of conserved membrane-associated proteins called the Soluble N-ethylmaleimide sensitive factor Attachment protein (SNAP) Receptors (SNAREs). They are found throughout the secretory pathway, participating in membrane-trafficking, docking, priming and fusion of vesicles (loaded with cargo like secretory enzymes, neurotransmitters etc) (Fig. 2). 36 human SNAREs have been identified. SNAREs belonging to the different families have specific localization and function and their dynamic cycle of assembly and disassembly is regulated by NSF, SNAP and Rab-GTPases [84,85]. SNAREs were originally classified functionally as v-SNAREs or vesicular membrane SNAREs (Synaptobrevin / Vesicle Associated Membrane Protein 2 (VAMP2)) and t-SNAREs or target membrane SNAREs (Syntaxin 1, SNAP-25) on the assumption that each SNARE is localized either on the donor or the acceptor membranes [86]. As this classification is not applicable for homotypic fusion (fusion of structurally and functionally similar vesicles), SNAREs are then classified structurally as R- and Q-SNAREs depending on whether they contain the highly conserved Arginine (R) or Glutamine (Q) residues in the SNARE motif [87]. The core of the SNARE complex is composed of four intertwined coiled coil parallel α-helices with each helix provided by one SNARE motif. The centre of this bundle consists of 16 stacked layers of interacting side chains and most of these layers are hydrophobic except for the central '0' layer which consists of highly conserved three Q residues and one R residue [87]. Accordingly, these are subdivided into Qa-,

Qb-, Qc- and R- SNAREs [88]. Each of these has different N-terminal domains, one respective Qa-, Qb-, Qc- or R- SNARE motif and mostly a C-terminal transmembrane domain. In addition there is also a subclass of SNAREs, which have one Qb- and one Qc- SNARE motif, hence they are called the Qbc- SNAREs, such as SNAP-23, SNAP-25, SNAP29 and SNAP-47. It is necessary that at least one SNARE (having a transmembrane domain) that is participating in the formation of the SNARE complex should be contributed by each of the fusing membrane during the assembly of a trans-SNARE complex [89]. These SNARE motifs assemble and intertwine starting from the N-terminal domain and zippering inwards towards the C-terminal membrane anchor domain which causes the fusion of the participating membranes [90-92]. The following table 1 shows the classification of members into various SNARE families [93]:

Qa-SNARE	Syntaxin 1 (Stx1), Stx2, Stx3, Stx4, Stx5, Stx7, Stx11, Stx13, Stx16, Stx17, Stx18
Qb-SNARE	Vti1a, Vti1b, GS27, GS28
Qc-SNARE	Stx6, Stx8, Stx10, GS15, BET1, SLT1
Qbc-SNARE	SNAP-23, SNAP-25, SNAP29, SNAP-47
R-SNARE	VAMP-1, VAMP-2, VAMP-3, VAMP-4, VAMP-5,VAMP-7, VAMP-8, SEC22b, YKT6
unclassified	D12,SEC20,SEC22a and SEC22c

Table 1: Classification of SNAREs

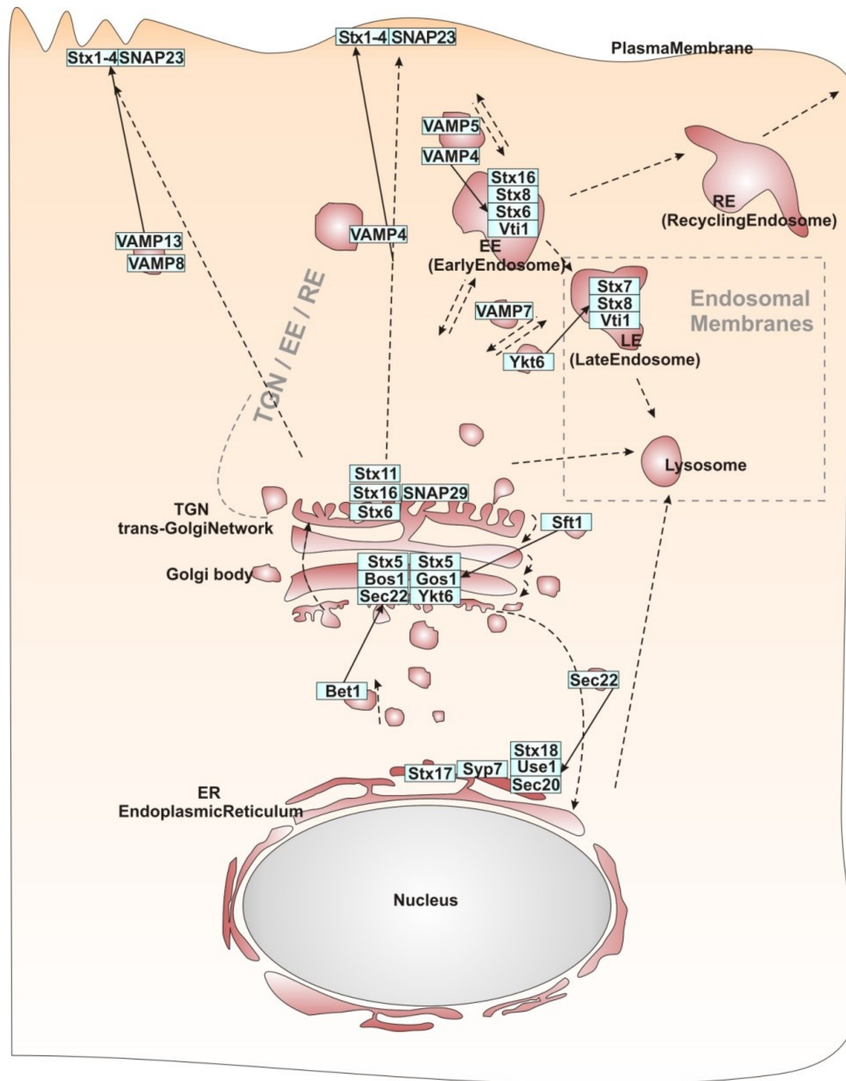

Figure 2: SNAREs involved in the endosomal trafficking pathway of a representative animal cell Different SNAREs are expressed and participate in the trafficking of proteins through the endosomal pathway. The proteins synthesized in the ER are transported through the trans-Golgi network to the early, late and lysosomal compartments for release or are recycled back by the recycling endosomes. (Figure modified from Kanehisha Laboratories, 2010).

1.6. SNARE and related proteins in immune cells

SNAREs have been extensively studied for their role in neurotransmitter release in neurons. Studies in immune cells showed the presence and formation of SNARE complexes for membrane fusion and exocytosis of secretory vesicles, but the interacting SNARE partners differed from those seen in neuronal cells. Mast cells,

eosinophils, basophils, macrophages, NK cells, $CD4^+$ cells and CTLs are some of the immune cells where SNAREs were detected to be present. VAMP7 and not VAMP2 was found to be crucial for NK cell mediated target killing and also for exocytosis in neutrophils and eosinophils [94,95]. It has been shown in NK cells that SNAREs are essential for the polarized exocytosis of intracellular recycling vesicles (like TCR vesicles) at the IS. t-SNAREs like Syntaxin 4 and SNAP-23 and v-SNARE VAMP-3 (and maybe VAMP-2) forming the SNARE complex that mediates vesicle fusion are involved in the TCR targeting to the IS [96]. Munc family proteins bind to the amino terminal domains of Syntaxins and regulate the availability of Qa-SNAREs during the formation of the trans-SNARE complex. The qRT-PCR conducted with naïve and anti-CD3 / anti-CD28 coated bead stimulated $CD8^+$ T lymphocytes showed that some SNAREs like Syntaxin 1A (Stx1A), Stx1B, Stx19, Vti1a, SNAP-25 are not expressed in human CTLs. While SNAREs like Stx2, Stx5, Stx11, Stx17, Stx18, SNAP-23, SNAP-25 and VAMP1, 2, 5, 7 and 8 are expressed in human CTLs. Other SNAREs expressed in human CTLs are Stx3, Stx4, Stx6, Stx7, Stx8, Stx13, Vti1b, VAMP3 and VAMP4 that colocalized with CD3 and Vti1b, Stx8 and Stx16 that showed highest degrees of colocalization with lytic granules. Out of the 4 members of the Munc13 family (Munc13-1, Munc13-2, Munc13-3 and Munc13-4) only Munc13-4 expression was detected in human CTLs [1]. Deficient of several SNARE or related proteins (Stx11, Munc13-4, Munc18-2, Rab27a) in key steps of vesicle trafficking, priming and release leads to life-threatening immune disorders [48,97]. Qa-SNARE, Syntaxin 7 has been demonstrated to be important for TCR trafficking [98] and a Qb-SNARE, Vti1b is required for the tethering of lytic granules with TCR endosomes which leads to the efficient docking and release of the lytic granules at the IS in CTLs [45]. The importance of SNARE and Munc proteins in CTL functioning has been proven by the

genetic diseases caused by mutations in the genes coding these proteins. Some of the diseases caused by mutations in SNARE and SNARE related proteins are mentioned as follows: Griscelli syndrome type 2 (GS-2) is an autosomal recessive disorder which is also called as Chédiak-Higashi like syndrome, where altered Rab27a GTPase causes defective pigmentation and impairment in lytic granule docking at the IS in NK cells and CTLs [97]. HLH is a severe hyper inflammatory syndrome caused by uncontrolled but ineffective immune response. Primary HLH, also known as Familial Hemophagocytic LymphoHistiocytosis (FHL) is a heterogenous autosomal recessive disorder affecting the granule dependent exocytosis pathway [99]. Five different FHLs have been identified based on the mutations at five different loci. Mutation in chromosome 9q21.3-22 leads to FHL1 [100]. Defect in the PRF1, perforin gene causes reduction in perforin synthesis impairing the perforin / lytic granule dependent cytotoxicity of NK cells and CTLs [101]. FHL3 is a result of mutations in UNC13D gene that codes for Munc13-4 protein required for the priming step of lytic granule secretion preceding vesicle membrane fusion at the IS [102]. FHL4 is caused by defect in the encoding and expression of Syntaxin 11 protein in NK cells and CTLs resulting in partial impairment in granule exocytosis without hindering granule polarization. A partial repair of this defect is possible by IL-2 restimulation [103]. Mutation in STXBP2 gene encoding Munc18-2 causes FHL5 were in defective exocytosis of the lytic granules in NK and CTLs is observed. Munc18-2 is known to bind with Syntaxin 11 to bring about the release of lytic granules [104]. All these establish the fact that SNARE and SNARE related proteins are indeed very significant for NK and CTL cytotoxicity.

1.7. Syntaxin 8: the protein of interest

Syntaxin 8 is a Qc- SNARE of 236 amino acid length with 27 kDa molecular weight. PC12, Cos7 and HeLa cell lines, Chinese Hamster Ovary (CHO) cells, Normal Rat Liver (NRL), Normal Rat Kidney epithelial cells (NRK) [105,106], murine lymphocytes are some of the cell types in which Syntaxin 8 has been studied but its function has not yet been studied in human CTLs. Syntaxin 8 protein is encoded by a STX8 gene that maps to chromosomal band17p12. N-terminal region of the Syntaxin 8 protein contains a coiled coil domain, which is followed by another coiled coil domain that is highly conserved and is called the coiled coil domain 2 or t-SNARE domain. The t-SNARE domain is followed by a hydrophobic trans-membrane anchor at the C-terminal region [107]. Two major groups of sorting signals that efficiently sort different membrane proteins to various post Golgi compartments have been identified to be tyrosine based motifs and di-leucine / dihydrophobic motifs. The tyrosine-based motif carriers out the indirect route, that is, transport to the plasma membrane and then to the endosomes whereas the di-leucine motif mediates the direct transport to late endosomes [108]. But the presence of two distinct di-leucine motifs on a protein can independently mediate transport by both direct and indirect pathways. Syntaxin 8 has been shown to comprise this functionally distinct two di-leucine motifs at amino acids 77-83 and 184-190 and that these independently function for exocytosis and endocytosis respectively. Thus indicating that Syntaxin 8 is internalized from the plasma membrane by the indirect pathway and also directly delivered from the TGN to endosomal / lysosomal population. This can also be explained by the hypothesis that Syntaxin 8 may be delivered from the TGN directly to endosomal / lysosomal populations, rather than being transported to the plasma membrane but then recycled to endosomes by the plasma membrane endosome route [109]. This is supported by the

studies showing the localization of Syntaxin 8 (in murine cells and cell lines) in early endosomal [105], late endosomal compartments [110] and also in TGN. It has also been shown that in CHO cells, Syntaxin 8 mainly colocalizes in non-clathrin coated vesicles, thus ruling out its involvement in clathrin mediated endosomal pathway [106]. It has been predicted that Syntaxin 8 is one of the interacting partners of the SNARE complex formed by VAMP7, Syntaxin7 and Vti1b [111-113]. In Cos7 cells Syntaxin 8 has been shown to be localized in the recycling endosomes [114]. Some studies have shown that Syntaxin 8 is one of the SNAREs expressed in human CTLs and that it may play a role in CTL function. It has been shown to colocalize with lytic granules in CTLs [1]. It has also been shown to co-immunoprecipitate with a Qa-SNARE, Syntaxin 7, that is required for the TCR trafficking to the IS in human CTLs [98]. But the functions of Syntaxin 8 in human CTLs still remain to be identified.

1.8. Aims of this study

SNARE and SNARE related proteins have already been shown to participate in the vesicle trafficking pathway in various cell types. The screening of expression of different SNAREs in primary human CTLs proved the expression of many SNAREs in human CTLs. Among them, Syntaxin 8 was highly expressed in naïve and activated $CD8^+$ T cells. Furthermore, some SNAREs like Vti1b, Stx8 and Stx16 showed highest degrees of colocalization with lytic granules in CTLs [1]. One of the mechanism by which the CTLs kill their targets is by lytic granule release at the IS. SNAREs and SNARE related proteins are believed to be involved in the trafficking of cytotoxic molecules to the IS in CTLs. Up to date, Qa-, Qb-, R-SNARE haven been reported to be involved in CTL function, however, no Qc-SNARE protein has been revealed to regulate CTL function. Therefore in this work the main focus is on

Syntaxin 8, a Qc-SNARE, which partially colocalizes with lytic granules and accumulates at the IS, indicating a potential promising role in CTL function. Thus we aim to study the significance of Syntaxin 8 in human CTL cytotoxicity. In this study, we try to answer several problems, of which first of all we try to determine the localization of Syntaxin 8 in human CTLs. Then we determine its effect on the CTL cytotoxicity and also try to identify the mechanisms it may be involved in, that might influence CTL function. To answer these, we use different techniques like immuno-cytochemistry, imaging, various molecular biological, biochemical and functional assays.

2. MATERIALS AND METHODS

2.1. Antibodies and Reagents

Antibodies used are Alexa^{488}-labelled anti-CD3 mAb (UCHT1, Biolegend), Alexa^{647}-labelled anti-CD3 mAb (UCHT1, Biolegend), Alexa^{647}-labelled anti-Perforin mAb (dG9, Biolegend), Alexa^{488}-labelled anti-LAMP1 mAb (H4A3, Biolegend), mouse anti-EEA1 mAb (BD Biosciences), Alexa^{568}-, Alexa^{647}- andAlexa^{488}- labelled goat anti-rabbit secondary Ab and Alexa^{568}- and Alexa^{488}- labelled goat anti-mouse secondary Ab (Life Technologies), rabbit polyclonal anti-Syntaxin8 Ab (Synaptic Systems), rabbit anti-γ-tubulin Ab (Sigma), rabbit polyclonal anti-Perforin Ab (H-315, Santa Cruz), rabbit polyclonal anti-Granzyme B Ab (Cell Signaling), rabbit polyclonal anti-CD178 Ab (FASL-Q20, Santa Cruz), horseradish peroxidase anti-rabbit secondary antibody (Amersham), mouse anti-CD28 Ab (BD Biosciences), mouse anti-CD3 Ab (AbD seroTec), FITC labelled anti-human Perforin (dG9 clone, BioLegend), FITC Mouse IgG2b (MPC-11) κ isotype Control, rabbit polyclonal anti-mouse Immunoglobulins (DakoCytomation).

All reagents used, if not specifically mentioned, are from Sigma (highest grade).

2.2. Peripheral blood mononuclear cell (PBMC) isolation

Human blood from healthy donors collected in leukoreduction system chambers (LRSCs) by the Department of Clinical Hemostaseology & Transfusionmedicine, Homburg, was used to isolate PBMCs. LRSCs are used to eliminate the contaminating leukocytes from blood products at the point of blood collection to

prevent allo-immunization and associated risks during transfusion [115-117]. These LRSCs are a source of viable human PBMCs.

Leucosep™ filtered tube (227290, Greiner) containing 15-17 ml lymphocyte separation media-1077 (LSM) (PAA, J15-004) was prepared for the PBMC isolation by centrifuging at 1000 g, 30 sec at room temperature (RT). Then the blood in the LRSC was flushed with 20-25 ml HBSS (Hank's BSS, PAA labs, 15–009) into the prepared Leucosep™ tube. The leukocytes, plasma and erythrocytes were separated by density gradient centrifugation at 450 g (Hettich - Centrifuge 32R) for 30 min at RT (Break = 0, Acceleration = 1). The leukocyte white ring, found just below the plasma layer, was transferred into a falcon tube and the volume was made up to 50 ml with HBSS and centrifuged at 250 g for 15 min at RT (Break = 9, Acceleration = 9, Hettich-Centrifuge 32R). Supernatant was discarded and if necessary the erythrocytes in the red ringed pellet were lysed, depending upon the size of the pellet, using 1 to 3 ml of lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) to resuspend the pellet for 60-120 sec. 50 ml HBSS was added to stop the lysis and then centrifuged at 130 g for 10 min at RT (Break = 9, Acceleration = 9, Hettich-Centrifuge 32R) after which the supernatant was discarded to obtain an erythrocyte free PBL pellet that was resuspended in 20 ml PBS (Phosphate Buffer Saline)/0.5 % BSA solution and stored at 4° C or stimulated with Staphylococcal enterotoxin A super antigen.

2.3. Stimulation of PBLs with Staphylococcal enterotoxin A

Staphylococcal Enterotoxin A (SEA) is a bacterial (Staphylococcal aureus) toxin which is secreted to impair host humoral responses and immune recognition. In

organisms it rapidly elevates many cytokines like interleukin-2 (IL-2), interferon-γ (IFN- γ), and tumor necrosis factor α (TNF-α) to toxic levels [118].

It belongs to a family of superantigens (SAg) as it is a strong mitogen which activates a large fraction of T cells. It can interact with major histocompatibility complex (MHC) II molecules leading to the activation of $CD4^+$ and $CD8^+$ T lymphocytes [118-122], subsequently causing SAg induced T cell mediated B cell depletion leading to SAg-dependent cell-mediated cytotoxicity (SDCC) [122,123].

PBMCs isolated from healthy donors were stimulated with SEA (0.05 μg/ml) at a density of 1.5×10^8 cells/ml, for 1 hr at 37°C. These PBMC were resuspended at a density of 4×10^6 cells/ml in AIMV medium (Life Technologies, Cat. No: 12055) supplemented with 10 % FCS (Life Technologies, Cat. No: 10270-106) and 100 U/ml of recombinant human IL-2 (Life Technologies, Cat. No: PHC0021). After 5 days, SEA-specific CTL were positively isolated.

2.4. Positive isolation of $CD8^+$ T lymphocytes

Synthetic magnetic beads that are coated with anti- CD8 antibody are used to fish the $CD8^+$ CTLs out of the PBMC mixture. 5 days after the SEA stimulation, PBLs were positively isolated with magnetic Dynabeads (Life Technologies). $1x10^7$/ml of SEA stimulated PBLs were centrifuged at 220 g (Hettich-Centrifuge 32R) for 8 min at 4°C. Pellet was then resuspended in 1 ml ice cold buffer 1 (PBS/0.5 % BSA). Anti-CD8 antibody coated magnetic dynabeads bind specifically to $CD8^+$ T cells, thus selectively separating them from the PBL mixture. 25 μl of dynabeads were washed 3 times with 1 ml buffer 1 for 1 min each before use. These washed beads were then added to the ice cold cell suspension and incubated at 4°C for 20 min on a rotator. The $CD8^+$ T cells now bound to the dynabeads were separated from the rest of the solution

using a magnetic separator for 2 min. These bead bound cells were washed 3 times with 1 ml ice cold buffer 1 for 1 min each using a magnetic separator. This step allows the removing of cells that are nonspecifically bound to the beads.

The bead bound cells were then resuspended with 100 µl of buffer 2 (RPMI 1640/ 1 % FCS) and 10 µl of DETACHaBEAD (Life Technologies) and incubated at room temperature for 45 min on a rotator. The DETACHaBEAD detaches the $CD8^+$ T cells from the dynabeads. These detached $CD8^+$ T cells were separated from the beads using a magnetic separator for 1 min. The supernatant containing the $CD8^+$ T cells was collected in a separate tube. The beads were washed 3 times for 1 min each with 50 µl buffer 2, to collect any remaining $CD8^+$ cells that were still binding to the beads. The collected cell supernatant was resuspended and the volume was made up to 1 ml using buffer 2 and centrifuged at 400 g (Hettich-Centrifuge 32R) for 6 min at room temperature, to remove the DETACHaBEAD. Pellet obtained was resuspended in AIMV/10 % FCS with 100 units/ml IL-2 (Stock 1000 units/µl). The $CD8^+$ T cells were then plated at a density of $1.5x10^6$ cells/ml and cultured until further use.

2.5. Negative isolation of $CD8^+$ T lymphocytes

CD8 negative isolation kit (Life Technologies) is used to isolate naïve $CD8^+$ T cells from PBMCs. $1x10^7$ PBMCs were centrifuged at 220 g (Hettich-Centrifuge 32R) for 8 min at 4°C and the pellet obtained was resuspended in 100 µl cold PBS/0.5 % BSA (buffer 1). 20 µl FCS and 20 µl antibody mix (provided in the kit) were added to the cells and incubated at 4°C for 20 min on a rotator. Magnetic depletion beads provided in the kit are used to bind and remove all PBMCs other than $CD8^+$ T cells, thus isolating untouched $CD8^+$ T cells. 200 µl of depletion beads were washed 3 times with at least 1 ml of ice cold buffer 1 for 1 min each before use. After the 20 min

incubation, the cells were resuspended well in 2 ml cold buffer 1 and centrifuged at 300 g (Hettich-Centrifuge 32R) for 8 min at 4°C. Pellet obtained was resuspended in 800 µl cold buffer, mixed with pre washed 200 µl depletion beads and incubated for 15 min at RT on a rotator. These cells were gently resuspended for 5 times before adding 1 ml of cold buffer 1. The unwanted PBMCs bound to depletion beads were separated by placing in a magnet for 2 min. The supernatant containing untouched $CD8^+$ T cells was collected in a new tube. The beads were washed twice with buffer 1, placed in the magnet and supernatant collected as said earlier. The supernatant containing the untouched $CD8^+$ T cells, was centrifuged at 200 g (Hettich-Centrifuge 32R) for 5 min at RT. The pellet was resuspended in AIMV/10 % FCS and the cells were plated at a density of $3x10^6$ cells/ml until further use.

2.6. siRNA transfection of CTLs

To knockdown the expression of Syntaxin 8, we used RNA interference by small/short interfering RNA (siRNA). siRNA are small double stranded RNA molecules of 20-25 nucleotides in length. They interfere with the transcription of specific mRNA by binding of complimentary nucleotide sequence thus inhibiting the protein expression [124]. Modified control and Syntaxin 8 siRNA (Qiagen) were used for transfection. 2’-OMe modification stabilizes the siRNA by making it more resistant to nucleases thus increasing the siRNA interference efficacy [125]. Control siRNA (Qiagen) used here is a scrambled siRNA with no homology to any known mammalian genes.

These siRNA were transfected into primary human CTLs through nucleofection [126,127] by Amaxa technology (Lonza). Lyophilized modified siRNA is reconstituted by dilution in resuspension buffer or dH_2O provided by the company (Qiagen). 100 µl

resuspension buffer or water was added to prepare a dilution of 200 µM solution. From this 30 µl was taken and added to 270 µl resuspension buffer to get 300 µl of 20 µM solution (1:10 dilution).

$6x10^6$ CTLs were centrifuged at 100 g (Hettich-Centrifuge 32R) for 8 min at RT and pellet was resuspended in 100 µl of nucleofector-supplement solution (Lonza Human T cell nucleofactor kit (Cat. No: VPA-1002) and P3 Primary Cell 4D-Nucleofector™ X Kit (Cat. No: V4XP-3024)). 10 µl of 20 µM modified Syntaxin 8 siRNA was added to this, resuspended well and electroporated in special Amaxa (Lonza) vials with the programme optimal (T-023 in the Lonza nucleofactor II and E0-115 programme in the Lonza 4D Nucleofactor) for primary human $CD8^+$ T lymphocytes. Immediately after the electroporation, 500 µl warm AIMV media with 10 % FCS was added to the transfected cells, which were then transferred to 12 well cell culture plate (BD falcon) making the volume of the media upto 1.5 ml per well. 12 hr after transfection, the cells were centrifuged at 100 g (Hettich-Centrifuge 32R) for 8 min at RT. Fresh AIMV/10 % FCS media with 50 U/ml IL-2 (Stock 1000 units/µl) was added to the cells which were then plated at 1.5 $x10^6$ cells/ml and incubated in 37^oC till further use (36 hr after transfection).

The siRNAs used are from Qiagen unless mentioned otherwise. The target sequences of the different siRNAs used are as follows:

unmodified Syntaxin 8 siRNA, Hs-Stx8_5 (Cat. No: SI03071929): CAG GTC CAG CCT GAT GAG TGA and modified Syntaxun 8 siRNA, Hs_Stx8_5 (SI03071929, Cat. No: 1021962): 5' r(OMeC-OMeA-GGU CCA GCC UGA UGA) d(GUG) d(OMeA-OMeT-OMeT) 3'; 5' (dU r(CA CUC AUC AGG CUG GAC) d(OMeC-OMeU-OMeG) 3'.

Unmodified negative control siRNA (Cat. No: 1027310): 5’ UUC UCC GAA CGU GUC ACG UdT dT 3’; 5’ ACG UGA CAC GUU CGG AGA AdT dT 3’) and modified control siRNA (Cat. No: 1021962): 5’ r(OMeA-OMeA-UUC UCC GAA CGU GUC) d(ACG) d(OMeU-r(OMeT-OMeT)) 3’; 5’ A r(CG UGA CAC GUU CGG AGA) rA d(OMeU-OMeU-r(OMeT-OMeT)) 3’. Modified Vti1b siRNA (Cat. No: 4479): 5’r (OMeA-OMeA-GGA CCU UGC UAA ACU) d(CCA) d(OMeU-OMeT-OMeT) 3’; 5’ d(U) r(GG AGU UUA GCA AGG UCC-OMeT-OMeT)3’ and unmodified Syntaxin 6 siRNA (Dharmacon, Cat. No: L-017164-00-0005, clone NM_005819) which is a pool of 4 different unmodified siRNAs as follows:

J-017164-05,Stx6: G.C.A.G.U.U.A.U.G.U.U.G.G.A.A.G.A.U.U.U.U;

5’-P.A.A.U.C.U.U.C.C.A.A.C.A.U.A.A.C.U.G.C.U.U

J-017164-06, Stx6: C.A.G.C.A.U.A.G.U.U.G.A.A.G.C.A.A.A.U.U.U;

5’-P.A.U.U.U.G.C.U.U.C.A.A.C.U.A.U.G.C.U.G.U.U

J-017164-07,Stx6: G.C.C.C.A.G.G.G.A.U.U.G.U.U.U.C.A.G.A.U.U;

5’-P.U.C.U.G.A.A.A.C.A.A.U.C.C.C.U.G.G.G.C.U.U and

J-017164-08,Stx6: U.A.U.C.U.C.A.U.A.U.G.A.C.C.A.G.U.G.A.U.U;

5’-P.U.C.A.C.U.G.G.U.C.A.U.A.U.G.A.G.A.U.A.U.U

Perforin-mCherry, Rab7-mCherry and Rab11-mCherry were the constructs used in this study. 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 with BglII and Age1 restriction sites. After AgeI and BglII restriction digestion, perforin was ligated to mCherry vector to yield a C-terminal tagged perforin-mCherry.

Rab 7 and Rab 11 were also amplified from human cDNA with Eco R-1 and Bam H-1 restriction sites. The primers used for these are as follows: Rab 7: 5' TAT ATG AAT TCT ATG ACC TCT AGG AAG AAA GTG T and 5' TAT ATA GGA TCC TCA GCA ACT GCA GCT TTC TG. After Eco R-1 and Bam H-1 restriction digestion, Rab 7 was ligated to mCherry vector to yield an N-terminal tagged Rab 7-mCherry.
Rab 11: 5' TAT ATG AAT TCT ATG GGC ACC CGC GAC GAC and 5' TAT ATA GGA TCC TTA GAT GTT CTG ACA GCA CTG. After Eco R-1 and Bam H-1 restriction digestion, Rab 11 was ligated to mCherry vector to yield an N-terminal tagged Rab 11-mCherry.
The mCherry construct was a kind gift from Roger Tsien (Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA) [1,45].

2.7. RNA isolation, reverse transcription and Quantitative Real Time-Polymerase Chain reaction (qRT-PCR)

Total RNA was isolated from cells treated with TRIzol® reagent (Life Technologies, Cat. No: 15596018) including 1 µl Glycogen (5 µg/µl, Life Technologies, Cat. No: 10814-010) according to the following protocol:
Cells (1-1.5 × 10^6 cells for each sample) were harvested by centrifugation at 200 g for 5 min and then the pellet was stored in 800 µl TRIzol and stored at -80°C until further use. To isolate the RNA from these cell samples, the pellet dissolved in TRIzol was centrifuged at 12000 g for 10 min at RT. The supernatant was transferred to a fresh eppendorf tube and incubate for 5 min at RT. 200 µl of chloroform per 800 µl TRIzol is added and shaken vigorously for about 15 sec and left at RT for 2-3 min. This is then centrifuged at 12000 g for 15 min at 4°C. The aqueous phase obtained is transferred to a fresh tube to which 1 µl Glycogen (5 µg/µl) is later added. To this 500

µl of Isopropanol is added and incubated for 10 min at RT and then centrifuged at 12000 g for 10 min at 4°C. The supernatant is removed and pellet is resuspended in 1 ml of 75 % ethanol (prepared with DEPC-treated H_2O) and centrifuged at 7500 g for 5 min at 4 °C. The RNA pellet is left to dry at RT and then the RNA is dissolved in 10 µl of DEPC-treated H_2O. The concentration of RNA is determined using 2 % agarose gel.

0.8 µg total isolated RNA were then reverse transcribed into cDNA using SuperScript™ II reverse trancriptase (Life Technologies, Cat. No: 18064-014) with 1 µl RNaseOut, (Life Technologies, Cat. No: 10777-019) and 1 µl oligo dT Primer (0.5 µg/µl, Life Technologies, Cat. No: 18418-012) as per the manufacturer's instruction. RealTime PCR was carried out in either MX3000 instrument from Stratagene or CFX96™ Real-Time System C1000™ Thermal Cycler (Software Biorad CFX Manager, Version 3.0). 1 µl of the cDNA prepared by reverse transcription was amplified using 300 nM of each primer were set into PCR reactions (25 µl) using Quanti Tect SYBR green kit (Qiagen, Cat. No: 204145). The conditions used in the thermal cycle of this PCR were: initial denaturation, 15 min, 94°C; 45 cycles: denaturation, 30 sec, 94°C; annealing, 45 sec, 58°C; elongation, 30 sec, 72°C followed finally with a dissociation curve cycle (60 sec, 95°C; 30 sec 55°C; 30 sec 95°C; 30 sec 25°C). Primers were designed using Primer3 program-170 [128] available at http://frodo.wi.mit.edu/. PCR fragments obtained were confirmed by sequencing (MWG). In the qRT-PCR experiments, expression of Syntaxin8 was normalized to the average expression of two reference genes RNA Polymerase II and TATA box-binding protein.

The primer pairs used here are:

Syntaxin 8: 1) (Gene bank accession number NM_005819): (forward primer)

5’ GGAGCATAGAGTGGGATCTA 3’, (reverse primer) 5’ TCATGTCCCTGACAA CTTGC 3’; 2) (NM_004853): (forward) 5’ ACCGAAGACAGAACCTCTTG 3’, (reverse) 5’ CGCTTAGCCTCTTCACTCAT 3’; TBP (NM_003194): (forward) 5’ C GGAGAGTTCTGGGATTGT 3’, (reverse) 5’ GGTTCGTGGCTCTCTTATC 3’; RNAPol II (NM_000937): (forward) 5’ GGAGATTGAGTCCAAGTTCA 3’, (reverse) 5’ GCAGACACACCAGCATAGT 3’; perforin1 (NM_005041 variant 1, NM_001083116 variant 2): (forward) 5’ ACTCACAGGCAGCCAACTTT 3’, (reverse) 5’ CTCTTGAAGTCAGGGTGCAG 3’ and Vti1b (NM_006370): (forward) 5’ AGGTGAGAAGCACACCTTTG 3’ and (reverse) 5’ GCATTGCCCTTTGAGAC TGT 3’.

2.8. Real Time killing assay

Raji is a human Burkitt’s lymphoma cell line [129] which is used as target cells in this study. These Raji cells (ATCC, Cat. No: CCL-86™) were cultured in RPMI 1640 medium (Life Technologies, Cat. No: 21875) supplemented with 10 % FCS.

Killing of target SEA pulsed Raji cells, by SEA stimulated CTLs over a time period of 4 hr is measured by real time killing assay. Here the target cells were loaded with fluorescent Calcein AM ester which on entering the cells, is enzymatically converted to Calcein. On being killed by CTLs, the target cell membrane loses its integrity, releasing the dye [130]. This reduction in fluorescence is measured over time of 4 hr for every 10 min.

Target cells (Raji cells) were pulsed with SEA (10 µg/ml, 1 µl stock solution in 100 µl of AIMV, up to $5x10^5$ cells/well in a 96-well plate BD Biosciences, Cat. No: 353948 and 353219, black/transparent bottom) and incubated at 37°C for 30 min. Pulsed cells were resuspended and transferred to 1.5 ml EP-tube and the bottom of the

well is washed once with 100 µl of AIMV to take all the cells. Cells were centrifuged at 200 g (Hettich-Centrifuge 32R), 5 min, RT and supernatant discarded. AIMV+10 % FCS (or AIMV media without FCS) with 10 mM HEPES (AIMV-HEPES) was prepared. During (or before) the centrifugation, 500 nM Calcein solution was prepared in AIMV-HEPES and vortexed rigorously. $1x10^6$ cells were resuspended in 1 ml 500 nM Calcein solution (scale up or down according to the cell number needed). These Calcein-loaded cells were incubated in dark at room temperature for 15 min with tilting. Then the cells were spun down (flash spin for about 7-8 sec) and washed once with the same volume of AIMV-HEPES and spun again as before. The cells were resuspended in the desired volume with AIMV-HEPES. $3x10^4$ cells in 200 µl of AIMV-HEPES/well were plated in a 96 well plate and the cells were allowed to settle for at least 10 min. The killer cells (CTLs) were centrifuged at 200 g (Hettich-Centrifuge 32R) for 5 min, RT and the pellet was resuspended in the desired volume of AIMV-HEPES to make different dilutions (killer to target cell ratios used are: 10:1 and 20:1). 50 µl of CTL suspension was added to respective wells. The Calcein in the cells was excited at 485 nm and the emitted fluorescence was measured at 535 nm on a plate reader (GeniosPro, TECAN) which was pre warmed to 37°C. The instrument settings were as follows: bottom reading, no shaking, 37°C, optimal gain and 2 flashes of exciting fluorescence for each of the single points out of the 9 points measured for each well. The emitted fluorescence is then measured for every 10 min over a period of 4 hr, consisting of 25 cycles in total (Fig. 3) (Kummerow, C. et al, unpublished). Controls used were:

Total Target Cell fluorescence: 3×10^4 target cells/well added to wells containing AIMV-HEPES. The final volume was adjusted to the same as in the experimental wells. These unlysed targets were taken as negative control.

Total lysed Target Cell fluorescence: 3×10^4 target cells/well added to wells containing AIMV-HEPES. The final volume was adjusted to the same as in the experimental wells. 20 µl of the lysis solution (10 % Triton in AIMV-HEPES) was added to each well and mixed well. These Triton lysed target cells were taken as positive control for lysis.

Volume Correction Control: 20 µl of above mentioned lysis solution was added to a triplicate set of wells containing 250 µl of AIMV-HEPES. This control was used to correct for the volume increase caused by the addition of the said lysis solution.

Culture Medium Background: 250 µl of AIMV-HEPES was added to a triplicate set of wells.

The loss of fluorescence of the target cells is measured as cytotoxicity which is analysed as the percentage of target cells lysed as follows:

$$\%\,Target\ lysis\ = \frac{[(Triton\ lysed\ targets * r) - (effector\ treated\ target\ fluorescence)]}{[(Triton\ lysed\ targets * r) - (Total\ fluorescence\ of\ unlysed\ targets * r)]} * 100$$

where ‘r’ is ratio of initial fluorescence of target cells treated with effectors to the initial total fluorescence of unlysed target cells at the beginning of the assay.

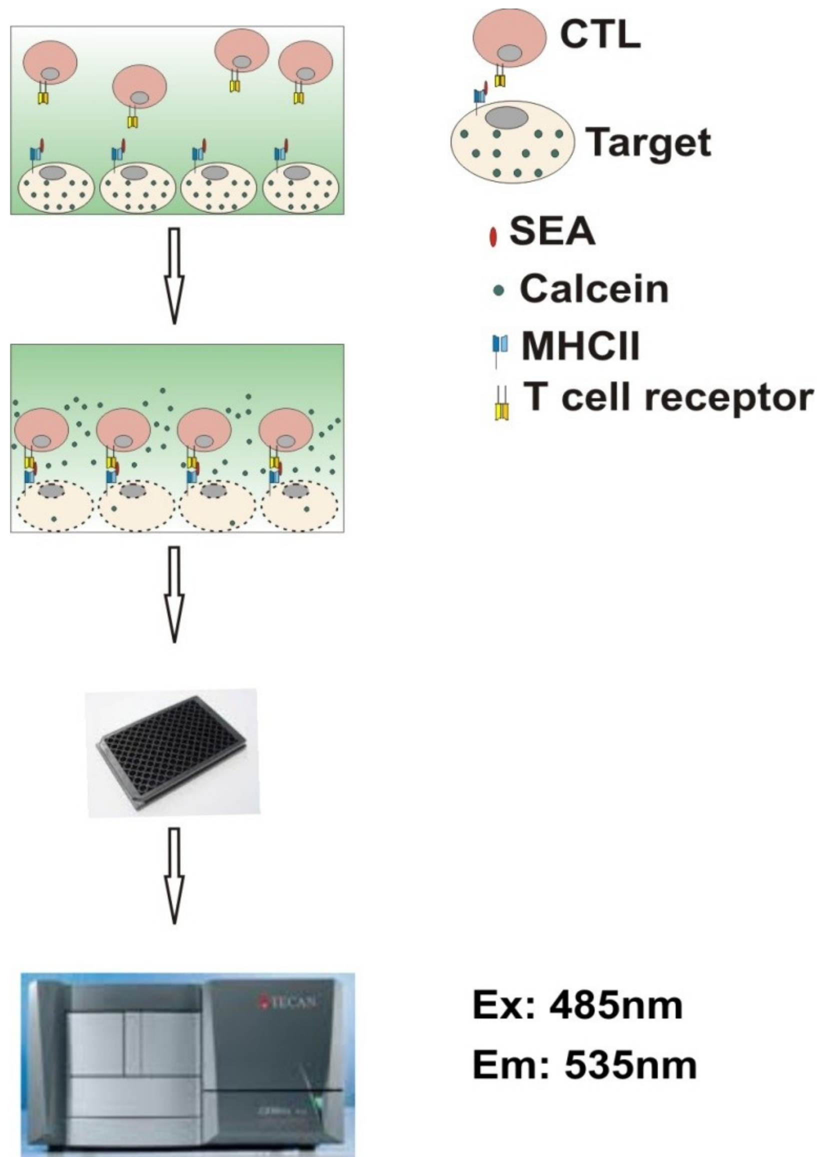

Figure 3: Diagramatic description of the protocol for real time killing assay
SEA stimulated CTLs were used for as effectors and Calcein loaded Raji cells were used as target cells in this real time killing assay. The loss of fluorescence of the target cells was measured as cytotoxicity, for every 10 min over 4 hr. Fluorescence excitation wavelength used was 485 nm and emission was measured at 535 nm.

For real time killing assays in Ringer solutions, Ringer solutions with different calcium concentrations were used to plate the Calcein loaded target cells (Raji cells) and further preceded as previously described to measure the effect of calcium on CTL cytotoxicity. The CTLs for different conditions were added in respective concentrations of calcium solutions.

Calcium solutions were prepared from the following stock solutions mentioned in table 2:

Individual stock solutions (solns)		1 Litre
3M NaCl	58.44 g/mol	175.32 g
1M KCl	74.55 g/mol	74.55 g
1M $MgCl_2$	203.3 g/mol	203.3 g
1M $CaCl_2$	147.02 g/mol	147.02 g

Table 2: Stock solutions of different reagents used in the preparation of calcium solutions

	For 500 ml		For 500 ml		For 500 ml
0 mM Ca^{2+} soln pH 7.4		1 mM Ca^{2+} soln pH 7.4		10 mM Ca^{2+} soln pH 7.4	
155 mM NaCl	25.835 ml	155 mM NaCl	25.835 ml	155 mM NaCl	25.835 ml
4.5 mM KCl	2.250 ml	4.5 mM KCl	2.250 ml	4.5 mM KCl	2.250 ml
10 mM Glucose	0.991 g	10 mM Glucose	0.991 g	10 mM Glucose	0.991 g
5 mM HEPES	0.596 g	5 mM HEPES	0.596 g	5 mM HEPES	0.596 g
3 mM $MgCl_2$	1.5 ml	2 mM $MgCl_2$	1 ml	2 mM $MgCl_2$	1 ml
$CaCl_2$	nil	1 mM $CaCl_2$	500 µl	10 mM $CaCl_2$	5 ml

Table 3: 0 mM, 1 mM and 10 mM calcium solution recipies

Similarly other solutions with different calcium concentrations such as 31.25 µM, 62.5 µM, 125 µM, 250 µM, 500 µM, 1 mM, 2 mM and 5 mM were prepared from the stock solutions mentioned in table 2. Note: The pH of the above mentioned solutions was adjusted to 7.4 with 1 N NaOH.

2.9. Cell lysate preparation for Western Blot

0.5×10^6 cells (siRNA transfected or untransfected CTLs) were taken in a 1.5 ml eppendorf tube, centrifuged at 200 g (Eppendorf-MiniSpin), 5 min, RT. Supernatant discarded and pellet resuspended with ice cold PBS, then centrifuged at 200 g (Eppendorf-MiniSpin), 5 min, RT. Pellet obtained was resuspended in 20 µl of 1 x gel loading buffer and stored at -20°C for 10-15 min (till frozen). This was then thawed to RT and sonicated to fully lyse the cells. These were heated at 90° C for 5 min. After a fast spin these cell lysates were stored at -20°C till use.

2.10. Western Blot

Western blot analysis was used to detect our proteins of interest. 4 % (stacking) and 12 % separating Tris-glycine gels were used for the separation of the proteins in the cell lysate by Bio-Rad gel electrophoresis system. The gels were run in 1x SDS buffer (that is prepared from 10x SDS buffer, pH 8.3). The proteins from the gel were electroblotted to a nitrocellulose membrane (Life Technologies) using transfer chamber (X-Cell SureLock^{TM}, Invitrogen Novex Mini-cell) in the presence of transfer buffer with 195 mA current and constant voltage for 135 min. After the transfer, the membrane was blocked with 5 % non fat dry milk in 1x TBST (20 mM Tris, 0.15 M NaCl (pH 7.4) with 0.05 % Tween-20) for 30 min at RT on a tilter. Then the blot was washed 3 times with 1xTBST for 5 min each. Later the blot was incubated overnight at 4°C with primary antibody (diluted in 2 % Bovine Serum Albumin (BSA) in 1xTBST).

Anti human rabbit polyclonal Syntaxin 8 antibody (SynapticSystems): 1:1000 dil

Anti human rabbit polyclonal gamma tubulin (γ-TBN) antibody (Sigma): 1:1000 dil

Anti human rabbit polyclonal perforin antibody (SantaCruz): 1:400 dil

Anti human rabbit polyclonal granzyme B antibody (Cell Signalling): 1:5000 dil

Anti human rabbit polyclonal FASL (CD178) antibody (SantaCruz): 1:100 dil

After the incubation with a primary antibody, the blot was washed 4 times with 1xTBST for 5 min each. Blot was then incubated with horseradish peroxidase anti-rabbit secondary antibody (Amersham, 1:40,000 diluted in 2 % BSA in 1xTBST) for 45 min at RT on a tilter. After washing the blot 4 times with 1xTBST for 5 min each, it was developed with ECL solution (1:1 mixture) (Pierce, ThermoScientific) for 1 min and the bands developed on the blot were imaged on BioRad imaging system and the bands were quantified and analysed by the QualityOne, ImageJ 1.45s and Exel softwares.

The different buffers used in the various stages of western blotting are:

1) Sample gel (SG, Loading gel) buffer pH 6.8:

18.905 g of 0.5 M Tris HCl

0.96 g of 0.4 % SDS   ad 240 ml

2) TG Buffer (Separating gel buffer) pH 8.8:

72.7 g of 1.5 M Trizma Base (Mol.Wt 121.14)

1.6 g of 0.4 % SDS (Mol.Wt 288.38)

while dissolving (stirring) the temperature is set to 50°C pH is adjusted with HCl and volume is made upto 400 ml with distilled H_2O.

3) Loading buffer:

   10 ml of 4x loading buffer is prepared with

   3.1 ml of distilled H_2O

   2.4 ml of 1 M Tris HCl pH6.8

   4 ml of Glycerol

   0.8 g of 1 % SDS

   0.5 ml of ß-Mercaptoethanol

   4 mg of 1 % bromophenol blue

When making 1X loading buffer from the above 4X buffer, 1 % (of the total volume of 1X buffer to be made) ß-Mercaptoethanol is added.

4) 10x SDS gel buffer, pH 8.3

   30.29 g of 250 mM Trizma

   144.15 g of 1.92 M Glycin

   10 g of 1 % SDS

   while dissolving (stirring) temperature is maintained at 50°C. The 1x SDS buffer was prepared by making dilution from the 10x SDS buffer stock with double distilled H_2O.

5) 10x Transfer buffer

   30.0 g of Tris Base

   144 g of Glycine

   The volume is made upto 1000 ml with distilled H_2O.

1x Transfer buffer is made from the 10x buffer as follows:

100 ml 10x Transfer buffer

200 ml Methanol

700 ml double distilled H_2O

2.11. Immunocytochemistry

SEA stimulated CTLs were incubated with Raji cells (pulsed with 10 µg/ml of SEA at 37°C for 30 min) at 37°C for different time points on glass coverslips coated with 0.1 mg/ml poly-Ornithine for 20 min. For resting cells, only CTLs without the target calls were plated on the coverslips. Cells were resuspended in a volume of 50 µl for one coverslip. Cells were fixed in ice cold 4 % PFA (freshly prepared from 10 % PFA stock by dilution with PBS (GIBCO)) for 20 min at RT [45]. After which the PFA is removed from the cover slips and the cells were washed with 0.1 M Glycine (prepared in PBS) for 3 min at RT to remove excess PFA. The cells were washed thrice with PBS for 5 min each. The cells were then permeabilized, before staining with primary and secondary antibodies, with PBS+0.1 % Triton for 20 min at RT and later blocked at RT with blocking buffer (PBS with 0.1 % Triton+2 % BSA) for 30 min at RT. All the primary antibodies and secondary antibodies used were diluted in this blocking buffer. The cells were incubated with primary antibody at RT for 90 min, then washed 3 times with PBS+0.1 % Triton for 5 min each and later incubated with the secondary antibody for 45 min at RT. Various proteins were stained using different antibodies. T cell receptors (TCR) were labelled with $Alexa^{488}$- or $Alexa^{647}$- labelled anti-CD3 mAb (UCHT1, Biolegend), lytic granules (LG) with $Alexa^{647}$- labelled anti-perforin mAb (dG9, Biolegend), lysosomes (representing late endosomes) with $Alexa^{488}$- labelled anti-LAMP1 mAb (H4A3, Biolegend) and early endosomes with mouse anti-EEA1

mAb (BD Biosciences). Rab7 constructs (transfected in CTLs) were used as late endosome markers. Endogenous Syntaxin8 was labelled with rabbit polyclonal anti-Syntaxin8 antibody (Synaptic Systems). $Alexa^{568}$-, $Alexa^{647}$-labelled goat-anti-rabbit or $Alexa^{568}$-, $Alexa^{488}$-labelled goat-anti-mouse secondary antibodies (Life Technologies) were used accordingly. After the secondary antibody incubation, the cells were washed thoroughly with PBS+0.1 % Triton twice for 5 min each and the last wash for 5 min with PBS. Coverslips were removed from PBS and carefully mounted with 3 µl mounting medium per coverslip, onto pre cleaned glass slides. The mounted glass slides were stored in dark at 4°C till further use for imaging.

In some cases, the control and Syntaxin 8 downregulated CTLs were incubated with CHX (Cycloheximide) and DMSO for 4 hr at 37°C and later used for fixing and staining. After mounting the cells, these were stored at 4°C until scanning.

Solutions used here as prepared as follows:

1) 10 % PFA stock solution in PBS:

   10 ml of 16 % Formaldehyde (ultrapur, polysciences Inv)

   1.6 ml of 10x PBS (Gibco)

   4.1 ml doubledistilled H_2O

pH was set to 7.4.and sterile filtered and stored at -20^0C after making aliquots. 4 % PFA was made just before use by diluting the 10 % PFA stock in PBS

2) Mounting medium

6 g of Glycerol was added to a 50 ml falcon tube to which 2.4 g Mowiol 4-88 was added and vortexed well. To this 6 ml double distilled water was added and let to stand for 2 hr at RT. Then 12 ml of 0.2 M Tris buffer (pH 8.5) was added and incubated at 53°C till the Mowiol is dissolved. This was mixed well from time to time and then centrifuged at 4000 - 5000 rpm for 20 min. The supernatant was aliquoted and stored at -20°C.

2.12. Epifluorescence deconvolution microscopy and analysis

Epifluorescence microscopy with subsequent deconvolution of the immunostained cell samples was done with Zeiss Cell Observer HS system with a 100x αPlan-Fluar objective (N.A. 1.45) and an AxioCam MRm Rev. 3. Images were acquired with a z-stepsize of 0.2 μm and 2 × 2 binning. Deconvolution with Classic Maximum Likelihood Estimation (CMLE) algorithm by Huygens Professional software 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, Life Technologies). These image files were then converted to tif images and if necessary edited with 'Resample' function of the AxioVision software to correct any pixel shifts present in the obtained images with respect to the fluorescent beads mentioned above. Later ImageJ 1.45s software was used to generate merged images and projections of stacks. For analyzing colocalization, Pearson's coefficient correlation factor was analysed using an ImageJ 1.45s plugin called Just Another Colocalization plugin (JACoP). The dependency and correlations of pixels from two channels are measured by Pearson's coefficient analysis. The fluorescence intensity of a pixel from one channel is plotted against the fluorescence intensity of a pixel from another channel to

form a scatter plot. The slope of this linear plot provides the rate of association of the two fluorophores. Pearson's correlation coefficient is the measure of strength of linear relationship between the two variables, which are the two pixels from two different channels. The value of the Pearson's coefficient factor 'r' can range from 1 to -1 with 1 for complete positive correlation of association and -1 for negative correlation and 0 for no correlation.

3D analysis of the perforin vesicles was done using the Axio vision 3D view software, where the scanned images were set to a low threshold for background correction to eliminate the noise and 3D measured for the selected channel (corresponding to the required wavelength) to obtain the volume of selected ROIs which represent the perforin vesicles.

2.13. anti-CD3/CD28 antibody prestimulation of CTLs

In a 24 well plate, each wells were coated with 200 µl of 1:1,500 dil (in PBS) rabbit polyclonal anti-mouse immunoglobulin (DAKO, Cat. No: Z0259) for 1.5 hr at 37°C and stored in PBS overnight at 4°C. Then they are washed twice with 1 ml PBS at RT and later they were incubated with 4 µg/ml each (prepared in PBS) of mouse anti-CD3 Ab (AbD seroTec, Cat. No: MCA463EL) and mouse anti-CD28 Ab (BD Pharmingen™, Cat. No: 555725) at 37°C for 1.5 hr. These wells were washed once with PBS. The pre existing perforin store was depleted from control and Syntaxin 8 downregulated CTLs by plating $2x10^6$ cells/ml in the Ab treated wells for 2 hr at 37°C. Then the cells were transferred to fresh wells without any Ab and are allowed to recover in AIMV + 10 % FCS with 20 U/ml IL-2 for 6 hr or 24 hr at 37°C.

2.14. Perforin ELISA

Control and Syntaxin 8 downregulated CTLs were first depleted of their preexisting perforin store by anti-CD3 / anti-CD28 Ab stimulation for 2 hr at 37°C. Then the cells were transferred to fresh wells without any Ab and are allowed to recover for 6 hr at 37°C as mentioned previously. After recovery, these cells were again incubated in anti-CD3 / anti-CD28 Ab pretreated wells (of a 96 well plate, BD Biosciences) in AIMV + 10 % FCS at 37°C for different time points after which the supernatants were collected from these into individual eppendorf tubes. These were centrifuged at 200 g at RT for 5 min and supernatants were collected into fresh eppendorf tubes and stored at -20°C until further use. The supernatants collected were thawed to RT and analysed for perforin using the perforin ELISA kit (Diaclone) as per the manufacturer's instructions. Perforin ELISA was also conducted for supernatants collected from CTLs which were not emptied of their pre-existing perforin pool. These CTLs were conjugated with either Raji cells as target cells or with anti-CD3 / anti-CD28 Ab coated activator beads (Life Technologies).

The different time points for preparation of the ELISA samples (after depletion of pre-existing perforin pool) are as follows:

Resting (or time zero, where the CTLs are incubated with target cells for 5 min and supernatant collected), 8 hr, 16 hr, 20 hr and 24 hr. Resting, 24 hr and 48 hr were the time points for ELISA samples without depleting pre-existing perforin pool.

2.15. FACS analysis

Fluorescence-activated cell sorting (FACS) is a flow cytometry technique which can be used for sorting cells, counting and analyses of microscopic particles. Multiple

physical and chemical parameters can be measured simultaneously by this technique, thousands of cells can be measured within minutes.

CTLs that were either depleted (using the anti-CD3 / antiCD28 Ab treatment) or not depleted of their preexisting perforin pool were incubated in anti-CD3 / anti-CD28 coated wells for different time points (like resting condition, 30 min, 2 hr, 3 hr, 4 hr and overnight conjugation). After each time point they were resuspended and collected for the assay. CTLs collected for the analysis are centrifuged at 200 g, 6 min at RT to remove the culture media and pellet obtained was washed with 1 ml PBS / 0.5 % BSA (Buffer 1) solution and centrifuged again to collect the pellet. The cells in the pellet were then vortexed for a few seconds and incubated with 4 % PFA (200 µl for 0.5 x 10^6 cells) for 15 min in ice, in the dark. After removing the PFA, the cells were washed 2 times with 200 µl wash buffer (PBS / 1 % FCS and 0.1 % NaN_3 (pH 7.4 – 7.6)). This was centrifuged at 690 g, 5 min, RT. Pellet obtained was resuspended in 1X 200 µl permeabilizing buffer (PBS / 1 % FCS +0.1 % Saponin (pH 7.4 – 7.6)) and centrifuged again at 690 g, 5 min, RT. Then these cells were fixed in 100 µl permeabilizing buffer and stained with 10 µl (per 0.5 x 10^6 CTLs) of FITC labelled mouse monoclonal perforin antibody (clone: dG9, BioLegend, Cat. No: 308103) or FITC labelled mouse IgG2b, κ Isotype Ctrl antibody (clone: MPC-11, BioLegend, Cat. No: 400309) and incubated at RT for 30 min in dark. These cells were then centrifuged at 690 g for 5 min at RT. The washing of the cells with permeabilizing buffer is repeated twice and centrifuged at 690 g for 5 min at RT. Supernatant was discarded, stained and fixed cells were resuspended in 200 µl PBS for further FACS analysis. BD Canto FACS analyser was used to analyze the perforin expressed in CTLs. Effector CTL population was gated and cells within these gates were analysed for perforin expression (FITC labelled mouse monoclonal perforin Ab) against an

isotype control labelled with FITC labelled mouse IgG2b monoclonal antibody using FlowJo software. Further analysis of the data was done using the FlowJo software, by gating the IgG control by setting a certain threshold % value and accepting all the values that exceeded this threshold in the FITC- A perforin labelled samples as the actual perforin fluorescence. These were represented as peaks plotted on a graph with FITC-A perforin labelling on the x-axis and counts (number of cells) on the y-axis. This gives us an idea of the number of cells labelled and also gives a rough quantification of the perforin content of the cell by giving us the perforin fluorescence intensity in these measured cells.

3. RESULTS

3.1. Syntaxin 8 is localized mainly in late and recycling endosomes and partially with lytic granules, TCR, Vti1b which are essential for normal CTL function

3.1.1. Syntaxin 8 is partially colocaized with lytic granules, TCR and Vti1b

SNAREs proteins, the members of intracellular trafficking pathway are known to be required for normal human CTL function [45,98,131]. Apart from Vti1b and Syntaxin 7, which are shown to be directly involved in CTL functions, several other SNAREs like Syntaxin 8, Syntaxin 11 are also enriched at the immunological synapse (IS), indicating that they could play essential roles in CTL cytotoxicity [98]. As no Qc-SNARE has been identified to be directly involved in CTL function, we set out to examine the significance of Syntaxin 8 in human CTL function. It has already been shown by us that Syntaxin 8 is expressed in both primary human naïve $CD8^+$ T cells and activated CTLs [1]. To study the function of Syntaxin 8 in CTL, we examined the sub-cellular localization of Syntaxin 8. SEA stimulated CTLs were conjugated with SEA pulsed Raji cells [129], fixed with PFA and later stained with CD3 Ab for labelling TCR (Fig. 4A), perforin Ab to label the lytic granules (Fig. 4B) and Syntaxin 8 Ab for labelling Syntaxin 8. These fixed samples were then scanned with epifluorescence deconvolution microscopy. The images obtained were deconvolved and anlysed for extent of colocalization by measuring the Pearson's co-efficient using JACoP function of ImageJ 1.45s.

The result showed that Syntaxin 8 partially colocalized with both TCR and lytic granules with Pearson's co-efficient of 0.596 ± 0.02 (Fig. 4A) and 0.481 ± 0.03 (Fig. 4B), respectively. We also found that Syntaxin 8 accumulated at the mature IS (indicated by enrichment of CD3 and lytic granules at the contact site facing the targets). As TCR activated CTLs kill their targets by release of lytic granules, both TCR and lytic granules are the functional components of a CTL. Therefore this finding, that Syntaxin 8 colocalizes with TCR and lytic granules, implies its involvement in the CTL functioning.

As Vti1b is required for the lytic granule and TCR tethering which facilitates the docking of lytic granules at the IS [45], we further investigated the localization of Syntaxin 8 and Vti1b. We observed that in resting CTLs, both Vti1b and Syntaxin 8 are dispersed throughout the cell whereas in conjugated CTLs both proteins accumulate at the IS. Furthermore Syntaxin 8 partially colocalizes with Vti1b in both resting and conjugated CTLs with a Pearson's co-efficient of 0.41 ± 0.02 and 0.45 ± 0.05 in resting and conjugated CTLs, respectively (Fig. 4C). These results indicate that Syntaxin 8 may be important for the normal functioning of CTLs.

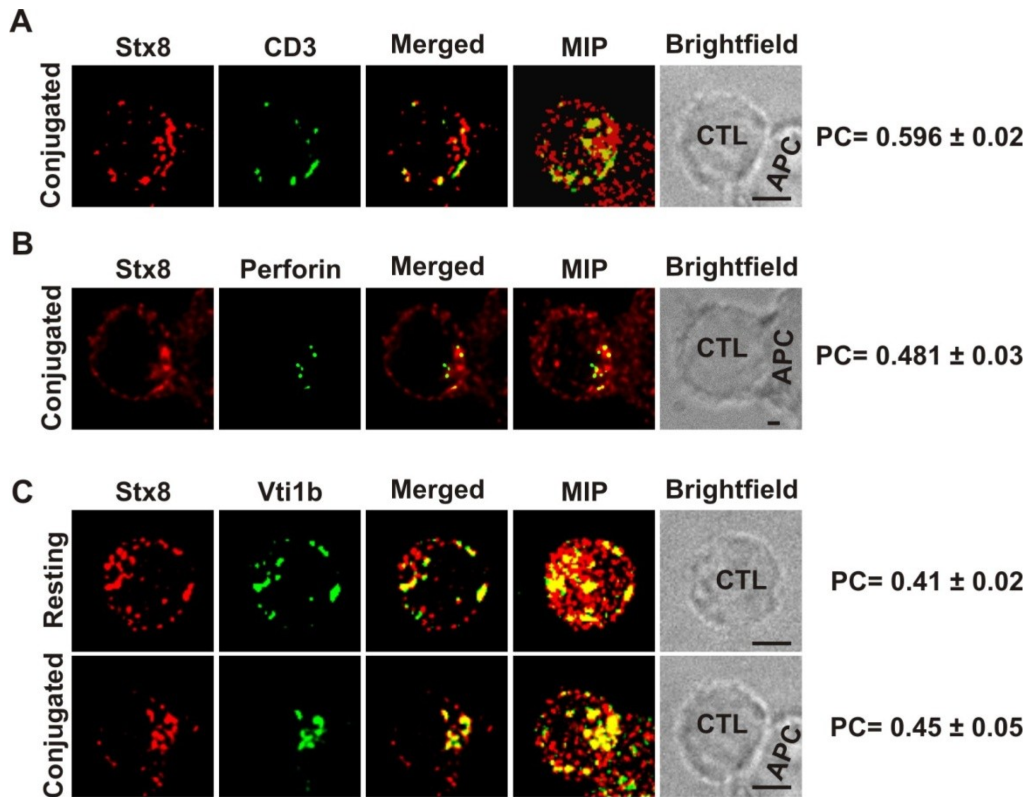

Figure 4: Syntaxin 8 partially colocalizes with TCR, lytic granules and Vti1b.
SEA stimulated CTLs were incubated with SEA pulsed Raji cells for 30 min at 37°C before fixation with ice-cold 4 % PFA. After permeabilizing these cells, Syntaxin 8 was labelled with rabbit polyclonal anti-Syntaxin 8 Ab; Alexa^{568} conjugated secondary goat anti-rabbit Ab (red). (A) TCR was labelled with Alexa^{647} conjugated anti-CD3 Ab (green), and (B) lytic granules with Alexa^{647} conjugated anti-perforin Ab (green). (C) Vti1b was labelled with mouse monoclonal anti-Vti1b Ab, Alexa^{488} conjugated secondary goat anti-mouse Ab (green). Scale bar = 3 µm. In each condition 10 cells were analysed for Pearson's coefficient (PC) [1 = complete co-localization, 0 = no co-localization]. MIP: Maximum Intensity Projection.

3.1.2. Syntaxin 8 is mainly localized in late endosomal and recycling endosomal compartments in CTLs

In order to further nail down the sub-cellular localization of Syntaxin 8 in CTLs, we examined the colocalization of Syntaxin 8 with early, late, recycling and lysosomal compartments, which were labelled with EEA1, Rab7-mCherry, Rab11-mCherry and LAMP1, respectively in both resting CTLs and CTLs conjugated with target cells. We found that Syntaxin 8 is mainly colocalized with the late lysosomal compartment

marker Rab7 in both resting and conjugated cells, with Pearson's coefficient of 0.72 ± 0.02 (resting cells) and 0.67 ± 0.01 (conjugated cells), (Fig. 5A, 6A) respectively. In resting cells we observed puncta like membranous distribution of both Syntaxin 8 and Rab7 mCherry (Fig. 5A) whereas in conjugated cells both the proteins are accumulated at the IS (Fig. 6A). Syntaxin 8 also colocalizes with LAMP1, a marker for lysosomal compartment with Pearson's co-efficient of 0.58 ± 0.01 for resting cells(Fig. 5C) and 0.63 ± 0.01 for conjugated CTLs (Fig. 6C) and with Rab11-mCherry which is a marker for recycling endosomes with Pearson's co-efficient of 0.73 ± 0.02 for resting CTLs (Fig. 5D) and 0.65 ± 0.03 for conjugated CTLs (Fig. 6D). In contrast to this a comparatively lower Pearson's co-efficient of 0.24 ± 0.01 for resting cells (Fig. 5B) and 0.35 ± 0.02 for conjugated cells (Fig. 6B) was observed with early endosome marker EEA1. Even though we see the accumulation of Syntaxin 8 at the IS, EEA1 does not accumulate at the IS in conjugated CTLs. In spite of lower overlapping of Syntaxin 8 in early endosomes than in late endosomes, the Pearson's co-efficient for early endosomes (in conjugated cells) is quite good and is considered as partial colocalization. By this we can conclude that Syntaxin 8 is localized mainly in the late endosomal compartments and in recycling endosomes and to a lesser extent in early endosomes of conjugated CTLs. The colocalization of Syntaxin 8 in lysosomal compartments and the other endosomal compartments indicates that it may participate in the trafficking of lytic granule components like perforin and granzymes to the lysosomal (lytic granule) compartments.

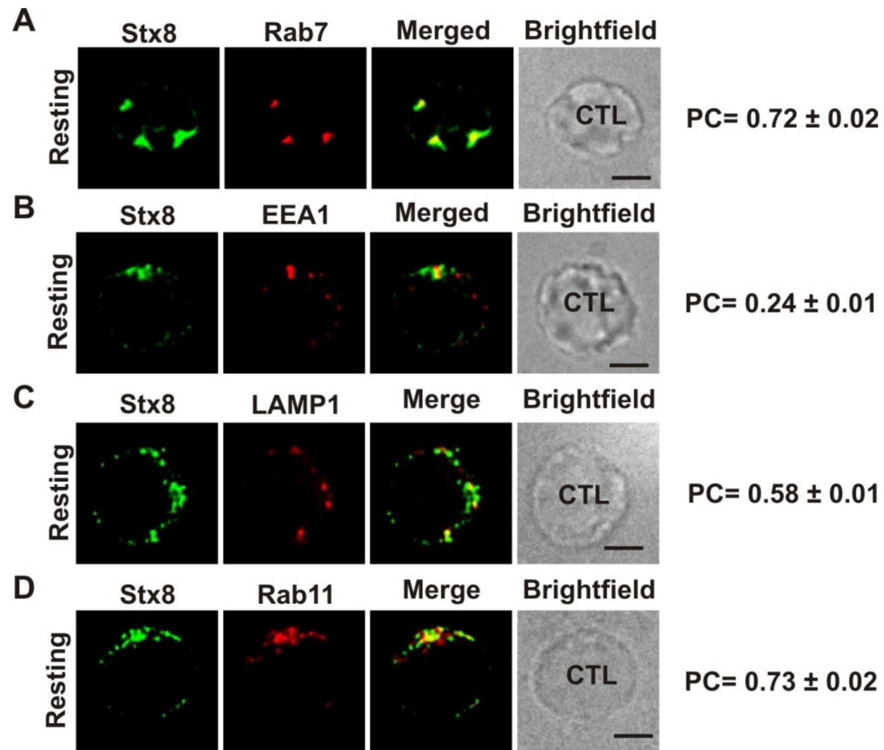

Figure 5: Syntaxin 8 localization in endosomal compartments in resting CTLs.

Syntaxin 8 labelled with rabbit polyclonal anti-Syntaxin 8 Ab, Alexa^{647} conjugated goat anti-rabbit secondary Ab (green) with different endosomal compartment markers. In resting CTLs. (A) Late endosomes were labelled with Rab7-mCherry construct (B) Early endosomes with mouse monoclonal anti-EEA1 primary Ab and Alexa^{568} conjugated goat secondary anti-mouse Ab (red) (C) Lysosomal compartments were labelled with Alexa^{488} conjugated LAMP1 (CD107) mouse monoclonal Ab (red). In each condition (Figs. A,B,C) 30 cells were analysed for Pearson's coefficient. (D) Recycling endosomes were labelled with Rab11-mCherry construct (red) and n = 6 cells for this condition. Scale bar = 3 µm.

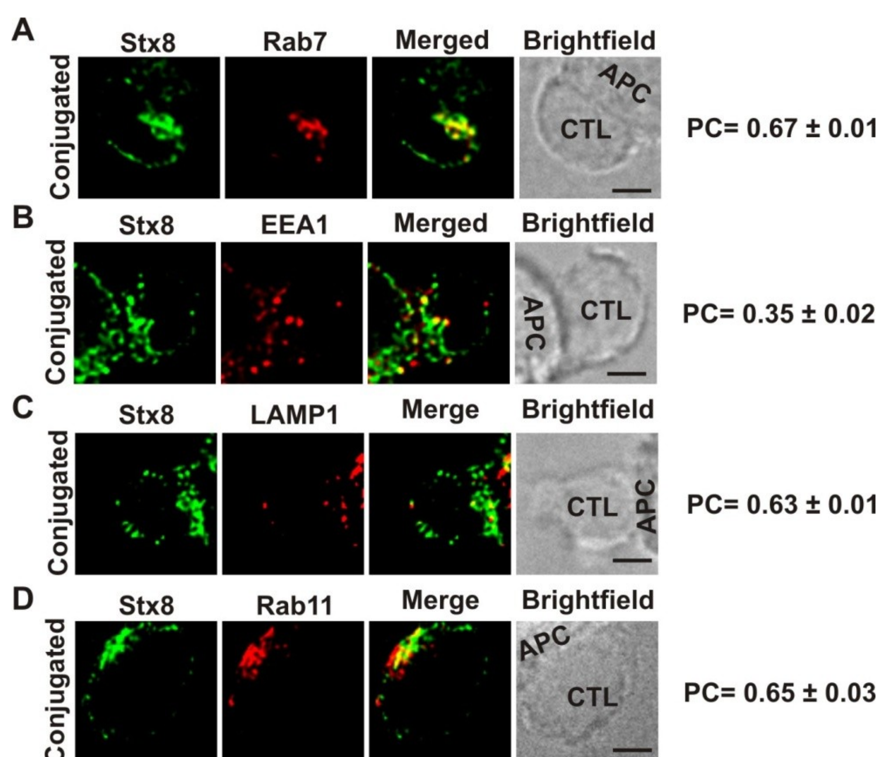

Figure 6: Syntaxin 8 localization in endosomal compartments in CTLs conjugated with targets. Syntaxin 8 labelled with rabbit polyclonal anti-Syntaxin 8 Ab, Alexa^{647} conjugated goat anti-rabbit secondary Ab (green) with different endosomal compartment markers in SEA stimulated CTLs that are conjugated with SEA stimulated Raji cells for 30 min at 37°C. (A) Late endosomes were labelled with Rab7-mCherry construct, (B) early endosomes with mouse monoclonal anti-EEA1 primary Ab and Alexa^{568} conjugated goat secondary anti-mouse Ab (red). (C) The lysosomal compartments were labelled with Alexa^{488} conjugated LAMP1 (CD107) mouse monoclonal Ab (red). In each condition (Figs. A,B,C) 30 cells were analysed for Pearson's coefficient. (D) Recycling endosomes were labelled with Rab11-mCherry construct (red) and n = 12 cells. Scale bar = 3 µm.

3.2. Syntaxin 8 is required for CTL cytotoxicity

3.2.1. Syntaxin 8 is efficiently downregulated in primary human CTLs by siRNA transfection

To understand the mechanisms by which Syntaxin 8 influences CTL cytotoxicity, we used siRNA to downregulate Syntaxin 8 in primary human CTLs. Using nucleofection with Lonza technology, we managed to achieve transfection efficiency of 97.5 ± 2.5 % (n = 3, mean ± SEM) of Alexa^{546}-labelled control siRNA (Fig. 7A).

To optimize the downregulation of Syntaxin 8, first we checked four Syntaxin 8 siRNA that are targeted against different regions of Syntaxin 8 mRNA. A scrambled unspecific siRNA was used as the control. Two out of the four siRNAs showed good downregulation at mRNA level, of which Sxt8_5 showed the highest downregulation (Fig. 7B). It is reported that a stable OMe- modification of siRNA can substantially prolong half-life of siRNA in primary T cells by stabilizing its structure [127]. Using this feature we modified Syntaxin 8 siRNA (Stx8_5) to maximize the downregulation of Syntaxin 8 (if not specified, the following used Syntaxin8 siRNA are modified Stx8_5). To determine the optimal time point of Syntaxin 8 downregulation, we collected siRNA transfected CTLs at various time points. Quantitative RT-PCR showed that Syntaxin 8 could be efficiently downregulated and only about 15 % of the mRNA could be detected between 12 to 36 hr after transfection (Fig. 7C). To balance between downregulation efficacy and effector status of CTLs, we chose 36 hr for the following functional experiments. Subsequently, western blot was carried out to confirm downregulation at protein level at 36 hr after transfection, which showed 75.1 % ± 3.8 % downregulation at protein level (Fig. 7F, 7G). The downregulation was further confirmed by immunocytochemistry which showed reduced Syntaxin 8 protein staining in Syntaxin 8 siRNA CTLs compared to control CTLs (Fig. 8A). The specificity of Syntaxin 8 siRNA was verified with the expression level of two other mRNAs, Vti1b and perforin, in Syntaxin 8 downregulated CTLs. It showed that only Syntaxin 8 and not Vti1b or perforin was downregulated by Stx8-5 siRNA (Fig. 8B). On the whole, 36 hrs after transfection is the time point optimized for downregulation and subsequent functional studies.

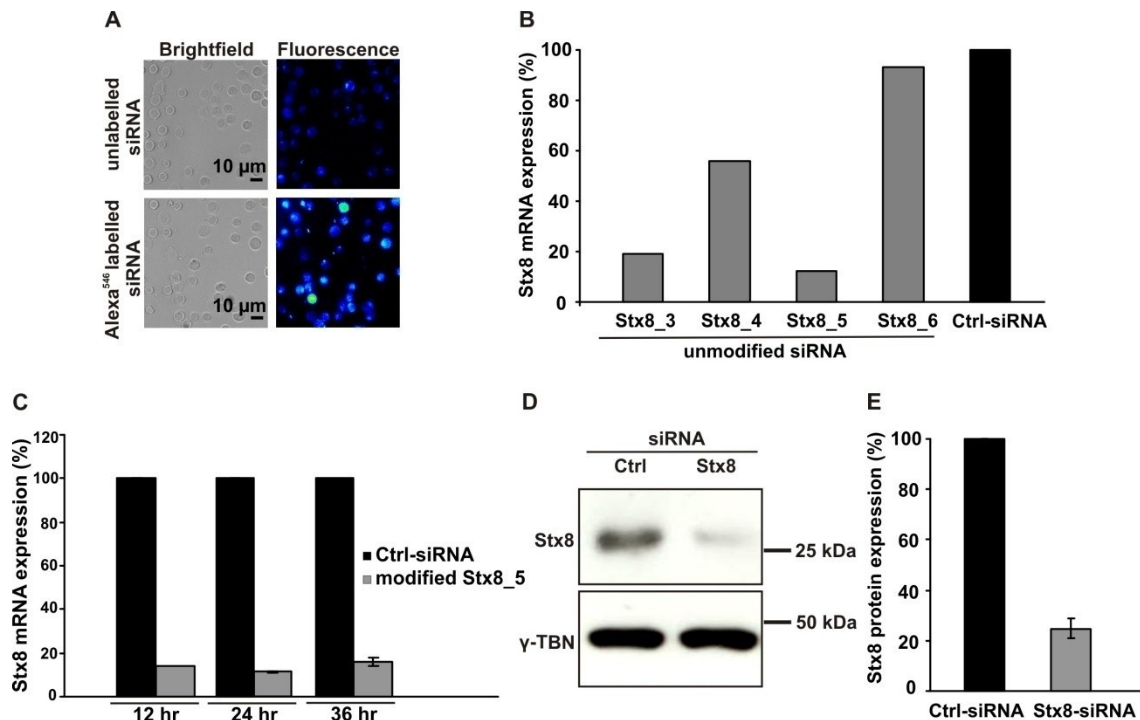

Figure 7: Syntaxin 8 is efficiently downregulated at both mRNA and protein levels by modified Syntaxin 8 siRNA (Stx8-siRNA).

Primary human CTLs were transfected efficiently with siRNA. (A) CTLs were transfected with Alexa^{546}-conjugated siRNA and control non-fluorescent siRNA. The transfection efficiency detected was 97.5 ± 2.5 % (n = 3, Mean ± SEM). (B) Four unmodified Stx8-siRNAs were tested for downregulation of Syntaxin 8 in primary human CTLs. Scrambled siRNA was used as control siRNA (Ctrl-siRNA). Stx8_5 (Syntaxin 8_5) showed the highest downregulation. (C) Optimization of the time point for the down-regulation. OMe- modified Stx8_5 siRNA was used to determine the downregulation efficiency at different time points after transfection. The down-regulation of Syntaxin 8 at 36 hr after transfection at (C) mRNA and (D,E) protein levels (n = 3, Mean ± SEM). (D) Western blot analysis of the proteins showed a band at 27 kDa for Syntaxin 8 protein and a band at 48 kDa for loading control γ Tubulin. Densitometry of the protein expressed in the western blot is shown in Fig. E.

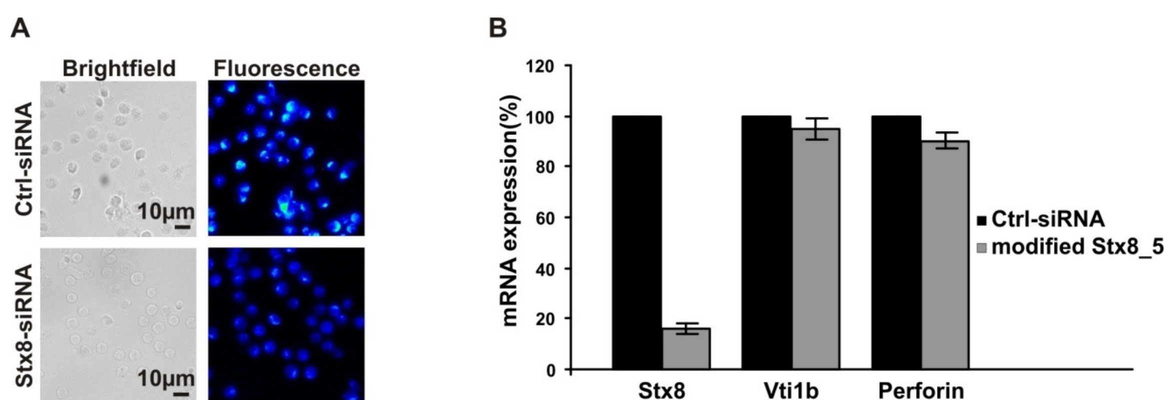

Figure 8: Syntaxin 8 is efficiently and specifically downregulated by modified Syntaxin 8 siRNA (Stx8-siRNA).

(A) Immunocytochemistry of control (Ctrl-) and Syntaxin 8 (Stx8- siRNA) si-RNA transfected CTLs showed efficient downregulation of Syntaxin 8, were Syntaxin 8 was labelled with rabbit polyclonal anti-Syntaxin 8 Ab and Alexa^{568} conjugated goat anti-rabbit secondary Ab. (B) Specificity of Stx8-siRNA was verified by testing the downregulation of Syntaxin 8, Vti1b and perforin in CTLs transfected with modified Ctrl- and Stx8_5-siRNA (n = 3, Mean ± SEM).

3.2.2. CTL mediated cytotoxicity is impaired by Syntaxin 8 downregulation

The ultimate read out for normal CTL functioning is its cytotoxicity efficiency. This can be measured at single cell level or at population level. The single cell level cytotoxicity can be measured by different microscopy techniques using fluorescent dyes, visualizing single killing events directly. However population killing assay offers an overview of the overall killing competence of CTLs at defined conditions, thus enabling the quantification of CTL cytotoxicity function. All the population killing assays reported till now are the end point analysis assays, in which only the end point or the time point at the end of the complete experiment can be measured. The most used end point killing assays are lactate dehydrogenase (LDH) release assay [132] and ^{51}Cr release assay [133]. These two assays measure the activity of LDH or ^{51}Cr, which are released from lysed target cells into the supernatant, 4 hours after the incubation.

We used a new technique developed in our lab, called the real-time killing assay, to measure the killing capacity of CTLs in real time. In this assay calcein-loaded target cells are measured every 10 min for 4 hr at 37°C, with the excitation and emission wavelength of 485 nm and 535 nm, respectively (Kummerow, C. et al, unpublished). In order to reflect the true killing event, we optimized the conditions for the media. We tried AIMV with (Fig. 9A) and without FCS (Fig. 9B). We found that though the same badge of effector and target cells were used, it showed different killing kinetics for AIMV with FCS (Fig. 9A) and without FCS (Fig. 9B). We further verified with high-content image setup BioImager that real-time killing assay done in AIMV without FCS represents the killing events at single cell level (Kummerow, C. et al, unpublished).

This assay was also tested with other media such as Ringer solutions with different calcium concentrations (Ca^{2+} conc) using different effector to target ratios (20:1 and 10:1) (Fig. 10).

In AIMV + FCS condition we found a final killing of 64.33 ± 3.54 % at 20:1 effector to target ratio. We also observed a gradual increase in target lysis over time (Fig. 9A). Whereas in AIMV no FCS condition, a much higher final killing of 91.33 ± 5.27 % at 20:1 effector to target ratio was observed. Also the increase in the target lysis is quite rapid even at the initial phase compared to that in AIMV+FCS condition (Fig. 9B). The 8 different Ca^{2+} conc used for CTL killing showed varied killing efficiencies. We showed an increase in the killing efficiency with increase in Ca^{2+} conc from 32.5 µM Ca^{2+} to 500 µM Ca^{2+} but at 250 µM Ca^{2+}, killing efficiency was the highest. Killing efficiency kept decreasing on increasing the Ca^{2+} concs to 1, 2 and 5 mM (Fig. 10). These results indicate the Ca^{2+}dependency of CTLs for their cytotoxicity.

To study the perforin killing pathway in CTLs, we blocked the H+ ATP hydrolase enzyme in CTL lytic granules by an inhibitor called conconamycin A (CMA) which compromises the acidic pH of the lytic granules by increasing the pH [134]. As an acidic environment is required to keep perforin and granzymes inactive by binding to proteoglycans, the increase in the pH degenerates these molecules. Another possibility is that the increase in the pH leads the activated granzymes and other proteases in the lytic granules to cleave and degrade the lytic granule contents thus blocking the lytic granule function. CTLs were treated with various concentrations of CMA for 2 hr at 37°C; DMSO was used as a solvent control. To rule out the possibility that the target (Raji) cells were affected by the CMA, we included controls of calcein loaded targets incubated with and without the CMA (highest concentration used), throughout the 4 hr time frame of the assay, which showed that the targets were unaffected by the CMA. We observed that CMA treatment blocks mainly the lytic granule pathway which can be seen by the concentration dependent reduction in cytotoxicity when compared to the cytotoxicity of CTLs treated with DMSO (Fig. 11). This strongly suggests that the major cytotoxic pathway in human CTLs is the perforin dependent lytic granule pathway.

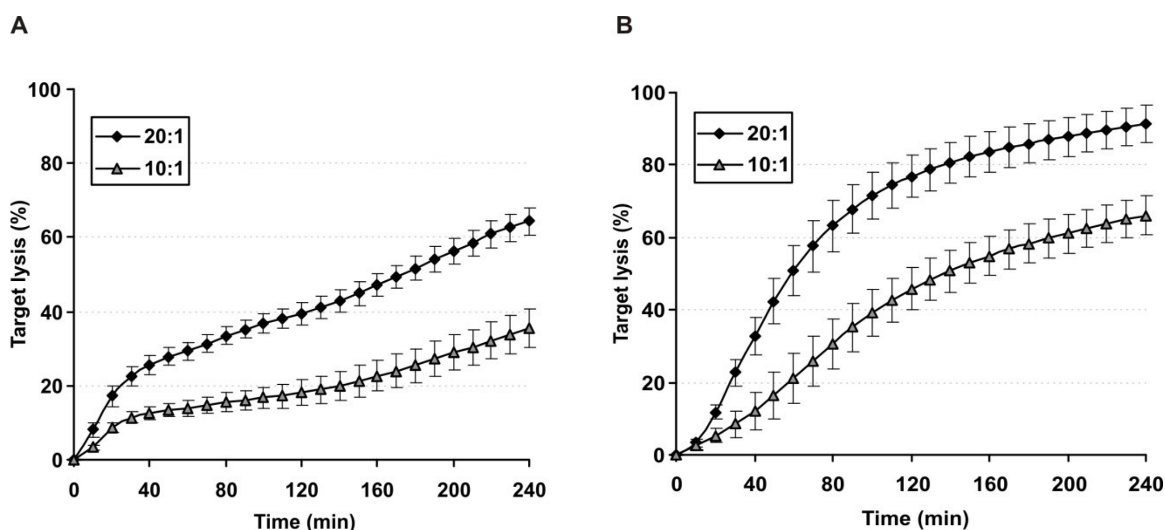

Figure 9: Real time Calcein killing assay.

(A) Real time Calcein killing assay using SEA stimulated CTLs and SEA pulsed Raji cells as targets was performed at 20:1 and 10:1 effector to target ratio in AIMV media with 10 % FCS. Total number of experiments, n = 10. (B) The same assay was performed again in AIMV media without FCS at 20:1 and 10:1 effector to target ratio. Total number of experiments, n = 4, Mean ± SEM. Fluorescence excitation wavelength used was 485 nm and emission was measured at 535 nm. Fluorescence measured for every 10 min over a period of 4 hr at 37°C.

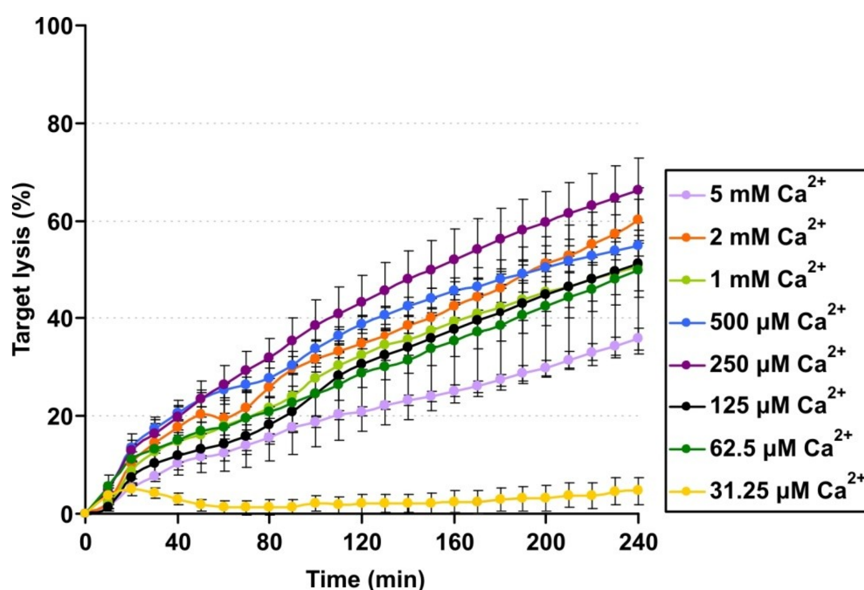

Figure 10: Calcium dependency of CTL mediated cytotoxicity.

Real time Calcein killing assay using SEA stimulated CTLs at 10:1 effector to target ratio in Ringer solutions with different Ca^{2+} concs (31.25 µM, 62.5 µM, 125 µM, 250 µM, 500 µM, 1 mM, 2 mM and 5 mM). Fluorescence excitation wavelength used was 485 nm and emission was measured at 535 nm. Fluorescence measured for every 10 min over a period of 4 hr at 37°C.

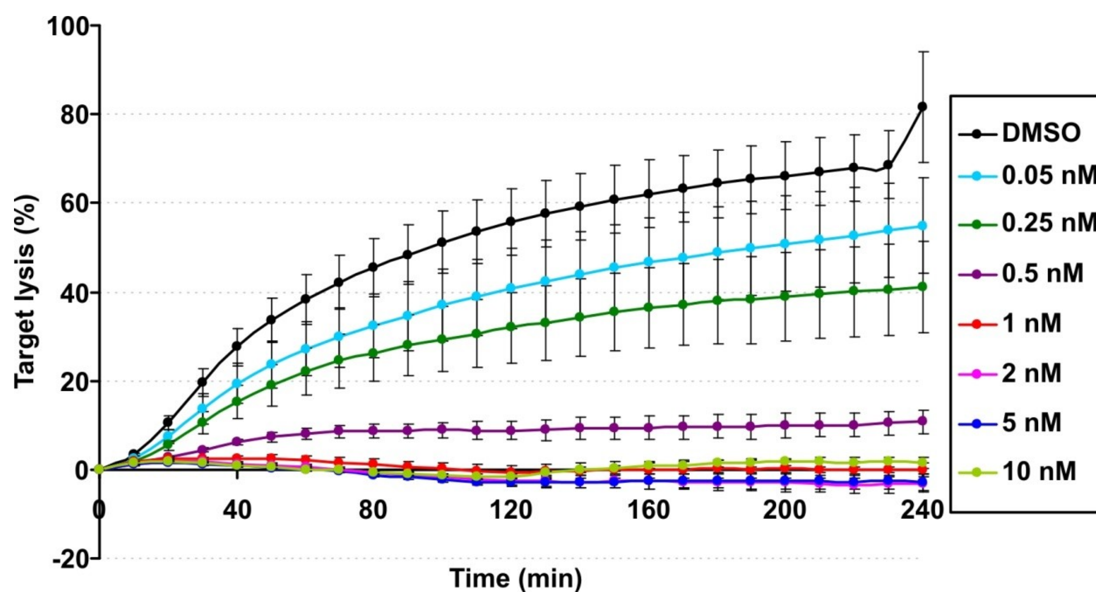

Figure 11: Effect of different CMA concentrations on CTL cytotoxicity.

CTLs treated with various concentrations (50 nM, 10 nM, 5 nM, 2 nM, 1 nM, 0.5 nM, 0.25 nM, 0.05 nM and 0.01 nM) of conconamycin A (CMA) were used in real time killing assay at 20:1 effector to target ratio in AIMV without FCS. Fluorescence excitation wavelength used was 485 nm and emission was measured at 535 nm. Fluorescence measured for every 10 min over a period of 4 hr at 37°C

Since Syntaxin 8 accumulated at the IS upon the recognition of target cells, we proceeded to determine if Syntaxin 8 is involved in CTL mediated cytotoxicity. Thus we downregulated this protein and checked if it effected the cytotoxicity efficiency of human CTLs. 36 hr after transfection with control or Syntaxin 8 siRNA, CTLs were used in real time killing assay. We found that, compared to the control CTLs, Syntaxin 8 downregulated CTLs showed reduced killing In killing assays, where CTLs in AIMV media with FCS were used, we start observing the difference in cytotoxicity of control and Syntaxin 8 downregulated CTLs from 60 min onwards but the difference between them is significant from 150 min onwards (Fig. 12A). Even in AIMV media without FCS, we see reduced killing by Syntaxin 8 downregulated CTLs compared to control CTLs. In this the killing is rapid even at the initial time points and the difference in killing between control and Syntaxin 8 downregulated CTLs is more evident from 100 minutes onwards (Fig. 12B).

Real time killing assay with Vti1b downregulated CTLs (which showed reduced cytotoxicty by LDH assay [45], was performed as positive control for cytotoxicity where we observed a reduction in cytotoxicity in Vti1b downregulated CTLs compared to the control CTLs (Fig. 13A).

To rule out the possibility that the cytotoxicity was affected by SNARE protein downregulation in general, we checked the target lysis in Syntaxin 6 (Stx 6) downregulated CTL, since Syntaxin 6 is also a Qc- SNARE like Syntaxin 8. We found no change in cytotoxicity by downregulation of Syntaxin 6 (Fig. 13B). These results suggest that Syntaxin 8 is involved in CTL cytotoxicity.

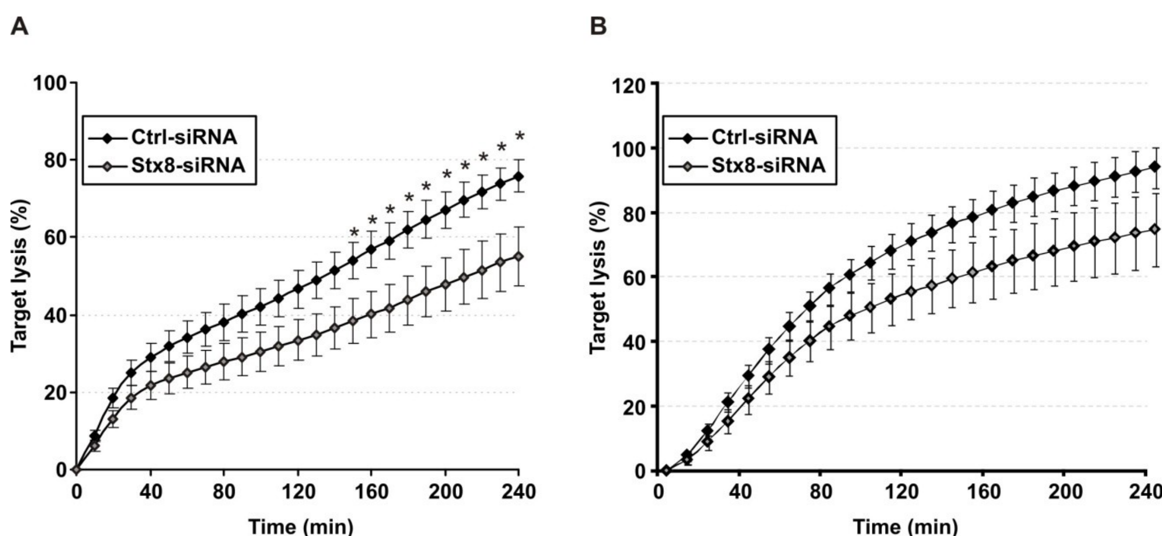

Figure 12: Syntaxin 8 downregulation impairs CTL cytotoxicity.

Real time killing assay using Ctrl-siRNA (control) and Stx8-siRNA (Syntaxin 8) CTLs with SEA pulsed Raji cells at 20:1 effector to target ratio showed that cytotoxicity of CTLs is reduced on Stx8 downregulation. The medium used for the assay is AIMV with 10 % FCS. SEA stimulated control and Syntaxin 8 siRNA transfected CTLs were used in this assay 36 hr after transfection. The results are shown as Mean ± SEM (n = 10, * = P < 0.05, ** = P < 0.01 and *** = P < 0.001). Fluorescence was measured for every 10 min over a period of 4 hr at 37°C.

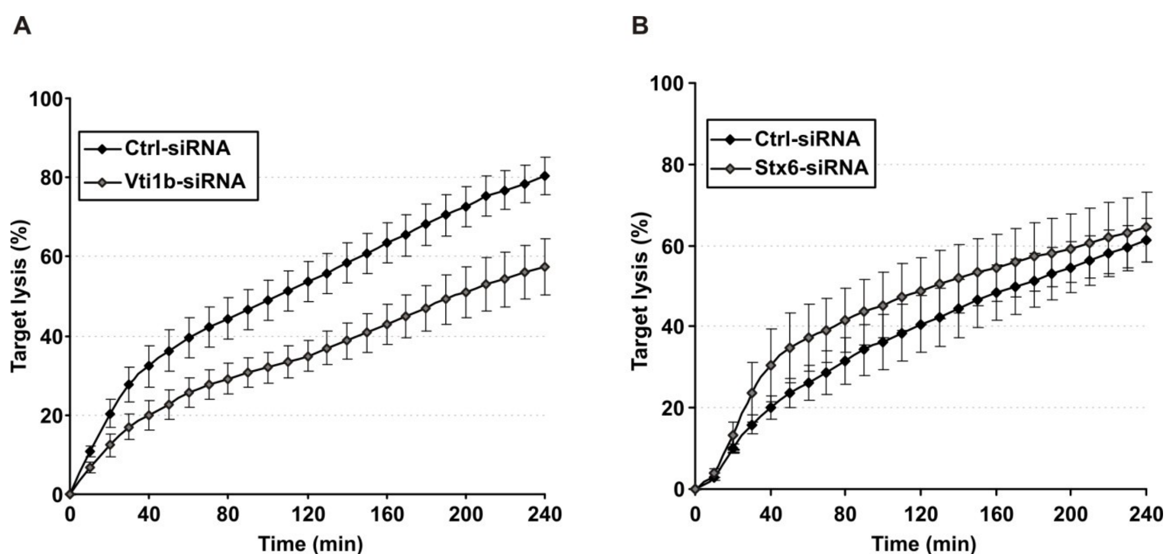

Figure 13: Vti1b and Stx 6 downregulated CTLs used as controls for CTL cytotoxicity.
(A) 36 hr after transfection of modified Vti1b-siRNA and Ctlr-siRNA in CTLs, real time killing assay was conducted with a total number of experiments, n = 6, Mean ± SEM. (B) 36 hr after transfection of unmodified Stx6-siRNA (Syntaxin 6) and Ctlr-siRNA in CTLs, real time killing assay was conducted. Total number of experiments, n = 5, Mean ± SEM. Fluorescence measured for every 10 min over a period of 4 hr at 37°C.

3.3. Syntaxin 8 downregulation does not inhibit exocytosis of lytic granules in CTLs

3.3.1. Decrease in perforin released by Syntaxin 8 downregulated CTLs results after emptying their preexisting cytotoxic molecule pool

To confirm if exocytosis of lytic granules is impaired by Syntaxin 8 downregulation, we analysed perforin secretion. This was examined by detecting the amount of perforin released to the supernatant upon CTL-target recognition using ELISA. CTLs were incubated with target cells like Raji cells (Fig. 14A) or CD3 / CD28 activator beads (Life Technologies) (Fig. 14B). The supernatant was collected at different times as indicated in the figures. We found that there was no significant change in the levels of perforin exocytosed by Syntaxin 8 downregulated CTLs (Fig. 14A, 14B). It implies that Syntaxin 8 might not have an impact on lytic granule release per se.

Next we were wondering whether Syntaxin 8 may play a role in the generation or sorting of new lytic granules. To address this question we depleted the pre-existing lytic granule pool by incubating the control and Syntaxin 8 downregulated CTLs in 96-well plates coated with anti-CD3 / anti-CD28 antibodies for 2 hr at 37°C. Once the pre-existing pools were depleted, these cells were kept in the incubation to recover for 6hr in fresh uncoated wells. Then the cells were again incubated with anti-CD3 / anti-CD28 antibodies (now to mimic the target cells) for various time points. The supernatant for ELISA were collected at each of these time points as indicated in the figure. We found that there was no significant difference in the perforin release at the initial time points, 0 hr and 8 hr; whereas at later time points (16, 20, 24 and 48 hr) the difference in perforin release was significant between control and Syntaxin 8 down-regulated CTLs (Fig. 15). It suggests that Syntaxin 8 is likely involved in steps prior to exocytosis like regulation in the synthesis of new cytotoxic proteins or in sorting and delivery of cytotoxic molecules into functional lytic granules through the endosomal pathway.

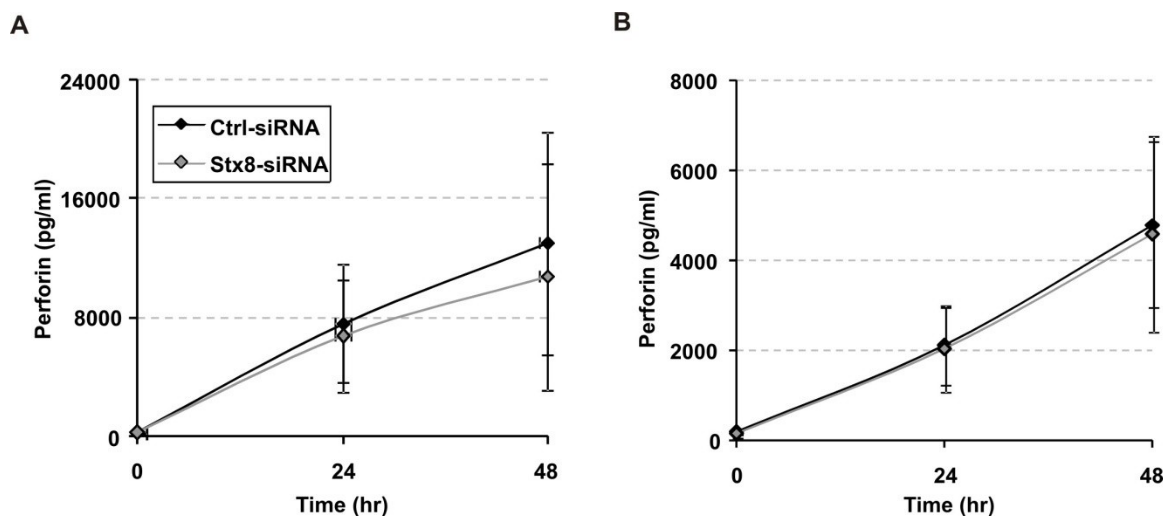

Figure 14: Control (Ctrl-) and Syntaxin 8 (Stx8-siRNA) CTLs without depletion of the preexisting lytic granule pool showed no significant difference in perforin release.

Perforin was measured by ELISA (Diaclone) in samples collected from siRNA transfected CTLs which were incubated with (A) Raji cells or (B) CD3 / CD28 activator beads (Life Technologies) for various time points at 37°C. These CTLs were not depleted of their preexisting lytic granule pool. n = 3, Mean ± SEM.

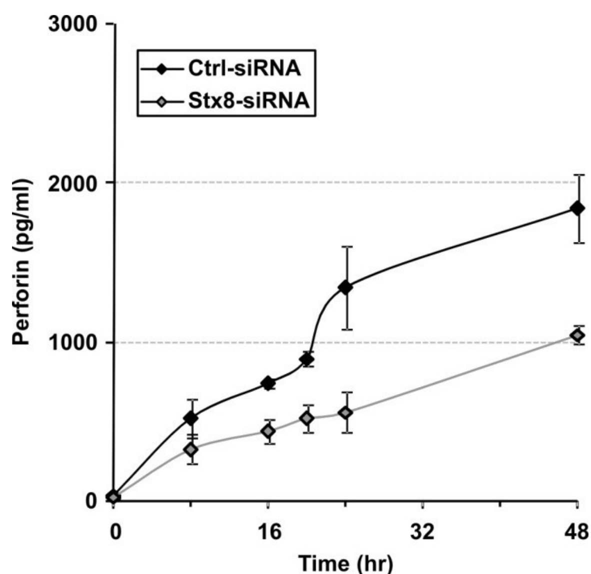

Figure 15: Syntaxin 8 downregulated CTLs show reduced perforin release on preexisting lytic granule pool depletion.

After releasing the preexisting lytic granule pool by anti-CD3/anti-CD28 Ab treatment and recovery, Ctrl and Stx8-siRNA transfected CTLs were incubated with anti-CD3/anti-CD28 Abs, mimicking target cells, for different time points at 37°C. Perforin ELISA (Diaclone) was used to detect perforin concentration in the samples collected from these CTLs. n = 3, Mean ± SEM (* $P < 0.05$).

3.3.2. Depletion of preexisting cytotoxic molecules increases the difference in cytotoxicity between the control and Syntaxin 8 downregulated CTLs

Along this line, we postulated that pre-depletion of the existing lytic granule pool could result in more substantial reduction in CTL-mediated killing by Syntaxin 8 down-regulation. To verify this, we performed real-time killing assay. The preliminary experiments with these conditions showed that indeed after the release of preexisting lytic granule pool, Stx8 down-regulated CTLs showed much lower cytotoxicity than the control CTLs (Fig. 16). We observed that there is an increase in the difference in cytotoxicity between control and Syntaxin 8 downregulated CTLs until 100 min but during the later time points the difference in cytotoxicity of antibody treated Syntaxin 8 and control CTLs is similar to that of antibody untreated CTLs. This also shows that not the lytic granule exocytosis itself but some other process upstream to exocytosis is affected, thus indirectly affecting and delaying the exocytosis.

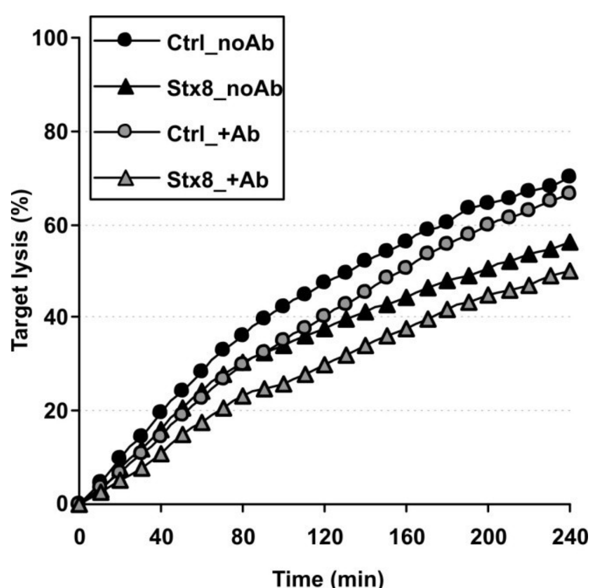

Figure 16: Increased reduction in cytotoxicity of Stx8-siRNA CTLs observed on depletion of preexisting pool of cytotoxic molecules.

Preliminary data of real time killing assay with control (Ctrl) and Stx 8 siRNA CTLs at 10:1 effector : target, in 1 donor with and without depletion of preexisting pool of cytotoxic molecules. The preexisting pool is emptied by treatment with anti-CD3/anti-CD28 Ab for 2 hr and allowed to recover overnight at 37°C in AMIV+10 % FCS.

3.4. Syntaxin 8 is involved in production and sorting of cytotoxic molecules to releasable lytic granules

3.4.1. Syntaxin 8 affects CTL cytotoxicity by influencing processes upstream of exocytosis of lytic granules

To identify which step of the cytotoxicity process is regulated by Syntaxin 8, we conducted the following experiments. First we inhibited the synthesis of new proteins in CTLs using cycloheximide (CHX), a protein synthesis inhibitor, which inhibits the protein elongation process [135]. First of all we tested the dose dependence of CHX, to check the optimal concentration suitable for our assay. At 10 µg/ml concentration of CHX the killing was inhibited after 60 min but not at the initial phase, which indicates that at this concentration most likely the synthesis of cytotoxic proteins was mainly

blocked but the killing machinery (transportation, exocytosis and so on) was still functioning, (Fig. 17A). Therefore we chose this concentration of 10 µg/ml for further experiments. DMSO was used as the solvent control in these experiments. We incubated the SEA stimulated Syntaxin 8 and control siRNA transfected CTLs with CHX and DMSO for 4 hr at 37°C.

The cytotoxicity of these CHX and DMSO treated CTLs was measured using the real time killing assay. As expected, the positive control, DMSO treated control CTLs showed highest killing as neither the protein synthesis nor Syntaxin 8 levels are affected. A significant difference in cytotoxicity of DMSO treated control CTLs and CHX treated control CTLs was observed from 30 min onwards. There was significant reduction of cytotoxicity between Stx8-DMSO and Ctrl-DMSO (10 min, 20 min = *, 80 to 120 min = *, 130 to 170 min = **, 180 to 220 min = ***, 230 and 240 min = **), Ctrl-DMSO and Ctrl-CHX (30 to 110 min = *, 120 to 150 min = ** and 160 to 240 min = ***). Here the * indicates the p value, the level of significance where,). *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$ (Fig. 17B). Also DMSO treated Syntaxin 8 siRNA CTLs, CHX treated control and CHX treated Syntaxin 8 siRNA CTLs showed reduced killing. At the initial phase of the killing all these three conditions showed similar reduction in killing, but from 180 min onwards, killing by DMSO treated Syntaxin 8 downregulated CTLs increases significantly compared to CHX treated Syntaxin 8 downregulated CTLs (180 to 240 min = *). Furthermore we found that after CHX treatment, the difference between Syntaxin 8 downregulated and control CTLs was diminished (Fig. 17B). Thus the recovery in cytotoxicity of DMSO treated Syntaxin 8 siRNA CTLs (where CHX mediated inhibition of protein synthesis is absent) in the later phase of killing (Fig. 17B) can be due to the effect on some processes after protein synthesis like sorting or trafficking of newly synthesized

cytotoxic molecules into releasable lytic granule. However we cannot exclude the possibility of Syntaxin 8 influencing the synthesis of new cytotoxic molecules in CTLs.

To rule out the possibility that CHX affects the initial level of cytotoxic proteins, we checked the levels of cytotoxic proteins in these transfected CTLs. We found no difference in expression levels of perforin and granzyme B in CHX or DMSO treated control and Syntaxin 8 down regulated CTLs (Fig. 17C). The Syntaxin 8 downregulation was also confirmed in these Syntaxin 8 siRNA transfected CTLs (Fig. 17C). Taken together, these findings indicate that Syntaxin 8 might be involved in new protein synthesis but also participates in the sorting and trafficking of cytotoxic proteins to the lytic granules.

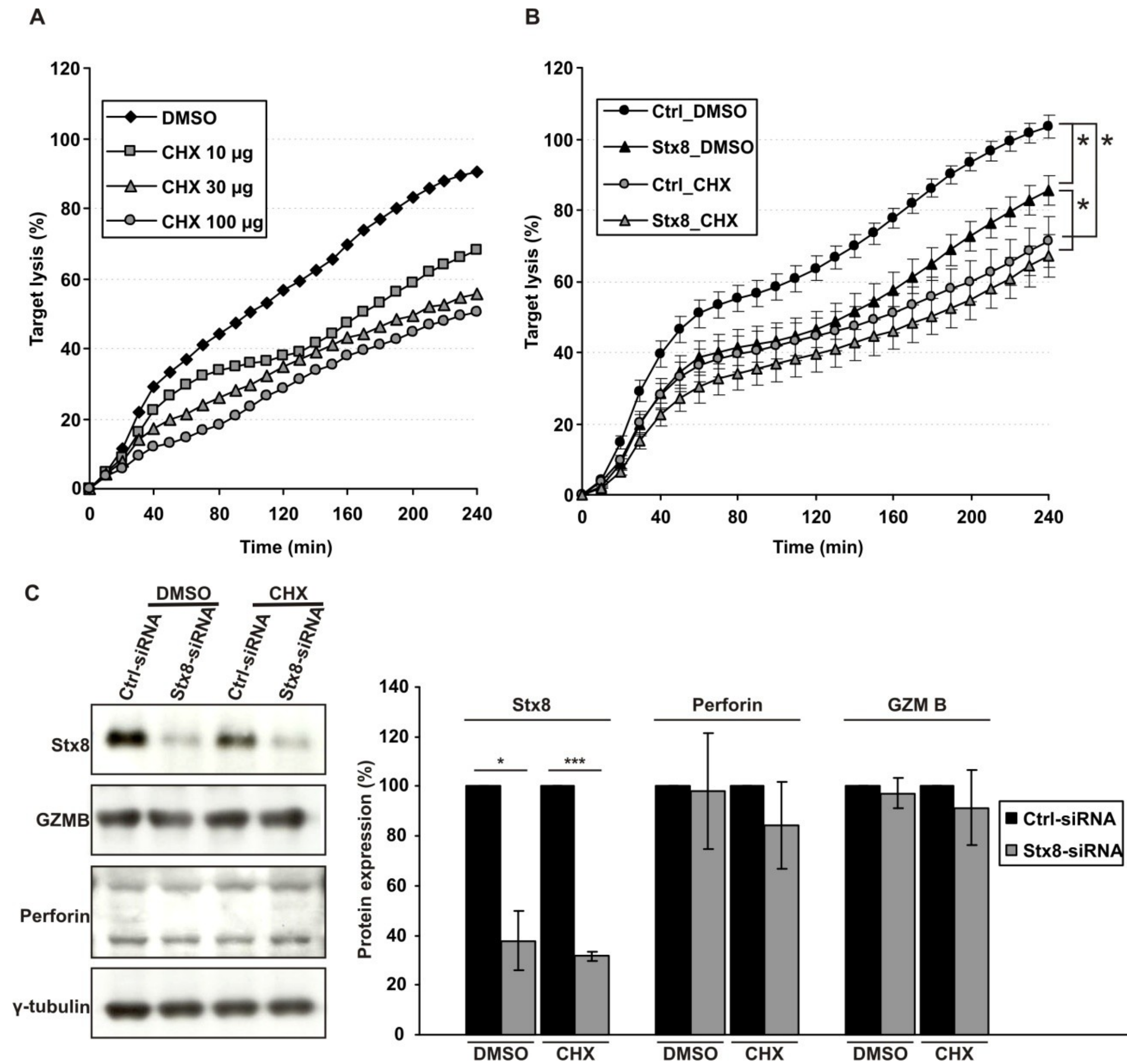

Figure 17: Syntaxin 8 knockdown does not alter the synthesis of cytotoxic molecules.

(A) Different concentrations of cycloheximide (CHX) were tested for dose dependence on cytotoxicity of human CTLs. 10 µg/ml, 30 µg/ml and 100 µg/ml were the concentrations tested. DMSO was used as solvent control. (B) SEA stimulated Ctrl- (control) and Stx8-siRNA (Syntaxin 8 siRNA) CTLs were incubated with CHX (Ctrl-CHX, Stx8-CHX) or DMSO (Ctrl-DMSO, Stx8-DMSO) for 4 hr at 37°C. These CTLs were used in real time killing assay at 20:1 effector to target ratio. n = 9, Mean ± SEM. There was significant reduction of cytotoxicity between Stx8-DMSO and Ctrl-DMSO from 10 min onwards, between Ctrl-DMSO and Ctrl-CHX from 30 min onwards. Even though the cytotoxicity of Stx8-DMSO, Ctrl-DMSO and Stx8-CHX were similar initially, the Stx8-DMSO CTLs started to recover their cytotoxicity and showed significant difference to Stx8-CHX cytotoxicity from 180 min onwards. (C) Western Blot showed downregulation of Stx 8 in both DMSO and CHX treated Stx 8-siRNA CTLs but all these conditions showed similar initial levels of perforin and granzyme B as the DMSO and CHX treated Ctrl-siRNA CTLs. Total number of experiments, n = 3, Mean ± SEM (* P < 0.05, ** P < 0.01 and *** P < 0.001).

3.4.2. Generation of new cytotoxic molecules may be influenced by Syntaxin 8

To further verify if Syntaxin 8 is involved in synthesis of new cytotoxic proteins, we examined after depletion of preexisting lytic granule pool, the change in the expression of total perforin protein by Syntaxin 8 down-regulation. As mentioned above the preexisting lytic granule pool is released by pre-incubation of the transfected CTLs within 96-well plates coated with anti-CD3/anti-CD28 Abs. Then the CTLs were recovered for 6 hr in the full medium at 37°C with 5 % CO_2. Afterwards the CTLs were incubated again with (Ctrl_+Ab, Stx8-+Ab) or without (Ctrl_noAb, Stx8_noAb) anti-CD3/anti-CD28 Ab coating and were collected at different time points for further preparations. Intracellular perforin was stained and the perforin expression was determined with the flow cytometry. IgG2b staining was used as an isotype control. We found that these CTLs showed no significant difference in the perforin levels in control and Syntaxin 8 downregulated CTLs in both conditions, with (Fig. 18B, 18D) and without (Fig. 18A, 18C) release of pre-existing cytotoxic protein pool. Nevertheless we observed a general tendency of lower perforin in Syntaxin 8 downregulated CTLs than in control CTLs in both the conditions (Fig. 18D and 18A, 18C). This indicates that the synthesis of new cytotoxic proteins may be influenced by Syntaxin 8 which needs to be confirmed by further analysis.

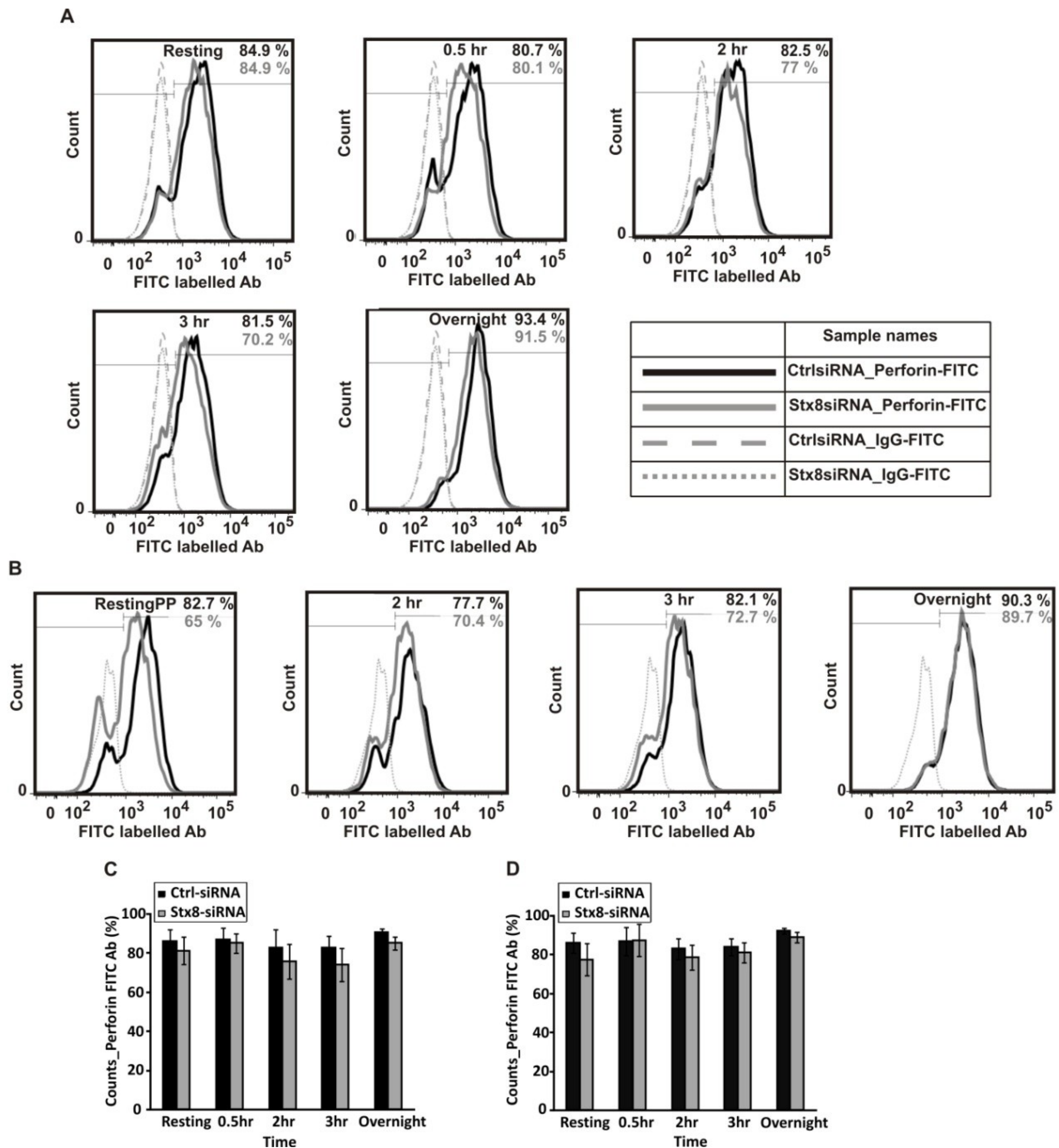

Figure 18: Perforin expression in Syntaxin 8 downregulated CTLs. Control (Ctrl-siRNA) and Syntaxin 8 (Stx8-siRNA) downregulated CTLs were stained for perforin (A) without and (B) with releasing the pre-existing pool of cytotoxic molecules after permeabilizing them.

These CTLs were collected after conjugation with anti-CD3/anti-CD28 Abs (mimicking target cells) at different time points [(A) without (Resting, 0.5 hr, 2 hr, 3 hr and overnight) and (B) with release of preexisting pool (RestingPP, 2 hr, 3 hr and overnight conjugation)]. Perforin was stained with FITC labelled perforin monoclonal Ab and as an isotype control, with FITC Mouse IgG2b Ab (MPC 11 clone). These stained CTLs were then analysed using a FACS analyzer for the internal perforin intensity. (C) The CTLs without releasing the pre-existing pool of cytotoxic molecules and (D) after release of the pre-existing pool showed no significant difference in perforin expression but a tendency of reduced perforin is observed in Syntaxin 8 downregulated CTLs in both conditions.

3.4.3. Syntaxin 8 is required for the regular sorting of cytotoxic molecules into lytic granules

As Syntaxin 8 is mainly localized in late and recycling endosomal compartments (Fig. 5A, 5D and Fig. 6A, 6D) and as it is indicated to influence the post protein synthesis processes according to the CHX treated real time killing assay (Fig. 17B), we proceeded to verify if Syntaxin 8 is involved in endosomal pathway, especially the sorting of cytotoxic molecules into functional lysosomes (lytic granules). We examined the morphology and numbers of lytic granules closely in Syntaxin 8 down-regulated and control CTLs. Lytic granules were labelled with fluorescence conjugated perforin antibody and the total number of perforin vesicles within the CTLs and at the IS were counted at various time points for resting (without target cells) and conjugated CTLs (30 min, 2 hr, 3 hr, 4 hr and overnight conjugation with the target cells) (Fig. 19). The scanned images of these CTLs were partitioned into 3 parts and the 1/3rd part facing the target cells is defined as the vicinity of the IS (Fig. 20A), as described before [45]. We found no significant difference in numbers of perforin granules (Fig. 19, 20C) and no change in perforin accumulation at the IS in control and Syntaxin 8 downregulated CTLs, no matter in resting or in conjugated CTLs (Fig. 19, 20D). However, at 2 hr time point we observed that in Syntaxin 8 downregulated CTLs the perforin vesicles were larger in size compared to the ones in control CTLs (Fig. 19). This was more evident in DMSO treated Syntaxin 8 downregulated CTLs at 2 hr time point, we observed blob like perforin vesicles (Fig. 21). Though this was not found in DMSO treated control CTLs or in CHX treated control and Syntaxin 8 CTLs (Fig. 21). Furthermore the 3D analysis of the perforin vesicles also showed that Syntaxin 8 downregulated CTLs had higher number of larger vesicles, compared to the smaller lytic granules in control CTLs at 2 hr (Fig.

22B) in contrast to this was the resting condition where Syntaxin 8 downregulated CTLs had smaller vesicles (Fig. 22A). This is observed again in the Syntaxin 8 downregulated CTLs that were pre-incubated with DMSO or CHX and conjugated with target cells for 2 hr and stained for perforin. In CHX treated conditions, at both resting and 2 hr conjugation (Fig. 21), we observe lesser number of perforin vesicles (as expected from the CHX treatment) and they appear to be of similar and smaller in size (Fig. 22E, F). Whereas the DMSO treated Syntaxin 8 downregulated CTLs, at 2 hr time point, showed more of large vesicles, almost double the amount found in DMSO treated control CTLs (Fig. 22D). These blob like vesicles were not observed at resting condition (Fig. 21, 22C). This phenomenon of larger blob like lytic granules at 2 hr time point may be due to a sorting problem where the cytotoxic molecules may be stuck at the earlier stages of sorting, thus affecting the sorting and delivery of perforin (from TGN to early endosomes and then to late endosomes and finally) to the lytic granules. This impairment in the sorting of the cytotoxic molecules can slow down all the downstream process like trafficking, docking, release of the lytic granules and hence the cytotoxicity itself causing much slower rate of killing in Syntaxin 8 downregulated CTLs than the control CTLs.

Another possible explanation for the impairment of CTL cytotoxicity by Syntaxin 8 downregulation is that Syntaxin 8 downregulated CTLs cannot form a functional immunological synapse (IS). Impaired accumulation of TCRs at the IS would be an indication for a nonfunctional or defective IS. To examine if the IS formation is effected in Syntaxin 8 downregulated CTLs, we checked for TCR accumulation at the IS of fixed conjugates. TCR was labelled with fluorescent conjugated anti-CD3 antibody. The enrichment of TCR was analysed by the relative fluorescence of CD3 at the IS, namely 1/3rd proximity to the IS (Fig. 20A). No significant change in the

accumulation of TCR at the IS on knockdown of Syntaxin 8 was found at 0.5 hr to 3 hr as well as overnight (OvN) conjugation with target cells (Fig. 19, 20B). It indicates that Syntaxin8 down-regulated CTLs are able to form a functional IS. Though at 4 hr after conjugation with target cells, we observed that the total fluorescence of CD3 measured at the IS in Syntaxin 8 downregulated CTLs increases when compared to control CTLs (Fig. 20B). But when we observed the scanned cells we detected that among the Syntaxin 8 downregulated CTLs, 66.7 % of CTLs had CD3 accumulated at the IS and the rest of 33.3 % of CTLs showed dispersed CD3 at 4 hr time point. Thus in Syntaxin 8 downregulated CTLs, the percentage of CTLs that showed CD3 accumulation at the IS was double than that of CTLs with dispersed CD3 whereas among the control CTLs, percentage of CTLs with CD3 at IS and that with dispersed CD3 were almost similar and were found to be 51.4 % and 48.6 % respectively at 4 hr time point. This can be explained by two hypothesis, one is that, at 4 hr time point, in Syntaxin 8 downreguated CTLs, due to the affected endosomal pathway, the recycling of the TCR is much slower leading to higher CD3 accumulation at the IS than in the control CTLs Another reason could be due to the slower degradation of TCR in these CTLs which is caused by less frequent killing events and conjugate formation by Syntaxin 8 downregulated CTLs that show reduced cytotoxicity. Hence the more active control CTLs degrade their TCR faster than the Syntaxin 8 downregulated CTLs which therefore show higher CD3 at the IS at later time points (Fig. 20B). This data proves that the IS formation is not impaired on Syntaxin 8 downregulation. All these above arguments lead to the conclusion that the formation of a functional IS does not depend on Syntaxin 8 but that Syntaxin 8 is essential for the sorting and trafficking of lytic granules in CTLs. Therefore Syntaxin 8 downregulation in CTLs, impairs and delays the sorting and trafficking of cytotoxic molecules in turn leading

to a slower and reduced killing of target cells. Thus Syntaxin 8 affects CTL cytotoxicity and is significant for CTL function.

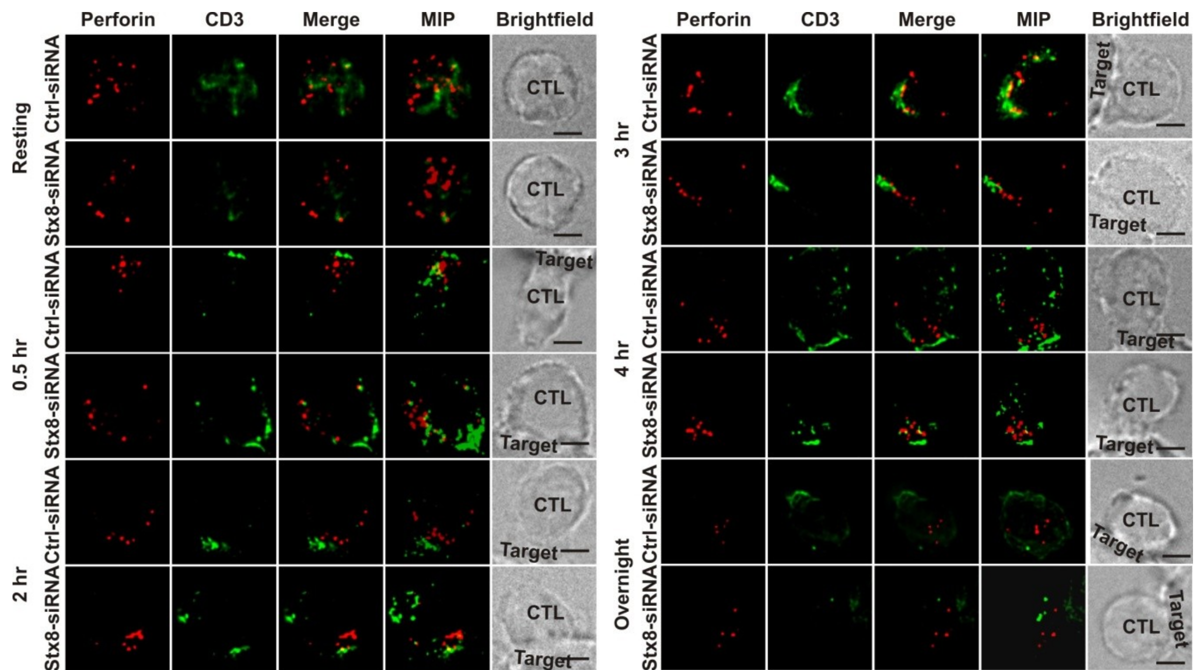

Figure 19: Syntaxin 8 downregulation does not affect normal IS formation but impairs the sorting and trafficking of lytic granules.

SEA stimulated control (Ctrl-siRNA) and Syntaxin 8 (Stx8-siRNA) downregulated CTLs were fixed in resting (without target / Raji cells) and in conjugation with SEA pulsed Raji cells for different time points (30 min or 0.5 hr, 2 hr, 3 hr, 4 hr and overnight) at 37°C and immunostained. TCR labelled with Alexa^{488} conjugated anti-CD3 Ab (green) accumulated well at the IS in Ctrl and Stx8-siRNA CTLs. Lytic granules were labelled with Alexa^{647} conjugated anti-Perforin Ab (red). On 2 hr conjugation with target cells, Stx8-siRNA CTLs showed larger perforin vesicles compared to their control CTLs.

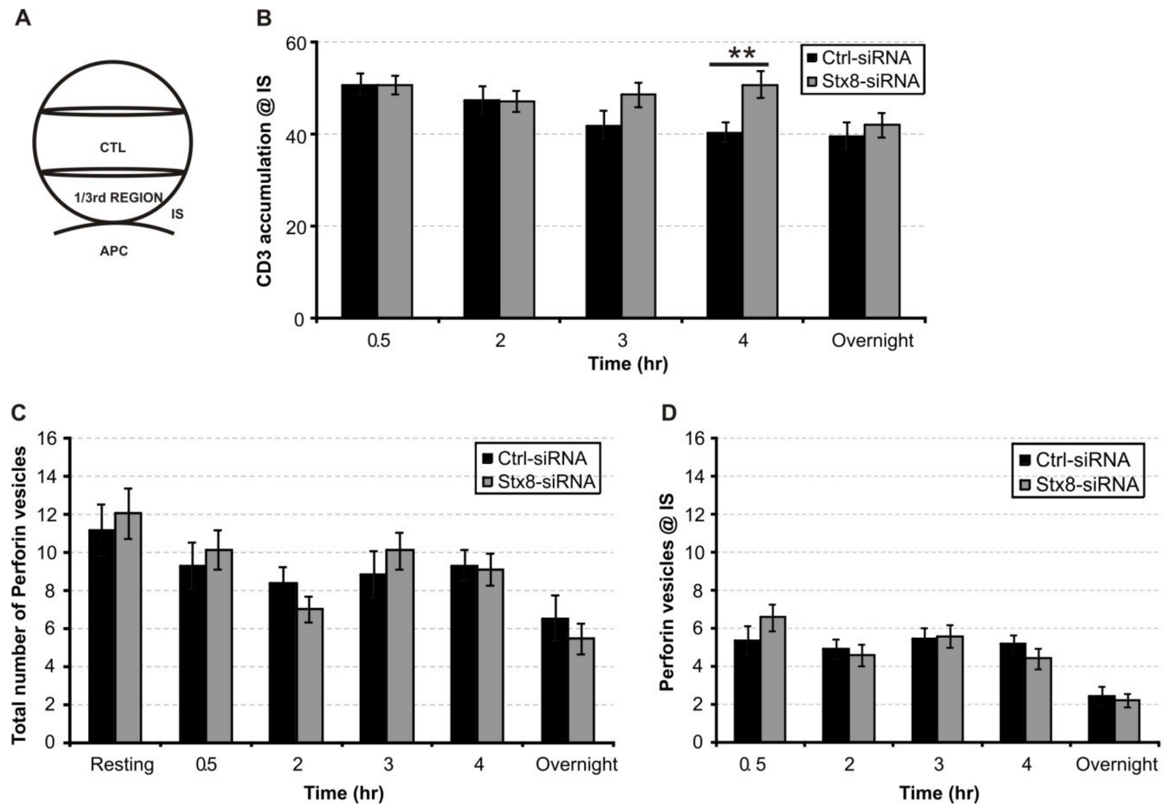

Figure 20: Syntaxin 8 downregulation doesnot impair normal CD3 and lytic granule accumulation at the IS (A) The CTLs were divided into 3 regions and the region closer to the IS and the target cell (the $1/3^{rd}$ region) was analysed for CD3 and perforin accumulation at the IS. (B) The total fluorescence of CD3 accumulated at the $1/3^{rd}$ region near the IS was measured for all the time points in conjugated CTLs. (C) The number of perforin vesicles were counted in resting and conjugated conditions in the complete CTL (total cell) and (D) also at the IS ($1/3^{rd}$ region of the CTL near the IS). Total number of cells, n = 25 to 30 cells for various conditions, Mean ± SEM (p values: * = P < 0.05, ** = P < 0.01 and *** = P < 0.001).

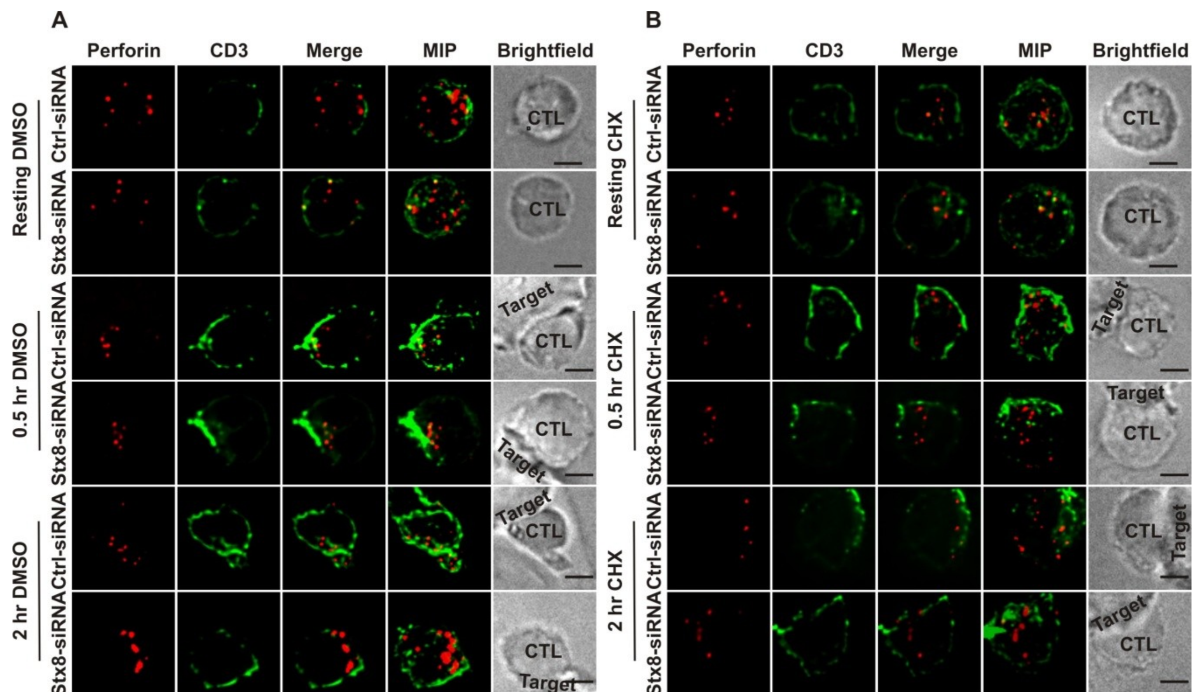

Figure 21: Syntaxin 8 is required for the sorting of the cytotoxic molecules to the lytic granules. SEA stimulated control (Ctrl-siRNA) and Syntaxin 8 (Stx8-siRNA) downregulated CTLs were treated with CHX or DMSO for 4 hr, at 37°C. These CTLs were fixed in resting (without target / Raji cells) and in conjugation with SEA pulsed Raji cells for different time points (30 min or 0.5 hr, 2 hr, 3 hr, 4 hr and overnight) at 37°C and immunostained. TCR labelled with Alexa^{488} conjugated anti-CD3 Ab (green) accumulated well at the IS in Ctrl and Stx8-siRNA CTLs. Lytic granules were labelled with Alexa^{647} conjugated anti-Perforin Ab (red). On 2 hr conjugation with target cells, Stx8-siRNA CTLs treated with DMSO showed larger perforin vesicles compared to their control CTLs.

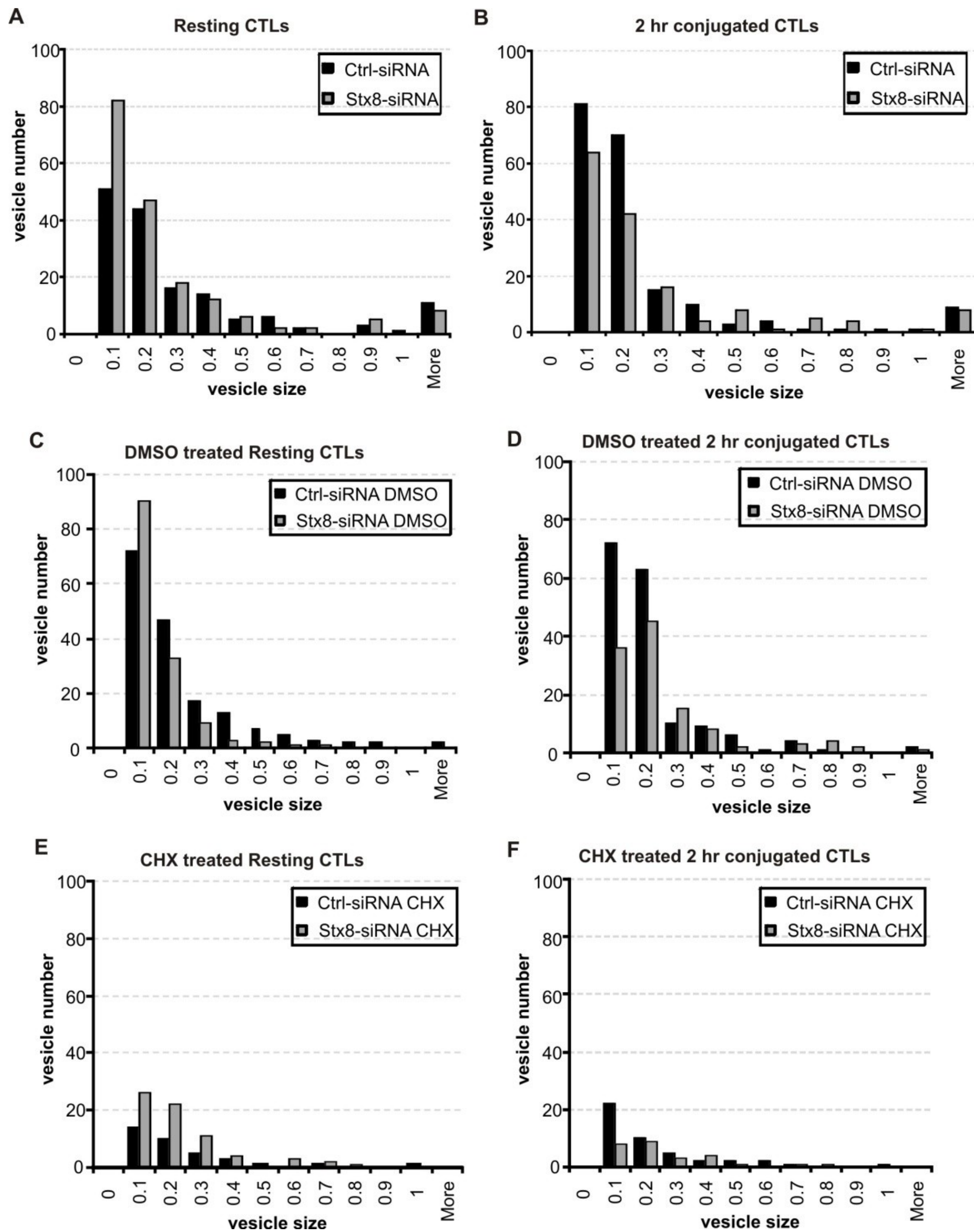

Figure 22: Syntaxin 8 downregulation impairs the sorting and trafficking of lytic granules.
SEA stimulated control (Ctrl-siRNA) and Syntaxin 8 (Stx8-siRNA) downregulated CTLs were fixed in resting (without target / Raji cells) and in conjugation with SEA pulsed Raji cells for 2 hr at 37°C and immunostained. Lytic granules were labelled with Alexa^{647} conjugated anti-Perforin Ab (red). (A) The 3D volume of the vesicles present in the control and Stx8-siRNA in resting (B) at 2 hr conjugation with target cells. (C) Perforin vesicle size measured in Ctrl-siRNA and Stx8-siRNA CTLs treated with DMSO in resting condition and (D) at 2 hr of conjugation with target cells. (E) The same was analsed for Ctrl-siRNA and Stx8-siRNA CTLs that are treated with CHX (Cycloheximide) during resting and (F) at 2 hr of conjugation with target cells. Total number of cells, n = 5 to 10 cells for various conditions.

4. DISCUSSION

In the current study, we have investigated the significance of Syntaxin 8 in the human CTL cytotoxicity. Our data show that Syntaxin 8 is prominently localized in the late and recycling endosomes, partially colocalizes with lytic granules and Vti1b. Downregulation of Syntaxin 8 resulted in reduced CTL cytotoxicity. Syntaxin 8 is not involved in the formation of a functional IS, whereas possibly play a role in the endosomal trafficking, especially sorting of cytotoxic molecules like perforin into lytic granules. These findings reveal previously unknown function of Syntaxin 8 in CTLs and further broaden our knowledge on the regulation of CTL cytotoxicity by SNARE proteins. In this study, some points remains to be further investigated and several new questions emerged, which will be discussed in detail below.

4.1. Syntaxin 8: an important Qc-SNARE in human CTL

The screening in CTLs has confirmed the expression of different Qa-, Qb-, Qc- and R-SNAREs in primary human CTLs [1]. In the present study, we confirm that in human CTLs, Syntaxin 8 accumulates at the IS and that it partially colocalizes with functionally significant proteins like CD3 and perforin (Fig. 4A & 4B). We also demonstrate that Syntaxin 8 partially colocalizes with Vti1b (Fig. 4C), a Qb- SNARE which is required for the tethering of lytic granules to the TCR and thereby docking and releasing the lytic granules at the IS. Previous studies have described the association of Syntaxin 8 with a Qa- SNARE Syntaxin 7 which is required for the accumulation of CD3 at the IS [98]. We postulate that like in the neuronal cell, Syntaxin 8 may act as Qc-motif interacting partner for the Qa- Syntaxin 7 in CTLs.

4.2. Syntaxin 8 as a player in the endosomal vesicle trafficking pathway

Earlier studies on Syntaxin 8 protein in different cell types other than human CTLs, have shown its involvement at different stages in the endosomal membrane trafficking pathway. Syntaxin 8 has been show to be localized in TGN, early endosome and late endosomal compartments in NRK, PC12, Cos7, CHO cells [105,106,110]. In this study, we showed that the Syntaxin 8 is localized predominantly in the late endosomes (Fig. 5A, 6A) and recycling endosomes (Fig. 5D, 6D) in human CTLs both in the presence and absence of target cells. We also observed some colocalization between lysosomes and Syntaxin 8 (Fig. 5C, 6C). Syntaxin 8 even colocalized with early endosomal compartments again in the presence and absence of target cells (Fig. 5B, 6B), though the colocalization was not as high as in late endosomes. The localization of Syntaxin 8 in these endosomal compartments indicates that it might participate in the sorting and trafficking of receptors like TCR, FasL and proteins like perforin, granzymes and other proteases and hydrolases of the lytic granules. The regulated secretory pathway is subdivided into direct and indirect pathways. Proteins with two di-leucine motifs participate in both direct and indirect pathways [108]. Syntaxin 8 has been reported to comprise two functionally distinct di-leucine motifs that can independently function, suggesting that Syntaxin 8 could participate in both direct and indirect pathways [109]. Syntaxin 8 can be internalized from the plasma membrane by the indirect pathway or rather than being transported to the plasma membrane, Syntaxin 8 can be involved in the direct pathway by being delivered from the TGN directly to endosomal / lysosomal populations and then recycled to endosomes by the plasma membrane endosome route [109]. This can be the reason for Syntaxin 8 localization in early and late endosomal and recycling compartments in human CTLs. This is supported by the studies showing the localization of Syntaxin 8 (in murine cells and cell lines) in early

endosomal [105], late endosomal compartments [110] and also in TGN. The significantly higher localization in late and recycling endosomes in CTLs can be an indication of the role of Syntaxin 8 in sorting of perforin, granzymes and other proteins to the lytic granules through this pathway. What needs to be further studied is the exact role the Syntaxin 8 plays in this pathway. Is it responsible for synthesis or transport or recycling of lytic granules, CD3 or any other receptors or molecules essential for CTL cytotoxicity. We attempt to investigate this further in this study.

4.3. Syntaxin 8 influences cytotoxicity elicited by CTLs

The siRNA silencing approach facilitated us to efficiently downregulate Syntaxin 8 (Fig. 7, 8A) in primary human CTLs thus enabling us to analyze its functional significance in CTLs. All the experiments were conducted 36 hr after transfection as Syntaxin 8 was downregulated at both mRNA and protein levels (Fig. 7C, 7D & 7E) and also retaining the effector function of CTLs during this time point.

Real time killing assay using Calcein dye (Kummerow, C. et al, unpublished) was the functional assay used here to measure the CTL cytotoxicity. All the cytotoxicity assays shown in this study were conducted with CTLs in AIMV media with 10 % FCS except for assays with Ringer solution (Fig. 10) and figures 9B, 11, 12B & 16. The reason for this is, the CTLs used in our lab are cultured in AIMV media with 10 % FCS. To keep the CTLs healthy and comfortable we used this media containing 10 % FCS, even in the cytotoxicity assays. This worked successfully initially, but with the later batches of FCS, we observed that FCS somehow influenced the cytotoxicity causing lower efficiency in target lysis. Therefore we tested this assay with AIMV media without FCS (Fig. 9B) which showed efficient target lysis. Hence all the further cytotoxic assays were conducted using AIMV media without FCS (Fig. 11 &

16). The calcium dependency of CTLs for their cytotoxicity was shown by using Ringer solutions with different calcium concentrations (Fig. 10). Different concentrations of perforin inhibitor conconamycin A (CMA) were used to inhibit perforin in CTLs. Cytotoxicity assays conducted with such perforin inhibited CTLs showed the dose dependency of the inhibitor and indicated that this cytotoxicity assay is more sensitive to perforin mediated killing as the cytotoxicty could be inhibited by even a very low concentration (0.5 nM) of CMA. With concentrations lower than 0.5 nM like 0.25 nM and 0.05 nM, the cytotoxicity is not completely inhibited but was reduced compared to the DMSO control. This can be due to either incomplete inhibition of all the perforin in CTLs or cyctotoxicity by another pathway called the FAS ligand pathway. Thus we conclude that this real time calcein cytotoxicty assay is a very sensitive assay which can be used to measure cytotoxicity that is mostly mediated by the perforin pathway.

Measurement of cytotoxicity of Syntaxin 8 downregulated CTLs by this method showed a reduction in cytotoxicity in comparison to the control CTLs (Fig. 12A & 12B). A significant reduction in the cytotoxicity of Syntaxin 8 downregulated CTLs was observed from 150 min onwards. This demonstrates that Syntaxin 8 influences CTL cytotoxicity. But why do we see a significant reduction only at a later time points? One of the reasons for this is that we use SEA stimulated CTLs for transfection of the Syntaxin 8 and control siRNAs. As these cells are already activated before transfection, both control and Syntaxin 8 downregulated CTLs have similar amounts of readily releasable, pre-existing pools of cytotoxic molecules (lytic granules). Therefore we hypothesized a more significant downregulation in the cytotoxicity of Syntaxin 8 downregulated CTLs, only after the preexisting pool of lytic granules is used up. To confirm it, we emptied the preexisting lytic granule pool

in both control and Syntaxin 8 downregulated CTLs (after transfection) by stimulating them with anti-CD3 / anti-CD28 Ab, which causes exocytosis of the lytic granules. After allowing them to recover, these CTLs were measured for their cytotoxicity with respect to control and Syntaxin 8 downregulated CTLs that were not emptied of their preexisting lytic granule pool. We observed that there is an increase in the difference in cytotoxicity of control and Syntaxin 8 downregulated CTLs during the initial time points until 100 min but during the later time points the difference in cytotoxicity of antibody treated Syntaxin 8 and control CTLs is similar to that of antibody untreated CTLs (Fig. 16A & 16B). The reduction in cytotoxicity of Syntaxin 8 downregulated CTLs compared to control CTLs established that Syntaxin 8 is crucial for human CTL cytotoxicity.

This is supported by ELISA detection of perforin released by control and Syntaxin 8 downregulated CTLs that were emptied of their preexisting lytic granule pool by anti-CD3 / anti-CD28 Ab stimulation. Though the perforin released initially at 8 hr by Syntaxin 8 downregulated CTLs was not significantly different than that released by control CTLs, from later time points of 16 hr to 48 hr, perforin released by Syntaxin 8 was significantly lower than the control CTLs. From this, one can deduce that the exocytosis of lytic granules maybe impaired in Syntaxin 8 downregulated CTLs. But we hold back this theory as the control and Syntaxin 8 downregulated CTLs which are not emptied of their preexisting perforin pool (in both conditions where we used Raji cells as target cells and CD3 / CD28 activator beads as target cells) showed no significant difference in their perforin release till 48 hr. Thus the final exocytosis itself is not deterred but some upstream processes prior to the exocytosis are affected by Syntaxin 8 downregulation. We cannot check further time points than 48hr, as the

Syntaxin 8 siRNA is stable only till this point where it starts to generate the Syntaxin 8 protein eventually.

When one observes the cytotoxicity assay data (measured for 4 hr) and the ELISA data (measured from 0 hr to 48 hr), there is a difference in the results of the two assays. As for the cytotoxicity assay, a difference in the cytotoxicty between Syntaxin 8 downregulated and control CTLs can be observed within the 4 hr (Fig. 12), whereas for ELISA we donot observe a significant difference till 16 hr (Fig. 15) or till 48 hr (Fig. 14). For Fig. 15, ELISA assay, the explanation is that as the pre-existing perforin pool is emptied, the perforin levels at the beginning of the assay are very low for both Syntaxin 8 downregulated and control CTLs making it very sensitive for the assay to pick up the difference. So we can only see a significant difference from the next time points, which is 16 hr onwards.

The difference in the results for cytotoxicity assay (Fig. 12) and ELISA assay for CTLs without the depletion of pre-existing perforin pool (Fig. 15), might be also due to the higher CTL to target ratio used in cytotoxicity assays (0.6 x 10^6 CTLs to 0.3 x 10^4 target cells) as opposed to the lower ratio in the ELISA assays (0.1 x 10^6 CTLs to 0.1 x 10^6 targets) which makes the chance of conjugation with the targets less frequent than in the much faster cytotoxicity assay. One must also remember that in both the assays (Fig. 12 and Fig. 15) the preexisting cytotoxic molecule pool is not emptied, thus in ELISA, due to less frequent conjugate formation, releasable perforin level maybe similar in both Syntaxin 8 downregulated and control CTLs and hence the difference between the perforin released by both is not significant. Whereas in the cytotoxicity assay, as the conjugates formed is more frequent, the release of the pre-existing perforin is much faster, making the CTLs dependent on newly synthesized and sorted cytotoxic molecules. Thus in the cytotoxicity assay, we can observe a

difference between the Syntaxin 8 downregulated and control CTLs within 4 hr (Fig. 12).

4.4. Generation and sorting of cytotoxic molecules to functional lytic granules require Syntaxin 8

We started by examining if Syntaxin 8 downregulation affected the synthesis of new cytotoxic molecules, as any impairment in the protein synthesis would result in reduced amount of cytotoxic molecules causing reduction in cytotoxicity of the CTLs [25]. Control and Syntaxin 8 downregulated CTLs that were incubated with a protein translocation inhibitor called cycloheximide (CHX) [135] were measured for cytotoxicity by the real time calcein cytotoxicity assay (Fig. 17B). All these CTLs were observed to have similar initial levels of perforin and granzymes (Fig. 17C & 17D). This is due to the pre-stimulation and activation of both control and Syntaxin 8 downregulated CTLs by SEA, which leads to the generation of a pre-existing pool of cytotoxic molecules. CHX treated control and Syntaxin 8 downregulated CTLs, where the new protein synthesis was blocked; showed significantly reduced cytotoxicity compared to DMSO treated control CTLs, which have normal protein synthesis machinery. DMSO treated Syntaxin 8 downregulated CTLs also showed significantly reduced cytotoxicity compared to the DMSO treated control cells. The reduction in cytotoxicity of DMSO treated Syntaxin 8 downregulated CTLs, CHX treated control and Syntaxin 8 downregulated CTLs were similar in the initial phase of killing but from three hours onwards, the DMSO treated Syntaxin 8 downregulated CTLs started to regain their cytotoxicity as we see a significant increase in cytotoxicity of DMSO treated Syntaxin 8 downregulated CTLs compared to that of CHX treated Syntaxin 8 downregulated CTLs. This recovery in the cytotoxicity of DMSO treated Syntaxin 8

downregulated CTLs in the later phases may suggest that some other upstream processes to protein synthesis, such as, sorting and transport of the cytotoxic proteins or exocytosis of the lytic granules is affected which causes a delay in the overall process of trafficking thus leading to delayed and reduced cytotoxicity. This experiment however does not exclude the possibility that Syntaxin 8 may affect the synthesis of new cytotoxic proteins, because in Syntaxin 8 DMSO CTLs, due to low amount of Syntaxin 8 proteins (by siRNA transfection) in the beginning of the assay causes the reduction in cytotoxicity similar to that in CHX treated control and Syntaxin 8 downregulated CTLs. But as the protein synthesis is not inhibited in the DMSO treated Syntaxin 8 CTLs, some new Syntaxin 8 protein maybe synthesized which may aid in the synthesis of new cytotoxic proteins leading to recovery of its cytotoxicity to some extent (Fig. 17B), though this is a little farfetched for Syntaxin 8 protein level in DMSO treated Syntaxin 8 siRNA transfected CTLs to increase within 4 hr. This is not possible in CHX treated control and Syntaxin 8 CTLs where protein synthesis is inhibited, thus maintaining the low Syntaxin 8 protein level in Syntaxin 8 CHX, eliminating the possibility of proposed Syntaxin 8 influenced synthesis of new cytotoxic molecules in CHX treated Syntaxin 8 CTLs. This indicates that Syntaxin 8 may be involved in the synthesis of cytotoxic molecules. This was further supported by intracellular staining of perforin in control and Syntaxin 8 downregulated CTLs with and without releasing their pre-existing pool of cytotoxic molecules. FACS analysis showed a slightly lower perforin in Syntaxin 8 downregulated CTLs than in control CTLs at 0.5 hr in one of the three donors (Fig. 17A) but there was no significant difference in the perforin levels in control and Syntaxin 8 downregulated CTLs in both the conditions that is, with (Fig. 18B, 18D) and without (Fig. 18A, 18C) releasing the pre-existing cytotoxic molecule pool. However we see a tendency of

lesser perforin in Syntaxin 8 downregulated CTLs than in control CTLs in both the conditions. This indicates that the synthesis of new cytotoxic molecules maybe influenced by Syntaxin 8, though this needs to be further elucidated in more donors, as the above conclusion is from the results obtained by three donors only.

The proper sorting and trafficking of cytotoxic molecules into mature releasable lytic granules is crucial for CTL cytotoxicity. The signal peptides bound to these cytotoxic proteins mark them for sorting into the secretory lysosomes (lytic granules) [47]. To detect if Syntaxin 8 played a role in the vesicle sorting pathway; control and Syntaxin 8 downregulated CTLs were fixed and stained for perforin and CD3 in both resting (no targets) and conjugated (with targets) conditions (Fig. 19) and the fluorescence of CD3 at the IS (Fig. 20B) and perforin in total cell area and also at the IS were quantified (Fig. 20C, 20D). We did not observe any significant difference in total perforin and also perforin vesicles accumulated at the IS between control and Syntaxin 8 downregulated CTLs in resting and conjugated conditions (0.5 hr, 2 hr, 3 hr, 4 hr and overnight time points) (Fig. 20D & 20E). However at 2 hr time point the perforin vesicles were larger in size in Syntaxin 8 downregulated CTLs compared to that in control CTLs (Fig. 19). The control and Syntaxin 8 downregulated CTLs that were treated with CHX showed similar vesicle sizes for resting and 2 hr conjugation (Fig.. 21). A prominent difference in size was observed in DMSO treated Syntaxin 8 downregulated CTLs that showed to have larger perforin vesicles that looked like blobs than in DMSO treated control CTLs at 2 hr (Fig. 21). Also the 3D volume analysis of perforin vesicles in these resting and 2 hr conjugated CTLs showed that at 2 hr time point, Syntaxin 8 downregulated CTLs had higher number of larger perforin vesicles compared to the smaller vesicles in control CTLs (Fig. 22B, 22D). This indicates a sorting problem wherein perforin (from TGN) may not be properly sorted

through early and late endosomal compartments to the lytic granules. This impairment in the sorting of perforin can rate limit all the downstream process like trafficking, docking, release of the lytic granules, thus affecting the cytotoxicity itself by causing much slower rate of killing in Syntaxin 8 downregulated CTLs than the control CTLs. This causes the control CTLs to be more active than the Syntaxin 8 downregulated CTLs. They conjugate with and kill more targets by lytic granule secretion than the Syntaxin 8 downregulated CTLs thus leading to the higher cytotoxicity than Syntaxin 8 downregulated CTLs.

TCR polarization and the accumulation of TCR at the IS is very crucial for the formation of a functional IS [136]. It has been reported that Vti1b downregulated CTLs that showed impaired cytotoxicity did not show any impairment in MTOC and Golgi polarization to the IS [45] (Fig. 23). This and the other important feature of normal CD3 accumulation at the IS proved the formation of a functional IS by Vti1b downregulated CTLs [45].

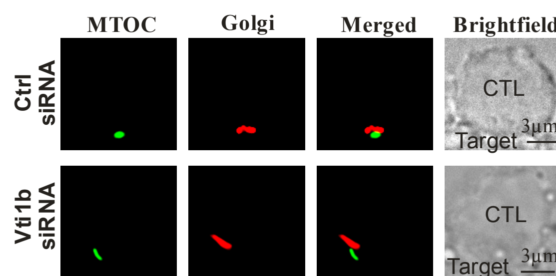

Figure 23: MTOC and Golgi polarization not impaired on Vti1b downregulation

The polarization machinery is unaltered in Vti1b downregulated CTLs despite their impaired cytotoxicity. MTOC was labelled with γ-tubulin GFP and trans-Golgi was labelled with GM 130 mouse monoclonal primary antibody and Alexa[568] conjugated secondary goat anti-rabbit Ab (red). Scale bar = 3 µm.

As we observed reduction in CTL cytotoxicity in Syntaxin 8 downregulated CTLs, we examined if the TCR (CD3) accumulation was altered in Syntaxin 8 downregulated

CTLs. Similar accumulation of CD3 at the IS was observed in Syntaxin 8 downregulated and control CTL during initial time points of 0.5 hr to 3 hr conjugation with targets. However at 4 hr, CD3 accumulation at the IS was higher in Syntaxin 8 downregulated CTLs than in controls (Fig. 20, 21B). On overnight conjugation, CD3 accumulation was again similar and limited in both Syntaxin 8 and control CTLs (Fig. 21B). One should note that at 4 hr not only was the CD3 accumulation more in Syntaxin 8 downregulated CTLs but the percentage of Syntaxin 8 downregulated CTLs with CD3 accumulated at the IS was double the percentage of cells with CD3 distributed throughout the cell. That is, we observed 66.7 % of Syntaxin 8 downregulated CTLs with CD3 at the IS and 33.3 % of them with dispersed CD3, This was not the case in control CTLs were the percentage of cells with CD3 at the IS (51.4 % CTLs) and that of cells with overall distributed CD3 (48.6 % CTLs) were almost similar. This shows that even though there is CD3 throughout the area of CTLs in both Syntaxin 8 and control CTLs, the accumulation at the IS is significantly higher in Syntaxin 8 downregulated CTLs than in control CTLs at 4 hr conjugation (Fig. 20B). The Syntaxin 8 downregulated CTLs showed higher CD3 accumulation at 4 hr than in control CTLs because Syntaxin 8 that is localized in early endosomes and mostly in the late endosomes and recycling endosomes, when downregulated, could limit the recycling of TCR from the IS to the early, then to late endosomes and finally either back to the plasma membrane or to lysosomes for degradation. Thus retaining more CD3 at the IS than the control CTLs at 4 hr time point. It is also possible that the impairment in sorting of perforin to the lytic granules causes the reduced cytotoxicity of Syntaxin 8 downregulated CTLs due to slower and lesser release of perforin compared to the control CTLs. Hence the more active control CTLs might degrade their TCR faster than the much slower Syntaxin 8 downregulated CTLs which could

in turn show higher CD3 at the IS at later time points. But on overnight conjugation, the Syntaxin 8 downregulated CTLs start recovering their Syntaxin 8 protein leading to faster recycling or degrading of the TCR. Another reason could be that the active control CTLs start generating and expressing new CD3 whereas the CD3 in Syntaxin 8 downregulated CTLs start recycling and also degrading thus showing similar CD3 accumulation at the IS as the control CTLs for overnight time point. Thus we could show that Syntaxin 8 downregulation does not impair the IS formation but could affect the CD3 endocytic pathway and by the likely participation of Syntaxin 8 in the sorting and trafficking of perforin, its downregulation leads to the delay and thus impairment of CTL cytotoxicity.

5. OUTLOOK

Our study proved that Syntaxin 8 is essential for CTL cytotoxicty and that it is involved in the endosomal pathway by influencing the sorting and trafficking of cytotoxic molecules like perforin to the lytic granules. It might also affect the generation of cytotoxic molecules, though this needs to be confirmed by further analysis. The actual stage in the endosomal trafficking pathway that the Syntaxin 8 is involved can be examined further. One can use electron microscopy to identify the endosomal compartment the perforin vesicles or lytic granules are trapped in at 2 hr conjugation of CTLs with target cells. Also as Syntaxin 8 siRNA only downregulates the protein, one can get a more efficient phenotype in a knockout mice which may give us more insights into the Syntaxin 8 function in CTLs. Syntaxin 8 has been reported to be one of the interacting partners in the SNARE complex formed by Vti1b, Syntaxin 7 and VAMP7 in cells other than CTLs like neuronal and NRK cells [111-113]. It has been shown that in human CTLs, Syntaxin 8 co-immunoprecipitates with Syntaxin 7 [98] suggesting that they may be interacting partners. It would be very interesting to determine which Qa-, Qb- and R- SNAREs interact with Syntaxin 8 if forming a SNARE complex in human CTLs. It would also be useful to examine if Syntaxin 8 interacted with or compensated for any proteins involved in the functioning of CTLs.

6. SUMMARY

By the results observed and discussed we can conclude that Syntaxin 8 is significant for the cytotoxicity in human CTLs. On efficiently downregulating Syntaxin 8 in human CTLs we observed that they influence cytotoxicity not by affecting the exocytosis itself but by affecting some stages prior to exocytosis. The reason for this could be due to the effect in generation and more importantly by delay in the sorting and trafficking of cytotoxic molecules into readily releasable lytic granules to the IS of the CTLs. It is indicated that Syntaxin 8 participates in the endosomal trafficking pathway as it colocalizes with early (partial), late, recycling and lysosomal compartments but predominantly with late and recycling endosomes. On 2 hr conjugation with target cells, Syntaxin 8 downregulated CTLs showed larger blob like perforin vesicles than control CTLs, indicating that perforin might be trapped in the early phase of trafficking probably in the trans-Gogli. This delay in sorting and trafficking in Syntaxin 8 downregulated CTLs, may cause the lytic granules to reach the IS and in turn get released at the IS very slowly compared to that in control CTLs. This also affects the CD3 (TCR) accumulation at the IS, at later time points. Thus the slower cytotoxic machinery in the Syntaxin 8 downregulated CTLs may lead to slower degradation of TCRs than in control CTLs, at longer time points. Also the recycling of TCR to the plasma membrane maybe slower in Syntaxin 8 downregulated CTLs, though the IS formation is not impaired in Syntaxin 8 downregulated CTLs. Thus Syntaxin 8 downregulated CTLs form normal conjugates with the targets but have delayed sorting and trafficking of cytotoxic molecules into readily releasable lytic granules, affecting the CTL cytotoxicity.

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8. PUBLICATIONS

1) Docking of lytic granules at the immunological synapse in human CTL requires Vti1b-dependent pairing with CD3 endosomes.

   Qu B*, Pattu V*, Junker C, Schwarz EC, **Bhat SS**, Kummerow C, Marshall M, Matti U, Neumann F, Pfreundschuh M, Becherer U, Rieger H, Rettig J, Hoth M.

   J Immunol. 2011 Jun 15;186(12):6894-904. PMID: 21562157

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2) SNARE protein expression and localization in human cytotoxic T lymphocytes.

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10. CURRICULUM VITAE

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