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Role of Syntaxin-11 and Munc18-2 in lymphocyte cytotoxic granule exocytosis

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Role of Syntaxin-11 and Munc18-2 in lymphocyte cytotoxic granule exocytosis

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To my family

ABSTRACT

Mutations in genes required for the exocytosis of cytotoxic granules by NK cells and cytotoxic T lymphocytes are associated with early-onset familial hemophagocytic lymphohistiocytosis (FHL). In this project, we examined how certain missense mutations in genes encoding syntaxin-11 and Munc18-2 abolish exocytosis and cause disease. Furthermore, we dissected the role of another protein suspected to play a role in cytotoxic granule exocytosis, VAMP8.

In the first study, we characterized an FHL5 patient (Munc18-2 p.Q432X and p.S545L) who developed Hodgkin lymphoma with underlying Epstein-Barr virus (EBV) infection. The tissue displayed high infiltrates of cytotoxic T lymphocytes responsive to EBV-derived peptides, yet the cells had dramatically reduced Munc18-2 protein levels and were unable to undergo cytotoxic granule exocytosis. The patient is an important example of how Munc18-2 missense mutations impairing exocytosis can lead to late-onset HLH and, due to decreased immunosurveillance, might result in cancer.

In the second study, we examined the pathophysiological mechanism underlying an N-terminal syntaxin-11 mutation (syntaxin-11 p.L58P) associated with FHL4 in three unrelated patients. These patients displayed defective cytotoxic granule exocytosis despite the functionally important SNARE domain of syntaxin-11 being intact. As the p.L58 is an evolutionary conserved amino acid, we hypothesized that the mutation interrupts interactions with Munc18-2. Further, we determined whether other conserved N-terminal syntaxin-11 residues also contribute to Munc18-2 binding. We found that the interaction is dependent on both an intact syntaxin-11 N-terminal peptide as well as Habc domain because mutations in either completely abolished binding to Munc18-2. It is thus plausible that syntaxin-11, analogous to the syntaxin-1 / Munc18-1 interaction, employs distinct binding modes to different domains of Munc18-2. The interruption of the syntaxin-11 / Munc18-2 interaction could explain the reduced syntaxin-11 expression levels.

Lastly, cytotoxic granule exocytosis is dependent on several proteins being part of the fusion complex between a cytotoxic granule and plasma membrane, yet the v-SNARE mediating granule fusion remains elusive. In the third study, we suspected VAMP8 to play a key role in this fusion process. However, VAMP8 did not localize to cytotoxic granules but instead to recycling endosomes. Upon T cell receptor stimulation, recycling endosomes were recruited to the immune synapse and fused with the plasma membrane. As VAMP8 knockdown blocked cytotoxic granule release, we hypothesized that VAMP8+ recycling endosomes might deliver an important component for the cytotoxic fusion machinery to the plasma membrane. Indeed, recycling endosomes transported syntaxin-11 to the plasma membrane, with VAMP8 knockdown hampering syntaxin-11 delivery.

In summary, these data provide a deeper understanding of FHL and the molecular mechanisms of cytotoxic granule exocytosis. We describe a possible link between FHL and cancer which may have diagnostic, prognostic and treatment implications for other FHL patients. Further, we show how N-terminal syntaxin-11 mutations can cause disease, with data hinting towards a meticulous series of interaction modes necessary for syntaxin-11 maintenance and cytotoxic granule secretion. Finally, we find that VAMP8 delivers syntaxin-11 to the immunological synapse in human cytotoxic lymphocytes, demonstrating an unexpected role of recycling endosomes in cytotoxic granule exocytosis.

LIST OF SCIENTIFIC PAPERS

I. **Development of classical Hodgkin's lymphoma in an adult with biallelic STXBP2 mutations.**

Machaczka M, Klimkowska M, Chiang SC, Meeths M, **Müller ML**, Gustafsson B, Henter JI, Bryceson YT.

Haematologica. 2013 May;98(5):760-4

II. **An N-terminal missense mutation in STX11 causative of FHL4 abrogates syntaxin-11 binding to Munc18-2.**

Müller ML, Chiang SC, Meeths M, Tesi B, Entesarian M, Nilsson D, Wood SM, Nordenskjöld M, Henter JI, Naqvi A, Bryceson YT.

Front Immunol. 2014 Jan 14;4:515

III. **VAMP8-dependent fusion of recycling endosomes with the plasma membrane facilitates T lymphocyte cytotoxicity.**

Marshall MR, Pattu V, Halimani M, Maier-Peuschel M, **Müller ML**, Becherer U, Hong W, Hoth M, Tschernig T, Bryceson YT, Rettig J.

J Cell Biol. 2015 Jul 6;210(1):135-51

CONTENTS

LIST OF ABBREVIATIONS

AID	Activation-induced deaminase
AP3	Adaptor protein 3
APC	Antigen-presenting cell
APC *(protein)*	Adenomatous polyposis coli *(protein)*
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BCR	B cell receptor
BZLF-1	BZLF1 [Human herpesvirus 4]
C. elegans	*Caenorhabditis elegans*
CD	Cluster of differentiation
Cdc42	Cell division control protein 42 homolog
CLIP-170	Cytoplasmic linker protein, CLIP-170
CRACC	CD2-like receptor activating cytotoxic cells
CRISPR / Cas	Clustered regularly interspaced short palindromic repeats / CRISPR-associated
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DNAM-1	DNAX accessory molecule 1
DOCK8	Dedicator of cytokinesis protein 8
EBERs	Epstein-Barr virus (EBV)-encoded small RNAs
EBV	Epstein-Barr virus
ER	Endoplasmatic reticulum
ERK	Extracellular signal–regulated kinase
FRET	Förster resonance energy transfer
GATA-2	GATA binding protein 2
Grb2	Growth factor receptor bound protein 2
GTPase	Guanosine triphosphate hydrolase
HA tag	Human influenza hemagglutinin tag
HCMV	Human cytomegalovirus
HEK293 cell	Human embryonic kindney cell *(The number 293 is derived from the experiment number of the scientist generating them)*
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

ITAM	Immunoreceptor tyrosine-based activation motif
KIR	Killer cell Immunoglobulin-like Receptor
KO	Knockout
LAT	Linker for Activation of T cells
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
Lyst	Lysosomal trafficking regulator protein
MAP(K)	Mitogen-activated protein (kinase)
MCMV	Mouse cytomegalovirus
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class I polypeptide‑related sequence
MTOC	Mictrotubule organizing centre
mTOR	Mechanistic target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor-[kappa] B
NHL	Non-Hodgkin lymphoma
NK cell	Natural killer cell
NKG2D	Natural killer cell group 2 D
NRK	Normal rat kidney epithelial cell line
NSF	N-ethylmaleimide-sensitive factor
Orai	Official full gene name: ORAI calcium release-activated calcium modulator 1. Official full protein name: Calcium release-activated calcium channel protein 1. *Commonly referred to as Orai*
PBMC	Peripheral blood mononuclear cells
PC12 cell	Pheochromocytoma cells
PHA-L	Phytohaemagglutinin-leucoagglutinin
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PID	Primary immunodeficiency
PKC	Protein kinase C
PLC-γ	Phospholipase C-[gamma]
PTK2B	Protein tyrosine kinase 2 beta *(formerly Pyk2)*
Rab (GTPase)	Ras-related in brain (GTPase) *(belong to the Ras superfamily of small GTPases)*
RAG	Recombination-activating gene
RBL cells	Rat basophilic leukaemia cells

RIM	Rab-3 interacting molecule
RSC	Reed-Sternberg cells
SAP	SLAM-associated protein
SEA	*Staphylococcus aureus* enterotoxin A
SIM	Structural illumination microscopy
siRNA	Short interfereing ribonucleic acid
SIV	Simian Immundeficiency Virus
SLAM	Signaling lymphocytic activation molecule
Slp-76	SH2 domain containing leukocyte protein of 76kDa. *Also known as LCP2 (Lymphocyte cytosolic protein 2)*
SNAP	Soluble NSF-attachment protein
SNAP-25	Synaptosomal-associated protein 25
SNARE	SNAP receptor (Soluble N-ethylmaleimide sensitive factor attachement protein receptor)
SOCE	Store-operated calcium entry
Src	Official full gene name: SRC proto-oncogene, non-receptor tyrosine kinase Official full protein name: proto-oncogene tyrosine-protein kinase Src *("Src" is derived from "sarcoma"; SRC is a proto-oncogene)*
STIM1	Stromal interaction molecule 1
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TGF-β	Transforming growth factor [beta]
TIRF(M)	Total internal reflection microscopy
TLR	Toll-like receptor
ULBP	UL16‑Binding Protein
UNC	Uncoordinated
VAMP	Vesicle-associated membrane protein
WAS(P)	Wiskott-Aldrich syndrome (protein)
wt (protein)	Wildtype (protein)
XIAP	X-linked inhibitor of apoptosis protein
ZAP70	Zeta-chain-associated protein kinase 70

“Climb the mountain not so the world can see you,
but so you can see the world.”
(David McCullough)

1 INTRODUCTION

1.1 THE SIGNIFICANCE OF REGULATED EXOCYTOSIS IN DIFFERENT CONTEXTS

Exocytosis is fundamental to various processes in life. The development of a regulated, secretory mechanism which mediates gradable information exchange between cells of same or different origin is instrumental for example for movement, memory, development, perception of environment, digestion, immunosurveillance – many mechanisms enabling multicellular forms of life. However, exocytosis is not only found in multicellular organisms. Some of the key exocytosis proteins have been identified in protozoans, with for example ciliates undergoing both, constitutive as well as stimulated secretion [2].

In multicellular organisms, the development is complex, and cellular specialization, translating into different cell types, is observed. Morphologically and functionally distinct organs can be formed which serve diverse, non-redundant purposes. All organs together maintain the whole organism but in order to do so, they need to communicate with each other. Exocytosis and, correspondingly, surface-receptor based recognition of various types of molecules is one way how cells communicate, even over far distances throughout the body. Exocytosis is a particular form of membrane-membrane fusion that delivers vesicle-stored molecules to the extracellular space (or for integration into the plasma membrane), based on the controlled fusion between an intracellular vesicle and the plasma membrane. Eukaryotic cells contain a variety of vesicles that serve different purposes. They are membrane-enclosed intracellular structures (organelles), which contain and carry specific molecules, and hence, different vesicle types have distinguishable molecular identities. Information exchange in the form of membrane fusion (and fission) has to occur intracellularly (between organelles), too, for example in order to sort molecules to a particular target compartment or pathway. There is a number of protein families involved in membrane fusion. Members of these families are implicated in both exocytosis and intracellular fusion events.

The molecular mechanisms of exocytosis have been studied intensively with respect to neurotransmission, insulin secretion, and some other exocytotic processes, representing vital physiological functions. Another intriguing process in which exocytosis is crucial is immune defense. A number of cell types are part of the immune system. For example, cytotoxic lymphocytes, comprising natural killer (NK) cells and cytotoxic T lymphocytes (CTL), are able to recognize and kill infected or transformed body cells. They execute this function by releasing specialized secretory lysosomes, termed cytotoxic granules, onto the target cell, in order to induce apoptosis in that target cell. Defects in lymphocyte cytotoxicity, both with respect to granule content and their release, are associated with life-threatening immune disorders. A deeper understanding of immune cell effector functions in such disease contexts has thus been a longstanding interest in research.

1.1.1 Basic components of exocytosis

Exocytosis is mediated by several families of proteins which all contribute important properties to the process. There are proteins which help to transport vesicles towards and settle them at the plasma membrane, governing their minute organization at the potential fusion sites. Others constitute the protein complex for fusion which can force the membranes in very close proximity and support fusion. Vesicle localization, fusion complex formation and also recycling of components for subsequent rounds of fusion are tightly controlled by a variety of structural, regulatory and accessory proteins.

The terminal fusion complex in exocytosis consists of proteins which bear specific domains (called SNARE domains, see chapter **1.1.1.1**) that can intertwine and form a tight helical bundle (called SNARE complex), promoting membrane merger. Such proteins (called SNARE proteins as they bear a SNARE domain) are for example members of the syntaxin family, VAMPs or members of the SNAP25 protein family. Amongst others, proteins of the Munc13 and Munc18 / SM families (no structural homologies) are implicated in vesicle docking and priming (preparatory steps typically required for membrane fusion), and they influence the interactions formed between SNARE proteins. In fact, for example syntaxins and SM proteins can undergo a series of distinct interaction modes which greatly determine if SNARE complex formation can occur. Also, there are proteins which serve to maintain the relative orientation of a vesicle to the exocytosis proteins and the plasma membrane. Such structural proteins comprise for example RIMs (Rab-3 interacting molecules), RIM-Binding proteins or liprins that can coordinate calcium channels, SNARE and Munc proteins and the vesicle via interaction with proteins on the vesicle (for example Rab3) or membrane lipids. Besides, there are numerous proteins implicated in other fundamental steps throughout exocytosis. These include for example calcium sensors which facilitate exocytosis upon calcium binding (for example Munc13 proteins or synaptotagmins), or proteins that aid in recycling SNARE complex components after fusion (for example NSF or SNAP proteins), and many more [3].

The general principles of membrane fusion are conserved in many cell types, with possible modifications according to the individual cell type and vesicle type to be exocytosed. To illustrate just one example, as opposed to pre-assembled hot spots for docking and exocytosis which are found in some cell types, a study in an insulin-secreting beta cells line (Ins1 cells) showed rapid *de novo* exocytosis protein cluster formation at subsequent granule fusion sites, and these clusters initiated with the proteins Munc18 and syntaxin-1, followed by recruitment of Rab3, SNAP25 and Munc13 [4].

1.1.1.1 SNARE complex formation and SM proteins

For many membrane fusion processes, a SNARE complex forms the basis for the merger reaction. The domains within the proteins that mediate this complex formation are called SNARE domains. These are alpha-helical stretches of around 60-70 amino acids, which can form a tight helical bundle, the SNARE complex, comprising four SNARE domains provided by several proteins. The SNARE domains

zipper up in an N- to C-terminal fashion [5, 6]. SNARE complexes are hydrophobic on the inside, except for one ionic layer in the middle of the bundle; where every SNARE domain contributes either an R or Q (arginine or glutamine), respectively. Accordingly, SNARE proteins can also be classified as R- or Q-SNAREs [7]. VAMPs and syntaxins each contain one SNARE domain and SNAP25 proteins possess two. One requirement for exocytosis is that the proteins (i.e. their SNARE domains) encounter each other in the correct orientation. At the time of fusion, typically, the SNAP25 and the syntaxin reside within the plasma membrane (hence also called target membrane-SNAREs, t-SNAREs) whilst the VAMP is integrated in the vesicle membrane (vesicular SNARE, v-SNARE). SNARE complex formation can force the SNARE domains from a previously rather perpendicular to a finally parallel orientation in respect to the plasma membrane, and that movement pulls the two membranes into very close proximity. This step does not require ATP. The force can suffice to overcome the hydrophobic barrier between membranes, and allow for fusion. Specificity of SNARE complex formation is not necessarily given by the SNARE proteins themselves, as it was observed with arrays of VAMP, SNAP25 and syntaxin family members that most of them can promiscuously bind each other [8, 9]. However, specificity is, at least in part, regulated by Munc18 proteins [10]. The interaction with SM proteins (see chapter **1.4**) possibly influences also the trafficking of various syntaxins, implicating that subcellular localization could also be a measure of specificity regarding syntaxin availability for SNARE complex formation. However, SM proteins can often bind several syntaxins. Thus, another aspect determining possible SNARE and Munc18 combinations for fusion events is the cell-type specific expression pattern of these proteins.

Munc18-1, -2 and -3 proteins belong to the highly conserved Sec1/Munc18 (SM) protein family. SM proteins do not possess membrane anchoring domains but they can fulfill their function due to interactions with other membrane-targeted molecules [11]. The role of SM proteins may exceed docking, as they also facilitate vesicle priming and membrane fusion complex formation (i.e. the SNARE complex) [12-16]. Munc18-1 for example can bind several proteins, including VAMP2 and VAMP3, and a number of other exocytosis-associated proteins [10, 17, 18]. One of the most important functions of SM proteins is to support the stability and guide the trafficking of syntaxins. The SM protein controls when the syntaxin can interact with other proteins of the fusion machinery. More specifically, it permits the syntaxin to adopt a more accessible conformation when SNARE complex formation has to occur. Thus, SM proteins are frequently termed chaperones for syntaxins, and they fulfil an essential role during exocytosis. Munc18-1, syntaxin-1 and a range of other proteins are central to neuronal exocytosis, and their absence can produce dramatic phenotypes. In immunology, exocytosis represents a central mechanism enabling for example eradication of tumors or infected cells. When genetic linkage analyses were performed on patients suffering from a severe hyper-inflammatory syndrome (familial hemophagocytic lymphohistiocytosis, FHL) [19-23], surprisingly, it turned out that some of the genes mutated in such patients encode homologues to some of the key exocytosis proteins identified in other model systems for exocytosis. In fact, mutations in the immunological pendants to the neuronal proteins Munc18-1, syntaxin-1 and Munc13-1 were all linked to FHL (see chapter **1.3**). Hence, it is likely that homologous molecular machineries operate in cytotoxic lymphocytes to regulate the release of cytotoxic components from such immune cells, and that these proteins are non-redundant for cellular cytotoxicity. It is essential to gain insights on the molecular processes governing cytotoxic granule exocytosis so we can better

understand disease-causing mechanisms of FHL mutations, and relate this specific type of secretion to other exocytotic processes.

1.2 CYTOTOXIC LYMPHOCYTES

Cytotoxic lymphocytes comprise cytotoxic T cells and NK cells. Both cell types are able to adhere to and directly kill aberrant cells in a cell-contact dependent manner. However, they differ in various ways, for example in regards to their development, the type of antigenic structure they recognize and, coupled to this, the pool of antigen specificities they encompass, their ability to form memory pools and some other criteria. Hence, they have historically been assigned to different branches of the immune system, with NK cells allotted to the innate immune system and CTL to the adaptive immune system.

1.2.1 Innate and adaptive immunity concepts

Our immune system is omnipresent, multilayered and highly adaptable. It protects the body from long-lasting consequences of tissue damage caused by injury, infection, inflammation, cancer or other pathologic conditions. The cell types that contribute to the immune system comprise amongst others megakaryocytes and their progeny (platelets, also called thrombocytes), granulocytes (basophils, neutrophils and eosinophils), dendritic cells, monocytes and macrophages, mast cells and lymphocytes (B, T and NK cells). But also other cells, for example endothelial cells, can shape and support immune responses, for example via secretion of soluble factors or by supporting or limiting migration from the blood stream into surrounding tissues. Classically, the immune system has been divided into innate and adaptive immunity. The distinction is based on whether a cell type is able to adapt to the antigen it recognizes throughout an immune response, and whether a response is specific for the antigen. Further, the ability to undergo clonal expansion (followed by contraction) and formation of a memory pool of these antigen-specific cells has been seen as a hallmark. Based on these broad definitions, granulocytes, dendritic cells, mast cells, monocytes, macrophages and NK cells were defined as innate immune cells. On the other hand, T and B cells were ascribed to be part of the adaptive system.

T and B cells rely on somatically recombined gene segments which are fused together and generate the T cell receptor (TCR), or B cell receptor (BCR) molecules, respectively. This process is known as the V(D)J gene segment recombination. The resulting TCR or BCR gene product is, simultaneously to end-joining of the V, D and J segments, further modified by random (non-templated) addition of individual nucleotides between the segments. Thus, an immense diversity of genetically rearranged TCR and BCR molecules is generated, providing a huge number of antigen specificities. The enzymes which mediate this DNA-modifying process comprise RAG enzymes and TdT. Also, during the course of an adaptive immune response, gene segments encoding BCR variable regions can undergo further modification known as somatic hypermutation. For that, key enzymes include the DNA deaminase AID and error-prone DNA polymerases. For B cells, the receptor affinity-based selection process occurs in germinal centers of lymph nodes and is assisted by follicular helper T cells. There, the B cells undergo further modification of the BCR by class switch recombination which turns the membrane-bound BCR into a soluble immunoglobulin with same antigen specificity but different isotype (distal

part of the antibody) [24, 25]. In the case of T cells, the TCR protein complex is not known to undergo somatic hypermutation in the same way as the BCR. However, T cells can undergo a process termed priming. It refers to naïve T cells being less responsive to stimuli than primed cells. Priming occurs when the naïve T cell is activated via TCR engagement with peptide-loaded MHC complexes (pMHC) and simultaneously via co-stimulatory receptor engagement, such as CD28 or CD8, as well as co-stimulation via pro-inflammatory cytokines such as IL-2 or IFN-alpha [26].

As far as is known today, NK cells do not undergo somatic recombination, hypermutation or priming processes and as such do not “adapt” to their trigger. However, in mice infected with murine CMV, responsive NK cells were able to mount memory-like expansion and recall responses [27]. Also, NK cells can mount an antigen-specific memory-like response in SIV-infected macaques [28]. Furthermore, in humans it has been shown that infection with CMV promotes expansion of specific NK cells subsets which present with differential signaling protein expression, altered epigenetic states and they can also display different functional capacities. Such cells can persist for a long time, and can even be protective against relapse of leukemia in patients who had undergone hematopoietic stem cell transplantation [29, 30]. Hence, memory formation may be an inherent property to NK cells. Based on such data, the classic definition of NK cells being part of the innate system has begun to be re-evaluated. Of note, other innate immune cells have also been observed to mount stronger recall responses without irreversible somatic changes in their DNA or clonal expansions upon antigen encounters [31]. The concept of trained immunity refers to a variety of innate immune cells which after pathogen encounter can undergo changes in metabolism and possibly epigenetic states which allows them to generate a stronger response to a pathogen if they encounter it again.

1.2.2 Cytotoxic T lymphocytes

T cells are derived from the common lymphoid progenitor in the bone marrow, and develop further in the thymus. T cell lineage commitment starts with recombination and surface expression of the TCR. Cells that recombine a productive TCR simultaneously upregulate the co-stimulatory receptors CD4 and CD8 and are then termed double-positive (DP) T cells. Depending on affinity for either MHC class I or II, T cells downregulate CD4 or CD8, respectively. Following commitment to the CD4+ or CD8+ T cell lineage, the cells undergo positive and negative selection aided by thymic epithelial cells which present self-antigens. This ensures that cells with a TCR interacting too strongly or too weakly are not released into the periphery and instead undergo apoptosis. For the organism this selection process is crucial as it ensures that no self-reactive or entirely non-reactive T cells are maintained. After selection, the naïve CD4+ and CD8+ T cells leave the thymus. In lymph nodes cell activation and maturation can occur. Antigen recognition by CTL is dictated by the TCR. After MHC class I –associated antigen presentation, a CD8+ T cell specific for that particular antigen can upregulate perforin and reach full cytotoxic capacity. Such a cell can then undergo clonal expansion and migrate to peripheral tissues in order to find the site of infection. CTL contain cytotoxic granules harboring perforin, granzymes and other molecules, similar to NK cell cytotoxic granules – although granule protein abundances can vary between cytotoxic NK cells and CTL. Besides, cytotoxic granule content also varies between differentiation stages, and,

corresponding to the gain in cytotoxic capacity, in mice it was reported to change with the number of proliferation rounds in CTL [32-34].

Just like healthy cells presenting self-peptides (not immunogenic) on their MHC molecules all the time, infected body cells can present cytoplasmic pathogen (or tumor) -associated peptides on their cell surface, typically bound to MHC class I molecules. Such peptides are 8-10 amino acids long. They are generated via intracellular processing of proteins through the proteasome and non-proteasomal peptidases [35]. The cytoplasmic peptides are transported through a transporter system (TAP) into the lumen of the ER where they can be loaded onto MHC class I molecules. The secretory pathway delivers the pMHC complexes to the plasma membrane, where they present the antigen to the extracellular space, for recognition by CTL.

Differences in the quality of TCR-pMHC interactions yield variable outcomes in different settings. The interaction strength for example influences T cell differentiation. It was shown that mice which have only few and weakly interacting TCRs had normal levels of T cells with a TCR composed of α and β chains, whereas $\gamma\delta$ T cells were reduced in numbers and showed impaired differentiation [36]. Another study found that low signaling strength of TCRs (measured in numbers of functional ITAM motifs in the associated CD3 molecules) yielded cytokine secretion whereas high signaling strength was needed to drive proliferation [37]. Along these lines, a study on CD4+ T cells showed that engagement of one single TCR was enough to mount cytokine production, and one interaction seemed enough to trigger a slow but long-lasting TCR clustering [38]. However, to form a stable synapse in a CTL, around ten TCRs needed to be engaged, whilst killing required engagement of only three TCRs, and thus a stable synapse may not always be required for killing a target cell [39]. Besides that, it was shown that signal propagation in the T cell is dependent on continuous engagement of the TCR as well as the actin cytoskeleton [40, 41]. There has been great interest in terms of TCR-pMHC interaction strength regarding prediction of successful cytotoxic events (for example [42, 43]). If the interaction is strong, supposedly cytotoxic granule exocytosis, followed by target cell killing could occur. However, despite some controversy in the field, one notion is that the bond lifetime is more critical than bond strength, although both parameters will determine the outcome. The bond strength was even found to negatively correlate with activation potency [42]. A possible explanation for this was discussed in the article: Following the concept of serial triggering [40, 44], the T cell has to quickly scan countless numbers of pMHC on the potential target surface, obviously also encountering many pMHCs loaded with peptides that the T cell is *not* specific for. It has been seen that pMHCs recognized by a TCR can engage and stimulate multiple TCRs serially. The authors speculated that there likely is an optimum combination of interaction strength and duration, with too short but numerous interactions being insufficient for TCR triggering, and fewer but longer-lasting interactions preventing serial triggering and initiating prosperous TCR signaling and synapse formation. However, prolonged TCR signaling is also well known to lead to internalization of the TCR signaling complexes which intriguingly was shown to be accompanied by prolonged signaling, up to 10 h after internalization [45]. The internalization is indispensable for T cell function, though hard to reconcile with continuous pMHC engagement supporting signaling. Dynamin-2 knockout mice (which cannot internalize TCR) presented with reduced TCR signaling strength and impaired proliferation [46]. Besides the complicated kinetics and internalization of TCR, a third layer of regulation is found over the different regions of the contact area [41]. The

authors observed that TCR clusters tend to move towards the center of the contact site, where they eventually stop signaling and get downregulated (for recycling or degradation) whereas peripheral TCR clusters keep forming and signaling. A suggested explanation could be the protection from excessive activation potentially yielding cell death. Due to high antigen sensitivity as well as massive signal enhancement the T cells need to control the signaling input carefully despite being dependent on continuous input for response generation.

1.2.3 Natural killer cells

The name “natural killer” cell originates from the observation that a subpopulation of lymphocytes could kill susceptible target cells without prior priming [47-49]. However, it is meanwhile known that NK cells can undergo various differentiation and education stages, and these do not harbor equal cytotoxic reactivity. The notion that NK cells are, besides their role in tumor surveillance, also pronouncedly connect to immunity against viruses (particularly Herpes viruses) was supported by various patient reports where NK cell dysfunction co-occurred with high susceptibility to viral infections (for example [50-57]. One important case is an NK cell deficient patient described in 1989 [58]. The patient had recurrent viral infections and was found to be devoid of NK cells and NK cell lytic activity and turned out to suffer from a heterozygous *GATA2* mutation. Haploinsufficiency of this hematopoietic transcription factor can cause variable clinical presentations, to a certain degree reflecting paucity in different immune cell subsets. Besides NK cells, deficiencies in monocytes, B cells and dendritic cells typically develop in *GATA2* patients [59].

NK cells develop in the bone marrow from common lymphoid progenitor cells. NK cell development comprises several phenotypically different stages. The NK cells that leave the bone marrow have lost early developmental markers such as CD34 or CD45RA, and most of them have acquired for example CD56 (frequently used as NK cell marker), granzymes and perforin. After this, they can differentiate further into functionally and phenotypically distinct subsets. Some models suggest that there is a progression from CD56bright NKG2A+ KIR- CD16- CD57- (uneducated, barely cytotoxic, hyporesponsive NK cells) towards CD56dim NKG2A- KIR+ CD16+ CD57+ (educated, cytotoxic, mature NK cells). The stages in between, broadly spoken, could comprise loss of NKG2A and gradual acquisition of one or more KIR receptors, CD16, perforin and later on CD57 [60, 61] although this is not completely clear to date. Of note, molecular barcoding of immune cells in macaques has raised the question if CD56bright cells in humans are precursors of CD56dim cells, or - as the macaque data suggests - rather an independent NK cell subset [62]. Further research will hopefully provide a deeper understanding of human NK cell development.

In order to sense aberrant cells, NK cells possess a variety of activating and inhibitory, germline-encoded surface receptors. The sum of all instant activating and inhibitory input determines how reactive an NK cell is towards a potential target rather than one receptor dominating every single encounter. It has been noted early on that cells deficient in ligands for NK cell inhibitory receptors (such as inhibitory KIR) are more susceptible to being killed by NK cells, although it became clear that absence of inhibitory interactions is mostly not enough to induce NK cell killing. NK

cell-resident inhibitory KIR molecules can bind to pMHC, on an opposing cell. Most, but not all body cells are protected through expression of MHC class I. For example, red blood cells do not express MHC class I. However, these possess other protective molecules, such as CD47, which mediate a “let me live” signal to myeloid and NK cells. CD47 additionally might have a role in NK and T cell homeostasis *in vivo* [63-65]. Due to the notion that, based on MHC class I expression, NK cells sense “self” and are prone to attack surfaces devoid of inhibitory ligands, the Missing Self-hypothesis was formulated [66]. It reminds of and remotely relates to the term Altered Self, which describes the concept that T cells, recognizing antigens via their T cell receptor (TCR), can only be activated through binding to self-MHC molecules which have been “altered” by the bound peptide. [67, 68]. However, it was also observed that certain KIR receptors show peptide selectivity in relation to MHC class I presentation. Thus, also for NK cells the concept of Altered Self has been discussed. [69]. Further, it has become clear that NK cells need to undergo an education process which is based on inhibitory (for example inhibitory KIR) receptor – ligand interactions. The quality and number of effector functions as well as the number of responding NK cells was shown to increase upon enhancement of inhibitory interactions in mice [70, 71] and humans [72, 73]. It should be noted that also other inhibitory receptors, such as the NKG2A/CD94 heterodimer, shape the KIR-MHC educated NK cell pool [74]. NK cells can not only express different numbers of KIR receptors (or none) but they can also differ in terms of which activating receptors they express (even though that is more homogeneous throughout the NK population). A mass cytometric screen of monozygotic twins and healthy controls revealed that the inhibitory receptor repertoire in human NK cells is mostly determined by genetics whereas the activating receptor expression is mostly shaped by environmental influences [75].

Activating NK cell receptors comprise the IgG receptor CD16 (FcγRIIIA, recognizes Fc parts of IgG molecules; mediates “antibody-dependent cellular cytotoxicity”, ADCC), NKG2D (recognizes stress ligands of the ULBP family, and MICA and –B), NKp30, -44 and -46 receptors (recognize viral, bacterial and parasitic epitopes; partly unknown), DNAM-1 (recognizes polio virus receptor (PVR) components), activating KIRs and NKG2C. NKp44 is only found on cytokine-activated cells though [76]. KIRs recognize classical molecules of the MHC class I family (HLA-A, -B, and –C molecules) whilst NKG2C binds to the non-classical MHC class I molecule HLA-E. [77]. Other activating receptors on NK cells include integrins such as LFA-1, and SLAM family receptors, for example 2B4 (CD244), CD48, NTB-A or CRACC. Some of these activating receptors can synergize to mount potent cell activation and cytotoxic responses [78, 79]. Of note, mutations in *FCGR3A* encoding CD16 [50, 51, 80] have been associated with immunodeficiency, as these patients suffered from recurrent viral (Herpes family) infections, and some had reduced NK cell numbers. Interestingly, none of the patients displayed impaired antibody-dependent cytotoxicity. However, some presented with reduced killing of the susceptible K562 cell line. One study [80] found *wt* CD16 to be associated with CD2 at the synapse, which was reduced for a mutant protein. The loss of CD2 association could account for lower adhesion and signal transduction in these patients, resulting in less efficient cytotoxicity.

As a side remark, it is worth considering that there are populations of NK cells presumably dedicated to other purposes than killing. Uterine (decidual, dNK) NK cells (and macrophages) were found to play important roles in remodeling the decidual vasculature [61, 81, 82]. dNK were shown to mediate poor cytotoxicity

against susceptible target cell lines and they formed contact sites which did not progress to cytotoxic granule polarization [83]. It is currently discussed which role dNK play in pregnancy, embryo survival and how the KIR-HLA gene setting contributes to implantation depth of the trophoblast / placenta which could in turn regulate nutrient and oxygen availability for the fetus [84].

1.2.4 Receptor signaling and synapse formation

Despite different modes of target cell recognition, the late steps leading to CTL or NK cell killing of a target cell are highly similar, or even equivalent, as they rely on the same molecular machinery. When cytotoxic lymphocytes recognize susceptible target cells, they form a tight conjugate with them and eventually induce apoptosis (intrinsic cell death) in the target. Such targets can be tumor cells or infected cells which can present pathogen-derived peptides on their surface, display an altered surface expression of MHC class I molecules or upregulate stress-induced surface proteins. If a pathogen does not interfere with surface presentation of MHC class I, a CTL may react to it, whilst if it down-modulates pMHC presentation, this could call to the attention of NK cells.

The TCR consists of several subunits. In CTL recognizing pMHC class I, it is composed of α and β chains. The TCR is associated with CD3, a multi-chain complex consisting of $\gamma, \delta, \varepsilon$ and ζ subunits. All intracellular CD3 complex chains harbor functional peptide motifs called ITAMs. How exactly the signal is initiated once the TCR is engaged, is still not clear, possibly several mechanisms act simultaneously [85]: TCR engagement can trigger a conformational change in the CD3 complex followed by release of the ITAM motifs from the plasma membrane, making them accessible for phosphorylation. Also, signaling is possibly initiated by the tight binding to the target cell surface which physically excludes large regulatory phosphatases from the contact site (such as for example CD45). The signal transduction over the membrane induces recruitment of non-receptor protein tyrosine kinases of the Src family which then phosphorylate the CD3 ITAM motifs. Similarly, NK cell receptors which are coupled to ITAM signaling, initiate Src kinase activation. Such NK cell receptors on are for example CD16, NKp30, Np44 or NKp46. ITAM signaling enables binding and phosphorylation of ZAP70 which can activate LAT and Slp-76, and also, via activation of Grb2, MAP kinase signaling can be initiated. This in turn for example leads to ERK phosphorylation and changes in gene expression liked to cell activation [86-88]. Other NK cell receptors which do not signal through ITAM motifs comprise the integrin LFA-1, 2B4 or NKG2D. These receptors can also initiate MAP kinase signaling, in the case of 2B4 PLCγ activation, or in the case of NKG2D activation of PI3K and mTOR [88]. ITAM signaling also leads to activation of PLCγ, and this enzyme can hydrolyze phosphatidyl-inositol-2-phoasphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 is soluble and diffuses to the ER membrane where it binds to IP3 receptors and opens them to allow calcium to pass through.

Calcium fluxes from the ER into the cytoplasm. IP3 is not opening plasma membrane resident calcium channels [89]. The ER calcium depletion is sensed by ER-resident transmembrane STIM1 proteins which oligomerize, change

conformation and reorient towards the plasma membrane site (where the activation occurred), where they open store-operated calcium channels consisting of Orai1 [90-93]. This results in further calcium influx from the extracellular medium. The process is termed store-operated calcium entry, SOCE. *STIM1* and *ORAI* deficiencies are associated with erroneous calcium sensing and SOCE. The lymphocytes can still recognize target cells, activate the integrin LFA-1 and polarize the cytotoxic granules, but exocytosis and target cell killing are reduced. Also cytokine production was shown to be reduced [94-97]. Calcium binds to several proteins, amongst others to calcineurin. This phosphatase then targets cytoplasmic NFAT leading to nuclear translocation of this transcription factor. Another protein activated by calcium binding is calcium calmodulin-dependent kinase, again initiating transcription factor activation [98].

DAG remains in the plasma membrane where it recruits protein kinase C (PKC). PKC activation at the site of cell activation has been linked to MTOC reorientation [99] which is important for polarized cytotoxic granule exocytosis in lymphocytes. DAG-activated PKCs were suggested to act through a reciprocal positioning of dynein and myosin proteins which ensure pulling/pushing forces that orient the MTOC towards the contact site [100]. PKC enzymes have also been implicated in regulating cell protrusions and microtubule stability in T cells under shear flow conditions [101]. That is interesting because it might implicate that PKC enzymes are important for cytoskeleton-dependent organelle positioning events in different settings. Of note, several genes regulating cytoskeletal elements, mostly actin, have also been linked to primary immunodeficiency. These are for example the genes *WASP* and *WIPF1* (associated with WAS), *DOCK8* (associated with autosomal recessive hyper-IgE syndrome and abnormal morphology and deformation during tissue migration of lymphocytes, [102]), *ITGB2* or *FERMT3* (associated with leukocyte adhesion deficiencies). Integrin as well as Src kinase signaling were both implicated in cytotoxic granule convergence to the MTOC, as well as the subsequent MTOC+granule polarization towards the immunological synapse [103-105]. The integrin-linked kinase (ILK) was found to transduce the integrin signal onto the MTOC via a network of signaling transducers comprising for example paxillin, PTK2B, Cdc42, APC and CLIP-170 [106]. Abrogation of components of this signaling network yielded abrogation of cytotoxic granule polarization. Also, the granule convergence could be triggered by pro-inflammatory cytokine stimulation (here IL-2), as well as target cell stimulation, and it was found to be independent of actin and microtubule reorganization events, but dependent on motor protein dynein. Further, cytotoxic granule recruitment to the MTOC was mechanistically not coupled to cytotoxicity [104, 105]. Synapse formation and recruitment of the MTOC with the associated cytotoxic granules is essential for polarized secretion.

Not all NK cell receptors trigger the entire cascade. Experiments activating single receptors have shown that for example pure integrin (LFA-1) engagement leads to granule polarization but not secretion, whilst isolated CD16 stimulation provokes cytotoxic granule release which is not polarized [107]. Also, it has been observed that many receptors cannot initiate cytotoxicity on their own in resting cells. Interestingly, they gained function when the cells were IL-2 stimulated. Of note, IL-2 also increases the expression level of many of these receptors. Such receptors, for example NKp46, NKG2D, 2B4, DNAM-1, or CD2 can also trigger cytotoxicity in resting cells when they are co-stimulated together with other receptors. One example is synergistic co-stimulation via NKp46 and NKG2D. Several other synergistic receptor combinations have been identified [79].

The signaling cascades initiating cytotoxic granule release can under some experimental conditions establish a large, organized landscape of signaling clusters at the site of activation: The immune synapse. A very schematic illustration of the immune synapse suggests that the synapse can be structured in a concentric fashion, with the rings being distinct supramolecular activation clusters (SMAC). The innermost area (central SMAC, cSMAC) comprises TCR (or NK cell receptor) signaling clusters which organize the actin mesh under the membrane. The next ring (peripheral SMAC, pSMAC) would then harbor adhesion molecules such as integrins, or proteins linking surface receptors with the cytoskeleton, for example talin. A more distal ring (dSMAC) would then contain spatially demanding, or regulatory molecules such as CD45 [108]. However, one should note that this model describes a structural frame for the subsequent exocytosis process, but not all synapses are perfectly round or adopt this exact architecture. Very low numbers of TCR molecules are actually needed to trigger exocytosis, and also some other observations demand a more careful synapse region definition. For example, CD45 was not only found in the peripheral synapse areas but was also observed in the very center of the cSMAC by some authors [109]. Also, TCR signaling might in fact preferentially occur in the distal synapse area and be reduced in the central domain [41]. It is thus possible that the cSMAC might mainly be the site of TCR downregulation and cytotoxic granule exocytosis. Spatial constraints could affect the cSMAC but maybe even more pronouncedly the pSMAC where tight adhesion is established. Novel TCR signaling clusters could continue to form in the dSMAC, cross the pSMAC and progress to down-modulation once they are in the cSMAC.
For NK cells, the immune synapse may also not always be as stringently organized, or the exclusive site of cytotoxic granule release. Some types of receptor-induced cytotoxic granule exocytosis may occur at regions outside of the contact site. This could for example be the case during the non-polarized degranulation following CD16 stimulation [107].

1.3 (FAMILIAL) HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS: CLINICAL ASPECTS

To date, there are numerous genetic aberrations documented which are causally linked to immunological disease and hence are classified as primary immunodeficiencies (PID). PID is a group of diseases which impair either development or function of the immune system. The genes as well as the disease phenotypes are variable, and most genetic variants or mutations affect more than one particular cell type. PID patient cells provide an important opportunity to gain essential insights into disease-causing mechanisms of aberrant proteins, and also do they often unveil crucial mechanistic details about the *wt* proteins.

Hemophagocytic lymphohistiocytosis (HLH) is a hyper-inflammatory syndrome which can manifest in variable symptoms. It can occur as consequence of an underlying genetic cause (FHL), but also as a secondary disease as complication of malignant, rheumatoid or infectious diseases. Epstein-Barr virus (EBV) infections are very often noted in HLH patients [110-112], but also other viruses possibly linked to the disease have been found in HLH patients, such as Herpes simplex viruses [113, 114], Varizella zoster [115], Dengue virus [116], parainfluenza virus [117], avian influenza virus or co-infections (for example with fungi or bacteria) in HIV-infected individuals [118, 119]. Also, some bacterial infections were suspected to be associated with HLH development, such as for example *Acinetobacter baumannii* [120] or *Mycoplasma pneumoniae* [121]. Similarly, some parasitic infections can trigger HLH, mostly reported in patients suffering from visceral leishmaniasis [122, 123].

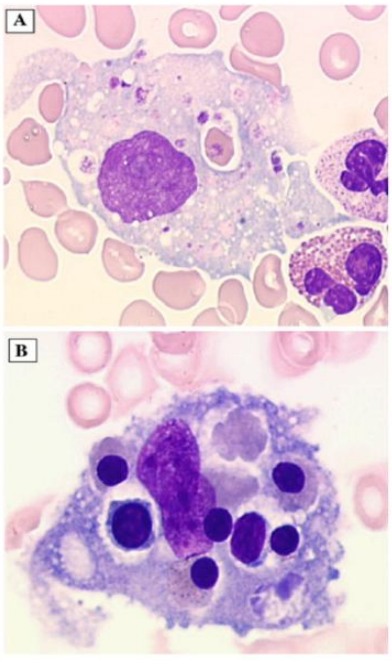

Figure 1. Hematoxilin-Eosin staining of bone marrow from HLH patients showing a) histiocytes phagocytosing erythrocytes and lymphocytes and b) phagocytic cells containing erythrocytes and platelets. [1]

The name of the disease is derived from the observation that in bone marrow aspirates of some HLH patients, activated tissue macrophages (histiocytes) engulf and digest erythrocytes, lymphocytes and other hematopoietic cells and precursors (Figure 1). However, this is not observed in all patients. The current list of diagnostic markers comprises prolonged periods of fever, splenomegaly, cytopenias in at least two blood cell lines, enhanced triglycerides, soluble CD25 and ferritin, reduced fibrinogen, low or absent NK cell activity [124]. Fulfilment of 5 of these criteria leads to the diagnosis HLH. This diagnostic system is necessary since none of the criteria is specific for HLH. Besides these, many HLH patients present with neurological or gastrointestinal abnormalities. Colitis, diarrhea, convulsions, loss of consciousness, confusion, irritability, cranial nerve palsies, hearing losses and seizures in the CNS have been reported, amongst others [125-129]. Of note, HLH patients with neurological involvement presented with a decreased life expectancy [130].

The treatment of HLH typically comprises immunosuppressive / chemotherapeutic drugs (eg. cyclosporine A, anti-thymocyte globulin, etoposide (also known as VP16), teniposide) in combination with corticosteroids, such as dexamethasone or prednisone [124]. The treatment aims at controlling activation and expansion of lymphocyte populations (and other hematopoietic cells, such as histiocytes) causing tissue pathology and enhancing the risk for ultimate multi-organ failure. Such treatments greatly improve the clinical symptoms of HLH. In the case of FHL, the only cure is allogeneic hematopoietic stem cell transplantation. A number of genes have been associated with FHL (`Table`) but the list might grow, incorporating genes that play non-redundant roles in cytotoxic granule exocytosis, but in which mutations are not embryonically lethal. Currently, four genes are associated with FHL. Some other genes are linked to similar clinical pictures as FHL, and thus they are often termed FHL-like. They comprise for example Chediak-Higashi syndrome or Griscelli syndrome type 2.

Primary Immunodeficiency	Chromosomal location	Gene	Protein
FHL-1	9q21.3-q22	Unknown	Unknown
FHL-2	10q21-22	*PRF1*	Perforin
FHL-3	17q25	*UNC13D*	Munc13-4
FHL-4	6q24	*STX11*	Syntaxin-11
FHL-5	19p13.2-3	*STXBP2*	Munc18-2
CHS	1q42.1-q42.2	*LYST*	Lyst
GS-2	15q21	*RAB27A*	Rab27a
XLP-1	Xq25	*SH2D1A*	SAP
XLP-2	Xq25	*XIAP*	XIAP
HPS-2	5q14.1	*AP3B1*	AP3

`Table`. Primary immunodeficiencies resulting in HLH and HLH-like disease. Abbreviations: FHL: Familial hemophagocytic lymphohistiocytosis, CHS: Chediak-Hiagshi syndrome, GS-2: Griscelli syndrome type 2, XLP: X-linked lymphoproliferative syndrome, HPS-2: Hermansky-Pudlak syndrome type 2. Modified from [131, 132].

In the case of FHL, the disease is presumably triggered by an immunological event, often an infection with viruses of the Herpes family. Many FHL patients are EBV or CMV-positive. Once an initial inflammatory response has been generated, a systemic HLH flare might follow and the patient deteriorates rapidly. HLH pathology is thought to be due to massive and continuous activation of cytokine- and chemokine-producing cells which cannot eradicate pathogenic triggers. One study in knockout (KO) mice showed that absence of granzymes A+B, or perforin, respectively, yields enhanced cytokine and chemokine release from CTL and NK, upon target encounter. Such supernatants were able to induce robust IL-6 production in syngeneic macrophages in an IFNg-dependent manner. The authors delineated that the increased activation and cytokine production is driven by the inability to detach from the target cell – as they cannot be killed by the KO lymphocytes. This yields prolonged conjugate time and repeated calcium flux [133]. These findings could potentially explain the abnormally high amounts of pro- and anti-inflammatory mediators found in plasma and tissues, and these in turn the fever flares. The lymphocytes would get overly activated upon an initial trigger, produce high amounts of cytokines which can stimulate various other cell types, start surface expression and at some point shedding of CD25, upregulate adhesive molecules and start invading different tissues such as liver, kidneys or even brain. A systemic state of pathologic inflammation can be the result. The cytokine storm does not only

lead to fevers, but some cytokines such as TNF or IFNγ, may impair hematopoiesis [131], which can contribute to the cytopenia seen in many patients. A study in perforin KO mice infected with LCMV has shown that the hyper-inflammation induces a shortage of free IL-2 [134]. That is a result of elevated CD25 surface expression on activated CD8T cells, shed CD25 and also decreased IL-2 production of CD4T cells. In the perforin KO mice, over the course of the disease, the IL-2 shortage lead to decreased surface CD25 expression and abnormal contraction of regulatory T cells. The residual IL-2 was shown to be consumed predominantly by activated CD8T cells, maintaining the high activation and proliferation state, and thus over time partially uncoupling the ongoing hyperinflammation from the (often infectious) initial trigger. Besides these processes, the elevated cytokines also activate macrophages to clear immune and red blood cells, which can lead to the phagocytic histiocytosis. This can further drive the cytopenia observed in blood, combined with the increased tissue invasion of lymphocytes and other immune cells. The elevated cytokines can also lead to inhibition of lipoprotein lipase and increase production and secretion of triglycerides [131]. Activated macrophages can secrete ferritin and other factors which can decrease fibrinogen levels. Overall, these processes sum up to imbalances in immune cell populations and an overall massive activation of multiple cell types.

Of note, another mechanism which could promote FHL pathology is a possible link between the massive cell activation and Toll-like receptor (TLR) activation. In a mouse model of FHL (Unc13d knockout (*jinx)* mice, infected with LCMV), one group studied whether the hyper-inflammatory state could be alleviated if certain proteins supporting inflammatory processes would be targeted. Interestingly, they found that a key signaling molecule implicated in TLR signaling, MyD88, was substantial for the development of the HLH-like syndrome [135]. In line with this, another report studying genetically normal mice found that consecutive stimulation of TLR9 results in an aggressive inflammatory state as well, resembling HLH [136]. More detailed, that stimulation led to macrophage activation syndrome, MAS. MAS shares several clinical features with HLH. Common are for example elevated cytokine levels, activated macrophages and a high inflammatory state. Hence, innate immune receptor signaling (TLR signaling) could powerfully contribute to the HLH pathology seen in FHL patients. Many mechanisms occur simultaneously in HLH and probably together drive the pathology which remains unsolved and is fatal unless treated. However, if the inflammatory trigger could be controlled in the beginning, a hyper-inflammatory state could be prevented. Thus, cytotoxic granule exocytosis is fundamental for FHL pathology.

1.3.1 Severity of FHL / HLH and association with malignancies

Interestingly, depending on which gene is affected, HLH onset and severity can have different kinetics. It was shown in patients and in FHL gene mouse models (infected with CMV) that on average there is a hierarchy of pathology and morbidity. There was a gradient of perforin > Rab27a > syntaxin-11, looking at age of onset (patients), survival, production of IFNγ, TNF and IL-1β (mice), with loss of perforin being the most severe. However, all mice presented with comparable target killing deficiency, CMV load, loss of body weight, overall decline of white and red blood cells as well as platelets [137]. This is in line with a report on genotype-phenotype correlations comparing syntaxin-11 and perforin deficient patients which found earlier onset for FHL2 as opposed to FHL4 patients [138]. Another study included mouse models for perforin, Rab27a, syntaxin-11, AP3 and Lyst deficiency as well as patient data. They found a similar gradient of severity of the affected proteins mentioned above, with the Lyst patients revealing later age of onset than syntaxin-11 and, dependent on the mutation, almost normal degranulation and cytotoxicity in some of the mice (*beige* mice). However, another Lyst mouse model (*souris* mice) was as detrimental as the *ashen* mice, which represents Rab27a deficiency. The mouse model for AP3 deficiency (*pearl* mice) displayed an intermediate severity [139]. A mouse model for Munc13-4 deficiency (*jinx* mice) displayed development of HLH-like symptoms upon mouse arena virus LCMV infection, but other pathogens which can trigger HLH in other FHL mouse models such as *Listeria monocytogenes* or herpes virus MCMV infection did not produce a robust representative HLH picture. Also, these mice did not suffer from neutropenia [140]. The *jinx* mice may thus only be a good model system in combination with a carefully chosen pathogenic trigger. Munc13-4 deficient patients, carrying homozygous or compound heterozygous mutations, present early in life with clinical symptoms (< 24 months), comparable to perforin homozygous / compound heterozygous patients [141]. Most of them have normal perforin expression, but the degranulation defect is comparably severe to Munc18-2 patients.

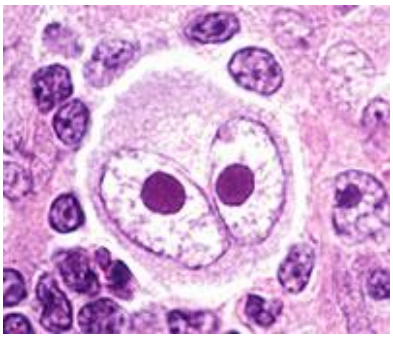

Figure 2. A classic Reed-Sternberg cell with two nuclei and enormous nucleoli.

From: Stanford Medicine, Dep. of Hematopathology. (http://hematopathology.stanford.edu/)

Besides the gene affected, there is evidence [141-143] that accumulation of heterozygous FHL mutations (in different FHL genes) increases the risk to develop HLH in mice and humans. Interestingly, when looking at the clinical records of FHL gene carriers (monoallelic relatives of FHL patients), one report found an increased risk of developing malignancies in female relatives of the first generation [144]. In fact, there are several reports possibly linking immunodeficiencies, malignancies and viral infections, such as EBV [145]. A co-occurrence between monoallelic mutations in perforin and lymphoma (Anaplastic large cell lymphoma) development was reported in about 30 % of these lymphoma patients [146] and a further report found that about 13 % of the lymphoma patients which shared clinical symptoms with HLH carried monoallelic perforin mutations, and a further 13 % carried biallelic perforin mutations [147]. Another study found a strong (50 %) incidence of diverse types of malignancies with homozygous perforin mutations in carriers which had not

developed FHL [148]. Thus, in mice and humans, subclinical heterozygous FHL variants could yield partly reduced immunosurveillance, marginally reduce control of infections, and / or allow for long-term subclinical chronic inflammatory processes which can build up over time to an enhanced risk for HLH and malignancy.

Occasionally, PID patients can develop Hodgkin (HL) and Non-Hodgkin lymphomas (NHL) which are malignancies of the lymphocyte compartment, and several cell lines can be affected. In HL, B cells are often transformed. The diagnosis of HL and NHL is based on the histological identification of Reed-Sternberg cells (RSC, Figure 2). If RSC are present, it is classified as HL. RSC are transformed, often EBV-infected and enlarged B cells, which can contain several nuclei. They tend to bear mutations in their rearranged Immunoglobulin genes and can present with aberrant signaling [149, 150]. Their development or pathogenesis is not clear. RSC are CD15+ CD30+ CD45-. However, also a few peripheral T-cell neoplasms were found to have similar phenotypes [151]. If they are infected by EBV, the RSC can be stained for EBERs which are small viral RNAs that are produced and secreted in high amounts by the infected cells. These support transformation, hinder apoptosis and drive immune activation [152]. Another factor sometimes abundantly present in RSC is PAX5, a transcription factor which promotes B cell lineage specific genes and its positivity in RSC was correlated with worse clinical outcome [153, 154]. A staining that can be used to visualize the accumulation of histiocytes / macrophages is CD68, a lysosomal protein highly expressed in macrophages, although this is not a specific staining [155]. It is the combination of histological stainings which allows for unambiguous classification.

One notion on development of HLH versus malignancies in individuals carrying FHL / FHL-like genetic predispositions could depend on the extent of the phenotype of the genetic variation. Accordingly, the organism would either directly progress to full-blown HLH once the trigger was set, if the mutation has full penetrance and / or severely compromises protein function. In the case of mild, heterozygous or lower penetrance variants, the body instead would succumb to lower, long-term chronic inflammatory processes which could eventually promote transformation. For the FHL / HLH field, it is important to report and understand the connections between hyperinflammation and the rise of malignancies, so that prognoses and treatments can be chosen with best accuracy and can be adapted and refined according to the patient.

1.4 THE MUNC18 – SYNTAXIN INTERACTION

The list of genes associated with FHL imparts that absence of non-redundant cytotoxic granule exocytosis proteins can rapidly evoke a life-threatening inflammatory state upon an infectious trigger. Syntaxin-11, Munc18-2 and Munc13-4 have all been identified as key mediators of exocytosis in cytotoxic lymphocytes. In general, the Munc18 / syntaxin interaction is central to vesicle exocytosis. However, we are only beginning to understand the molecular details about the Munc18-2 / syntaxin-11 interaction and their implications for protein stability, localization and function.

1.4.1 Munc18-1 and -2

Genetic variations in genes encoding Munc18 proteins, as well as syntaxins, have meanwhile been implicated in various diseases, for example obesity and diabetes [156, 157], immunological phenotypes (FHL, see Table), or neurological abnormalities such as epilepsy, anxiety-like behavior or changes in long-term potentiation and depression of neuronal circuits [158-162]. Munc18-1 (the corresponding neuronal syntaxin is syntaxin-1) is the major neuronal SM protein involved in neurotransmission as Munc18-1 null mice are entirely deficient in neurotransmission and suffer from substantial neurodegeneration [163]. Heterozygous mutations in Munc18-1 have, amongst others, been linked to development of (early infantile) epileptic encephalopathy (also known as Ohtahara syndrome) [164-168]. Munc18-2 is encoded by the gene *STXBP2* and has been associated with the development of FHL5, which dramatically affects the function of cytotoxic lymphocytes [23, 169]. Interestingly, both NK cells and CTL equally rely on the key components Munc18-2, syntaxin-11 and the hematological priming factor Munc13-4 in a comparable fashion, as cytotoxicity in patients lacking any of them was similarly impaired in both cell types [33]. Of note, FHL has mostly been considered to be an autosomal recessive disease, however, one heterozygous Munc18-2 mutation was reported to act in a dominant-negative fashion [170]. Munc18-2, like syntaxin-11, is broadly expressed throughout the immune system (additionally to some other cell types); and it has been implicated in exocytosis events in different immune cells, and also some non-immunological cells. For example, Munc18-2 was important in platelet secretion more so than other Munc18 proteins, although they express Munc18-1, -2 and -3 [171]. Munc18-2 also plays an important role in mast cell degranulation, supposedly together with syntaxin-3, but not -4 [172, 173]. Munc18-2 and -3 together with syntaxin-3 and -4 were shown to mediate exocytosis events in neutrophils [174]. Besides the immune system, Munc18-2 has also been shown to play roles in insulin secretion, where it likely acts in concert with other Munc18 proteins, such as Munc18-1 and -3, to regulate exocytosis [175-177]. In cytotoxic lymphocytes, Munc18-2 is mostly found on intracellular vesicular structures (Figure 3) and assumedly, similarly to syntaxin-11 (maybe even guiding syntaxin-11 trafficking), is recruited to the immunological synapse upon conjugate formation with target cells.

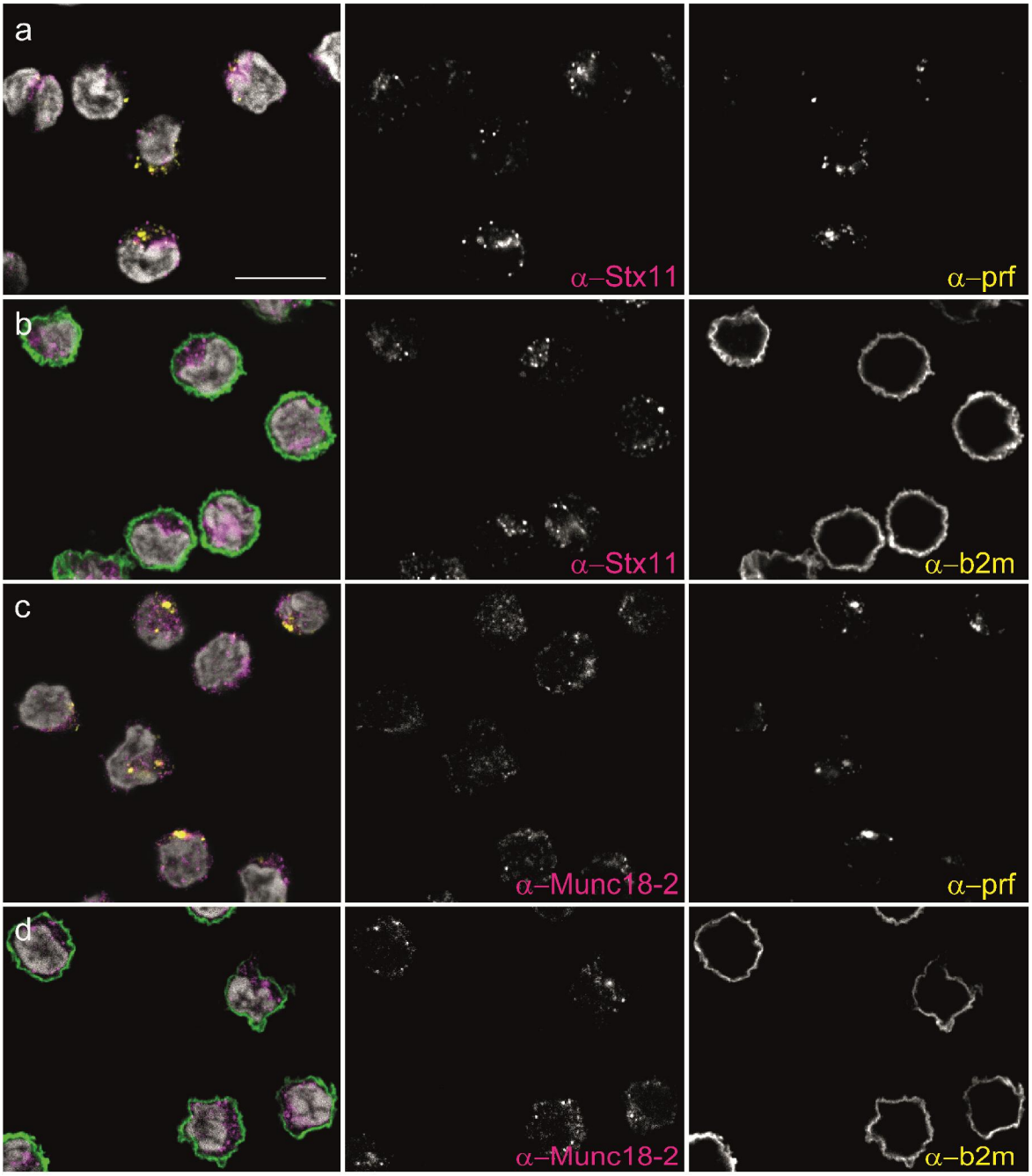

Figure 3. Syntaxin-11 or Munc18-2 do not pronouncedly overlap with cytotoxic granules, or the plasma membrane (indicated by perforin and β2microglobulin staining, respectively). NK cells from a healthy donor were seeded on poly-l-lysine-coated glass surfaces, fixed and stained for syntaxin-11 together with perforin (a) or β2microglobulin (b), or stained for Munc18-2 together with perforin (c) or β2microglobulin (d). The first image of each row depicts the overlay of DAPI stain (gray), syntaxin-11 or Munc18-2 (raspberry), and perforin or b2m (yellow), respectively. Size bar 10 μm. Stx11. Syntaxin-11; Prf, perforin; b2m, β2-microglobulin.

1.4.2 Binding modes

There is not much known about the details of the interaction between syntaxin-11 and Munc18-2. In fact, most knowledge about Munc18 / syntaxin interactions is derived from the neuronal counterparts, Munc18-1 and syntaxin-1. Some data suggest that Munc18 proteins can only bind the tightly packed, closed syntaxin conformation, having no effect on or even preventing it from forming SNARE complexes [178-182], and spatial segregation of SNARE complexes versus Munc18-syntaxin complexes had been reported in a mast cell line, analyzing Munc18-2 and syntaxin-3 [183]. However, there is data indicating that Munc18 could bind syntaxin also when the syntaxin is in a SNARE complex [184-186], and Munc18-bound syntaxin can readily undergo SNARE complex formation upon encounter of membrane-bound VAMP2. It was thus suggested that the Munc18-syntaxin interaction could possibly persists over the different binding stages of the SNARE cycle, and hence convey a sequence of differential interactions between the two proteins [187-189]. Such a complicated dynamic interaction is possible because of several interaction surfaces, on both proteins. First, the syntaxin-1 N peptide (comprising two or three very N terminal charged residues followed by hydrophobic L (Leu) or F (Phe) residues, often indicated as the first 20 amino acids [190-192] can bind to a hydrophobic pocket on Munc18-1 formed by several residues, for example F115 and E132 [193]. Second, the large internal groove of Munc18-1, formed by domains -1 and -3a, can bind to another syntaxin-1 domain residing further downstream in the N terminus: This syntaxin domain consists of three helical parts which together form the Habc domain. (Figure 4). These two types of interaction are not necessarily mutually exclusive and they could fulfill different functions.

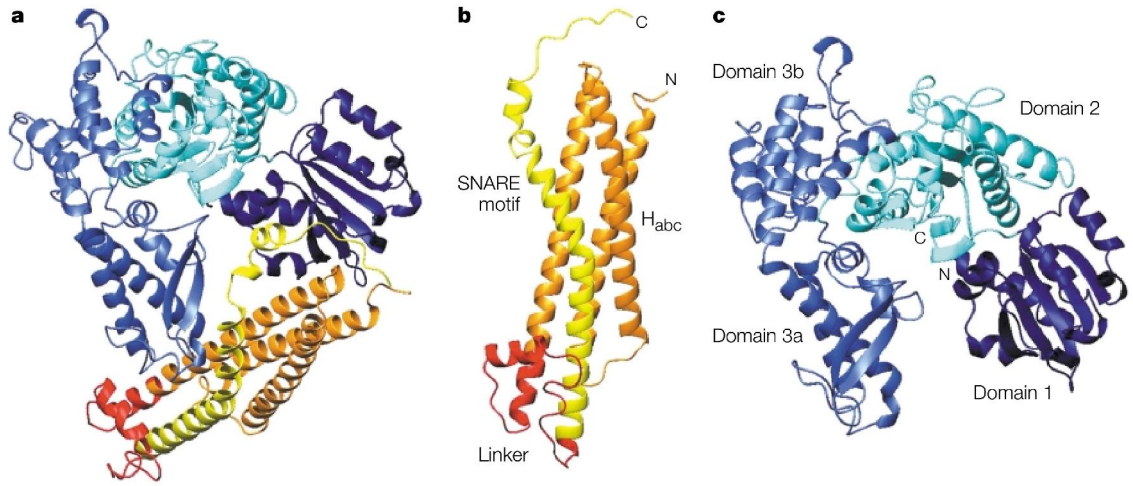

Figure 4. Structure of Munc18-1, syntaxin-1 and the binary complex. (a) The binary complex is of Munc18-1 (in shades of blue) and syntaxin-1 (in red, orange and yellow, according to the domains). (b) Syntaxin-1 in the closed conformation folds as a partly helical bundle, with the SNARE domain partially wrapped around the Ha, Hb and Hc helices. The linker domain (red) is partly unstructured and partly helical. (c) Munc18-1 consists of 4 bulky domains (1, 2, 3 a and 3b) which form a large internal groove that can bind to the folded Habc/SNARE domain bundle of the syntaxin, as shown in (a). The hydrophobic pocket resides in the outwards-facing surface of Munc18 domain 1 (not highlighted for clarity). From [13].

It was found that the Munc18-1 domain-1 is essential for syntaxin binding and mutations in that region confer aberrancies in vesicle priming because the Munc18-1

chaperoning function is impaired [194]. In line with this, it was seen that L46 (Leu46) and E59 (Glu59) in domain 1 (of Munc18-1 and -2) are important for the interaction with syntaxin-3, thereby mediating its chaperoning [195]. On the other hand, mutations in the Munc18 domain-3 abrogated exocytosis but they did not interfere with vesicle docking or syntaxin-1 localizing to the plasma membrane. This domain may thus serve a distinct or additional function to chaperoning, being implicated in syntaxin-1 stabilization and vesicle priming for fusion [196]. More detailed, some point mutations in the domain-3a suggested that this part has a role in granule exocytosis but less so in syntaxin localization, with a particular emphasis on amino acid P335 being key for chaperoning syntaxin-1 [197, 198]. Another study added that a stretch in the Munc18-1 domain-3a also provides the interaction surface for VAMP2 [199].

The interaction between the N peptide of syntaxin-1 and Munc18-1 was found to be conserved among several SM protein/syntaxin pairs, for example in the syntaxin4-Munc18-3, or syntaxin3-Munc18-2 interactions [200], but it is not clear how many of the syntaxin-SM protein interactions are characterized by both binding modes [201]. Despite considerable controversy in the field, many researchers appreciate that the interaction with the N peptide is not an optional binding possibility [202]. Hydrophobic pocket mutants on Munc18-1 or Munc18-2 both showed impaired interaction with syntaxin-11 [193], suggesting that syntaxin-11 might depend on its N peptide for proper Munc18 interaction. In *C. elegans*, lack of SM protein interaction (due to a mutation in the hydrophobic pocket) with the corresponding syntaxin homologue N peptide abrogated syntaxin binding, affected exocytosis and produced a classical uncoordinated (UNC) phenotype [203] with loss of locomotion in these worms [204]. Interestingly, in this study, a constitutively open syntaxin mutation ("LE mutation", syntaxin-1 p.LE165,166AA) [178] did not produce an UNC phenotype and was thus assumed not to play much of a role in exocytosis in *C. elegans*. Another study however [205] showed that syntaxin N peptide point mutations (L8A or D3R) did also impair binding to Munc18-1 but rescued secretion in syntaxin-1 deficient PC12 cells (a neuroendocrine cell line). On the other hand, the combination of these point mutants + LE mutation could not. In line with the previous study, in this study, the LE mutation alone rescued secretion well, too, despite considerable protein mislocalization. The N peptide was thus suggested to play an important role in interaction with Munc18-1 and trafficking of syntaxin-1 but it might be less important for exocytosis. Genetically modified worms subjected to the N peptide point mutants + LE mutation produced egg-laying defects, slower locomotion and slower growth rates. In Munc18-1 and -2 double knockdown neurosecretory cells, it was found that a mutant of Munc18-1 that cannot bind to the closed conformation of syntaxin-1 could not rescue syntaxin-1 expression, localization or secretion. This was in contrast to mutations which abolish N peptide binding. These could rescue the phenotype [206]. Thus, it was concluded that, in this system, the N peptide interaction was not essential for exocytosis. Another study showed that the minimal complement a syntaxin protein has to possess in order to mediate fusion (in an *in vitro* liposome fusion assay) is the N peptide and the SNARE domain; all other features might thus possibly have other roles for syntaxin. The authors suggested that the big groove found in Munc18-1 can in these complexes likely be occupied by the heterotrimeric SNARE four-helix bundle instead of the monomeric Habc-SNARE bundle provided by the syntaxin alone, and it may be plausible that these bundles might compete for the Munc18 groove also in a physiological setting [207]. Some authors suggested that the N peptide could compete with the closed syntaxin homologue conformation for binding to the Munc18 homologue protein [208]. These

and other studies could hint towards a scenario of interaction mode hierarchies, or sequential regulation of binding modes. For syntaxin-4, in one study the N peptide was found negligible for its targeting, but important for its stabilization at the basolateral membrane in epithelial cells and this was dependent on the interaction with Munc18-3 [209]. However, an *in vitro* study on Muc18-3 and syntaxin-4 found the N peptide was needed for binding, whilst for syntaxin-1 / Munc18-3 the N peptide was not necessary [210]. Thus, syntaxin-4 and syntaxin-1 could employ different modes of binding, with syntaxin-1 found in closed and open conformation (the N peptide would promote the open conformation), whereas syntaxin-4 would be mostly or only found in open conformation when bound to Munc18-3. Another study [211] interestingly found that Munc18-1 and -3 could also recognize different parts of VAMP proteins, here shown for VAMP2. One study showed that the N peptide can have a role in recruitment of SM proteins to the syntaxin [190]. The authors saw that the N peptide recruited the SM protein, also if it was fused to other SNARE proteins, or if the spacing between the SNARE domain of syntaxin-1 and the N peptide was changed. Surprisingly, when cleaving off the N peptide after the binary complex is formed, it did not manipulate fusion any more, hence once the complex is formed, the N peptide could be negligible for fusion. In total, they concluded that the N peptide functions as initiator for the SNARE-SM membrane complex. That can be true, however, *in vivo*, this initial recruitment of the SM protein to the syntaxin likely has to occur upstream of the plasma membrane stage of syntaxin-1 (see chapter **1.4.3**). How or if syntaxin-11 and Munc18-2 interaction is dependent on a functional N peptide is not known. Also, the mechanism of how syntaxin-11 Habc domain mutations can cause disease is not well understood, because very little is known about the exact interaction modes of this specific SM / syntaxin pair.

1.4.3 Trafficking of syntaxins: dependence on Munc18

In Munc18-1 deficient chromaffin cells, vesicle docking was abolished and syntaxin-1 levels were decreased [14, 16]. Interestingly, docking could be compensated for when expressing Munc18-2 instead, but the vesicle priming steps were then partially aberrant. Also, in this model, syntaxin-1 trafficking to the plasma membrane was dependent on co-expression of Munc18-1 or -2 [16]. Along those lines, Munc18-1 was also required to target syntaxin-1 to the plasma membrane in PC12 cells, but not for SNAP25 targeting [212]. The stimulatory function despite binding to the closed syntaxin conformation is in line with previously contradictory observations showing that Munc18 prevents SNARE complex assembly if incubated with compatible SNARE-vesicle populations before mixing the vesicle populations [10]. The binding to the closed conformation could thus stabilize the syntaxin when it is not implicated in membrane fusion, for example while it is trafficked to its terminal subcellular compartment. Throughout its trafficking the syntaxin mustn't undergo SNARE complex formation. In one study in which it was permitted so, syntaxin-1 did not reach the plasma membrane, and neither did SNAP25. The interaction syntaxin-1-Munc18 thus probably has to occur before it meets SNAP25 on the Golgi complex. The initial interaction was found to be dominated by Munc18 binding to the Habc domain of syntaxin, rather than the N peptide [213]. Regarding stability of syntaxin, also studies on baker´s yeast showed that the SM protein Vps45 was important to stabilize the syntaxin homologue Tlg2p as this was rapidly degraded by the proteasome when Vps45 was absent [214, 215]. Trafficking of syntaxin-1a was also

found to be dependent on co-expression of Munc18-1 in polarized epithelial cell lines. In this system, Munc18-2 could equally well traffic syntaxin-1a to the surface [216]. It is likely that syntaxin-11 depends on Munc18-2 for its trafficking but that has not been addressed in detail yet.

1.4.3.1 Concluding notes on Munc18

It should be noted that not all functions of Munc18-1 are in one way or another connected to syntaxin-1 interaction. Analysis of a series of mutations from Munc18-1 homologues from different model organisms showed that some mutations clearly produced a phenotype but they did not affect the syntaxin-1 interaction. Thus, it became clear that also other protein interactions could be important for Munc18-1 function [217]. It was concluded that Munc18-1 controls various stages of exocytosis, including syntaxin-independent steps. One study found that Munc18-1 must exhibit an essential mechanism for survival of neuronal cells. Munc18-1 mutant mice grew normal neuronal networks which formed synapses, but these degenerated over time. *In vitro*, neuron survival was prolonged for some days when adding external trophic factors but the degeneration occurred inevitably [218]. Long-term survival of neurons is thus not directly coupled to exocytotic activity, but to expression of Munc18-1.

1.5 MUNC13 AS POSSIBLE MODIFIER OF THE SYNTAXIN – MUNC18 COMPLEX

Munc13 proteins mediate a step immediately upstream of fusion, termed vesicle priming. Munc13 deficiency can still allow for vesicle docking to the plasma membrane but fusion is impaired. [219-221]. Munc13-1 and -2 deficient neurons can still form morphologically normal synapses [222]. An important insight came from a *C. elegans* study showing that a constitutively open form of syntaxin abolished the necessity of UNC13 in the priming of synaptic vesicles [223], which pointed towards a role of Munc13 proteins in supporting the opening of the syntaxin to render it capable of mediating fusion. This is in line with another report referring to another protein implicated in vesicle priming, CAPS1, which was found to bind well to “open” syntaxin whereas Munc13-1 was preferentially found to bind to the N terminal 80 amino acids of syntaxin-1, rather than the entire recombinant protein (without transmembrane domain) [224]. In line with the potential impacts on syntaxin conformation, Munc13 and Munc18 have both been shown to interfere with the SNAP25-syntaxin-1 binding stoichiometry. Addition of both promoted a binary 1:1 complex, as opposed to an aberrant 1:2 (SNAP25:syntaxin) stoichiometry; and this binary complex supports syntaxin-1 shifting towards an open, more accessible conformation [225, 226]. In summary, Munc13 could accelerate the transition of the binary syntaxin-Munc18 complex to the SNARE complex. Some authors mapped this function to a particular domain, the Munc13 MUN domain (Munc13-1 amino acids 859–1531, [227], which directly interacts with the syntaxin-1 SNARE domain. Similarly, Munc13-4 has been shown to interact with syntaxin-1, -4 and -11 SNARE domains. Physically, the Munc13-syntaxin interaction appeared not to be a very strong interaction, but it seems to be sufficient to fulfil this essential role in exocytosis [228-232]. What role this interaction plays during exocytosis is not certain, but a delicate scenario can be envisioned incorporating several findings. For example, the big Munc18 binding groove (between domains 1 and 3a), initially binding the syntaxin Habc and SNARE domain, could compete with the weak Munc13 interaction for the SNARE domain and eventually the Munc18 could allow the Munc13 to dissolve the SNARE domain from the folded Habc / SNARE bundle, allowing for subsequent SNARE complex formation. The big Munc18 groove could then possibly bind the terminal SNARE complex [207] instead of the syntaxin Habc domain, even if these interactions may not rely on exactly the same surfaces [233]. Throughout these steps, the N peptide of the syntaxin could continuously be bound to the hydrophobic pocket of the Munc18 protein, ensuring that the chaperone stays associated to the syntaxin, even if it may be negligible for fusion after the initial interaction [190]. Munc13 might thus promote the open syntaxin conformation and interfere with a re-closing of the syntaxin until it can form a SNARE complex with other SNARE proteins.

1.6 SYNTAXIN-11

Syntaxin-11 is encoded by the gene *STX11*. The syntaxin-11 amino acid sequence displays considerable homology to syntaxin-1, hence the basic protein architecture is probably similar. Syntaxin-1 comprises several functionally important domains. These are the N peptide, the Habc domain, a linker domain (not known to assume a particular secondary structure), a SNARE domain, and a membrane anchor domain, which in the case of syntaxin-1 is a transmembrane domain. Syntaxin-11 does not have a transmembrane domain, but it can get palmytoylated and this confers its membrane anchorage [234-237]. Syntaxin-11 in fact is tightly bound to membranes as it was not resolved from membrane fractions with high salt or high pH, and only partially (approximately 70-90% of total) with Triton-X-100. Recombinant syntaxin-11 can bind to VAMP2 and SNAP23, but not syntaxin-2 [234, 235]. In human platelets, that syntaxin-11 – SNAP23 interaction was confirmed, and it also bound to VAMP8 and VAMP3, but not syntaxin-4 [238]. Also, it was shown to interact with Vti1b, another SNARE protein implicated in intracellular fusion processes such as endosome – lysosome fusion [239].

Syntaxin-11 was first described and characterized around 1998. Syntaxin-11 mRNA was most abundant in human lung, placenta and heart, somewhat less in liver, pancreas, kidney and skeletal muscle, and almost absent in brain tissue lysate [240]. This tissue distribution was also seen in another syntaxin-11 study on mRNA and protein level [234]. Within immune populations evaluated to date, syntaxin-11 was observed in cytotoxic lymphocytes [32, 236], monocytes and macrophages [22, 239, 241], neutrophils [242], platelets [238], and B lymphoyctes [234]. Deficiency in syntaxin-11 has been associated with degranulation defects in the cytotoxic lymphocytes and development of FHL4 [22, 32, 243]. Of note, a few exceptional FHL4 patients were recorded with the ability to degranulate, albeit to a lesser extent than controls [244]. In healthy cells, cytotoxicity was suggested to correlate with syntaxin-11 expression levels, and that is for example mirrored in the functional maturation of CTL, which is accompanied by cell activation and this in turn has been shown to upregulate syntaxin-11 levels in human CTL [236, 245]. Syntaxin-11 (and Munc18-2) is part of the membrane fusion complex in cytotoxic lymphocytes [245]. In line with that, FHL4 (and FHL5) patient lymphocytes form normal conjugates with target cells and cytotoxic granule polarization towards the immunological synapse is morphologically normal [23, 32, 245]. FHL4 patient cells have been shown to partially gain back cytotoxicity upon IL-2 stimulation [32]. Presumably, most FHL4 patients are absolutely deficient of syntaxin-11 expression and / or function. However, theoretically, in individuals who might harbor milder mutations which preserve residual protein functionality, the IL-2 induced gain in function (also seen in healthy cells, Figure 5) could be supported by syntaxin-11 levels being upregulated upon thorough pro-inflammatory cytokine stimulation ([241, 246] and Figure 6).

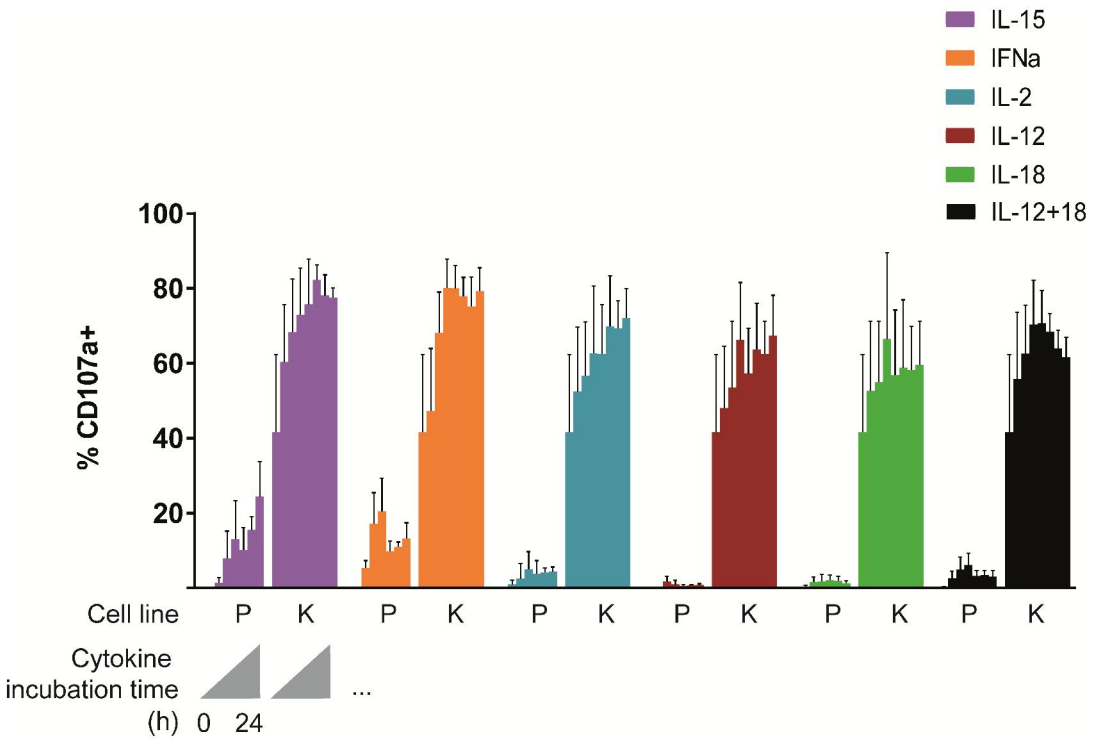

Figure 5. (Healthy) NK cell degranulation in response to K562 cells (K) after different pre-incubation times (schematically indicated by grey triangle) with various pro-inflammatory cytokines (color code). As control, a mouse cell line which does not induce NK cell degranulation by itself is shown (P815 cells, P). Whole PBMC were isolated from 3 healthy donors and pre-incubated with the cytokines for 8 different time points (0, 2, 4, 6, 8, 12, 16 or 24 h, respectively). Within the last two hours of the time course, the PBMC were mixed with either P815 or K562 cells and co-incubated for 2 h. The degranulation (CD107a exposure on the NK cell surface) assay was hence performed within the final 2 h of the time course. Each group of P815- or K562-stimulated PBMC shows one bar per time point, averaged over the three donors. Error bars indicate SD.

In general though, and this is not well understood, syntaxin-11 deficient cells are readily recovering their function upon pro-inflammatory cytokine treatment, much more so as compared to other FHL patient cells. It was suggested that this could contribute to the fact that FHL4 patients present with a later age of onset and sometimes a milder disease course (see chapter **1.3.1**) [138, 243]. A number of syntaxin-11 variants and mutations have been found meanwhile, spread over the entire protein sequence. One interesting mutation is for example syntaxin-11 p.Q268X, which is truncated immediately downstream of the SNARE domain (see Figure 8 and [22, 243]). This mutation is very useful to determine trafficking steps of syntaxin-11 and analyze protein-protein interactions, as it is linked to a profound degranulation defect, but the interactions of the syntaxin N terminal domains could be undisturbed.

A syntaxin-11 knockout mouse model provided data that loss of syntaxin-11 did not lead to developmental defects in immune cell populations, and similarly, our group has not observed any FHL4 patients with apparent immune system development abnormalities ([246] and unpublished obs). No impairment of macrophage or dendritic cell phagocytosis or cytokine secretion was observed. Mast cell degranulation was also not perturbed. However, an exocytosis defect was seen in these mice in the cytotoxic lymphocytes as well as in neutrophils, consistent with what is known from syntaxin-11 expression patterns and FHL4 patients.

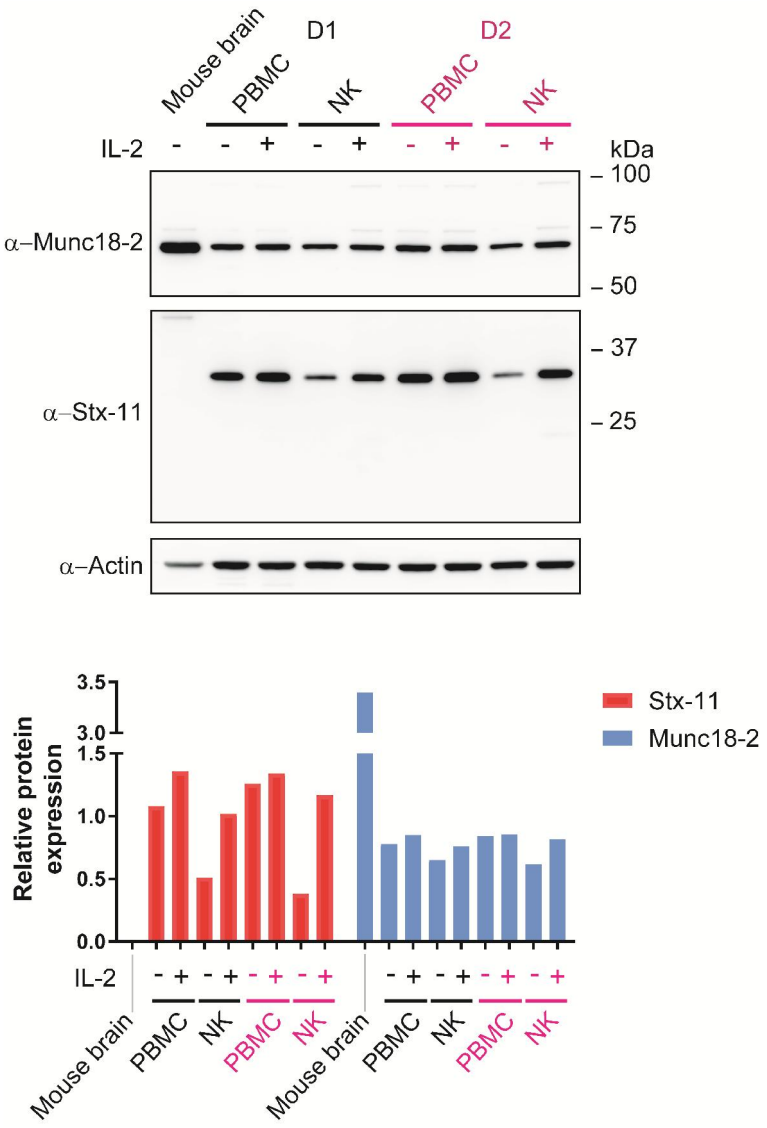

Figure 6. Western blot and corresponding densiometry on healthy PBMC and NK cells from 2 representative donors (black or raspberry, respectively). As indicated, some of the cells had been stimulated with 500 IU/ml IL-2 for 48 h. For comparison, a whole tissue lysate of a normal mouse brain was loaded, which does not express syntaxin-11. For densiometric analysis, protein levels were normalized to actin. D1, donor 1; D2, donor2.

Syntaxin-11 is also mediating degranulation in platelets [238]. However, in some immune cells syntaxin-11 can have different purposes. In human monocytes, it was reported that syntaxin-11 expression negatively correlates with phagocytic activity and TNF secretion [241]. This could be relevant for the HLH context, and also add to the observation that FHL4 patients can persist without developing the disease for a longer time than other FHL patients, as they might possibly clear necrotic debris in infiltrated tissues better than other FHL patients. Remarkably, in macrophages, syntaxin-11 was reported to regulate endosome-lysosome fusion events via sequestering Vti1b away from forming SNARE complexes with other syntaxins, such as syntaxin-6 and -7, or -7 and -8. This sequestration prevented accumulation of large endocytic compartments and lysosome tubulation in healthy cells. In syntaxin-11 knockdown cells, the late endosome-lysosome fusion (maybe also fission) was abnormal whilst trafficking and exocytosis of lysosomes was increased [239]. If syntaxin-11 possesses additional roles besides cytotoxic granule fusion in cytotoxic

lymphocytes is not known. The more we can learn about the syntaxin-11 interactome and the molecular details of these protein-protein interactions the better we can understand how syntaxin-11 acts, how it is regulated by its physiological environment, and how certain mutations but not others do impair particular aspects of its biology.

1.6.1 About syntaxin-11 localization

When overexpressed in NRK cells (a rat kidney cell line) the protein localized in a juxtanuclear region, and, surprisingly, when the C terminus downstream of the predicted SNARE domain was abolished, the protein still localized comparable to the *wt*. However, disposal of C terminus + SNARE domain caused a random cytoplasmic localization [235]. Another study using overexpression in HeLa cells found it to localize similarly as the cation-independent mannose-6-phosphate receptor (M6PR), and thus syntaxin-11 was ascribed to reside on the trans-Golgi network and late endosomes [234]. However, in activated human monocytes, syntaxin-11 was not found to associate with Golgi structures. Upon phagocytic activity, syntaxin-11 was seen at the plasma membrane [241]. How exactly it localizes in freshly isolated human cytotoxic lymphocytes, is not completely understood, but in intracellular stainings on endogenous proteins in non-conjugated cells, we have not observed it in the plasma membrane, and this is in line with observations on transfected NKL cells (an NK cell line) (see [247] and Figure 3). Similarly, in activated NK cell clones, the protein was reported on intracellular vesicular structures [236]. Besides, we have also not seen it pronouncedly co-localizing with cytotoxic granules (Figure 3), which was suggested in an overexpression system in human CTL [245]. However, these data could rather indicate exocytic protein cluster formation right under the arriving cytotoxic granule (see [4]), as it was imaged in TIRF microscopy in transfected CTL, employing stimulatory (anti-CD3 / -28) coated glass surfaces. The same article suggested that syntaxin-11 is mostly found on Rab11a+ recycling endosomes in such overexpressed CTL, and when a synapse with a target cell is formed, it is recruited to the synapse prior to cytotoxic granules [245]. This is a reasonable assumption, as a lymphocyte does not “know” where it will form the next synapse, and thus would instead deliver the necessary components on demand. In resting human neutrophils, syntaxin-11 distributed over plasma membrane, tertiary, secondary and azurophilic granule fractions. However, when the cells were activated with PMA, they degranulated and syntaxin-11 shifted more towards the plasma membrane and partially remained on azurophilic granules, whereas it left secondary and tertiary granules [242]. In macrophages, syntaxin-11 was found on endosomal and lysosomal membranes and a small fraction associated with the plasma membrane [239]. Insights into the trafficking of syntaxin-11 on subcellular compartments, if and how it is dependent on interactions with Munc18-2, and which fusion machinery targets syntaxin-11 to the plasma membrane on demand will provide important information about syntaxin-11 biology in cytotoxic lymphocytes, give clues on potential disease-causing mechanisms, possibly identify novel interaction candidates and maybe elucidate if the protein does fulfil other functions in these cells besides cytotoxic granule exocytosis.

1.7 VAMPS

To date, it is not clear which member of the VAMP family mediates fusion of the cytotoxic granules with the plasma membrane, and thus serves as the R-SNARE in fusion complexes. Several VAMPs were and are discussed as candidates; for example VAMP2, VAMP3, VAMP4, VAMP7 and VAMP8. VAMP2 was a strong R-SNARE candidate after a report on mouse CTL showed that it localized on cytotoxic granules and ablation or inhibition of VAMP2 abrogated degranulation [248]. In these cells, VAMP3, -4 and -7 did not co-localize with granzyme B and thus may serve other purposes in mouse CTL. However, VAMP2 protein was not detectable in human cytotoxic lymphocytes in our experiments (unpublished obs.). Similarly, a study on human platelets found no expression of VAMP1 or -2. Instead, VAMP3 and -8 were expressed, co-immunoprecipitated with syntaxin-4 and SNAP23, and both were required for granule secretion [249]. VAMP3 and Rab11a have also been implicated in cytokine release in an NK cell line (NK92). Of note, there was a reduction in perforin release upon inhibition of RE trafficking, but that was not significant [250]. Other authors found VAMP3, -4, -5 and -8 proteins expressed in human CTL, with VAMP4 and -8 showing a (partial) immunological synapse localization [251]. VAMP7 was found on the mRNA level, but no staining was shown. In an NK cell line (YTS), VAMP4 and -7 were both observed to be important for cytotoxic granule exocytosis, and interestingly, VAMP7 was also implicated in cytokine release. Moreover, VAMP7 was also found in areas partly overlapping with cytotoxic granules in non-conjugated cells, whilst VAMP4 was only found to overlap with perforin when the cells were in conjugates. VAMP7 might thus serve in several transport processes in cytotoxic lymphocytes, and VAMP4 may support a transport step specifically triggered upon and required for cytotoxic granule release. However, there was no evidence that particularly the cytotoxic granule-to–plasma membrane fusion is perturbed in these cells lacking either of these VAMPs, as the reduction in degranulation could in theory stem from other fusion processes for example preceding cytotoxic granule exocytosis [252]. VAMP4 has been implicated in intracellular transport processes, mostly trans-Golgi network to endosome transport, and it was additionally found on clathrin-coated and uncoated vesicles, endosomes and immature secretory granules in PC12 cells [253]. Also, VAMP7 was suggested to play a role in transport of triacylglycerol between ER and Golgi apparatus in intestinal endothelial cells [254].

Some of the VAMP proteins may thus serve several functions in the same cell. The exact roles of VAMP3, -4, -7 and -8 in human cytotoxic lymphocytes deserve revisiting, as it is still not completely understood what function each of them has, or if they are partly redundant. All of them were found to be expressed in a rat mast cell line (RBL cells), and VAMP8 was suggested to form a complex with syntaxin-4 and SNAP23 to mediate exocytosis of secretory granules (lysosome-related) [255]. VAMP8 was found to form a complex with syntaxin-11 and SNAP23 in platelets [238], and moreover, in mice, VAMP8 was co-localizing with cytotoxic granules, found to be important for their exocytosis and target cell killing, and on top of that also cytokine release in CTL [256, 257]. Cytotoxic granule and MTOC polarization in conjugates were normal in VAMP8 KO CTL, implicating that upstream granule maturation and recruitment steps were not affected. These findings raised the question whether VAMP8 is expressed in human CTL, where it localizes and whether it could function in cytotoxic granule exocytosis directly, for example as the R-SNARE in the fusion complex.

2 AIMS OF THE THESIS

This project focuses on gaining mechanistic insights on how variants and mutations in FHL genes can interfere with exocytosis, abrogate cytotoxicity and cause disease. In the known FHL genes, mutations have been found all over the sequences and also, the genetic states of FHL patients are highly variable. Thus, protein properties can be affected to a variable degree, which translate into altered protein interactions, localization, stability or other parameters determining efficiency of exocytosis. Besides the mechanistic understanding on how FHL variant proteins can lead to aberrant exocytosis, there is still a lack of knowledge about the *wt* proteins, regarding for example their trafficking. Thus, we also aimed at characterizing the trafficking of *wt* exocytosis-associated SNARE proteins VAMP8 and syntaxin-11.

Paper I

- Follow-up on a particular FHL5 patient case (Munc18-2 p.Q432X and p.S545L)
- Evaluate Munc18-2 protein levels in patient PBMC
- Histopathology to clarify diagnosis of classical Hodgkin lymphoma
- Characterize functionality of tumor-infiltrating and peripheral lymphocytes

Paper II

- Evaluate cytotoxicity and cytotoxic granule integrity of FHL4 patients
- Understand how N-terminal mutations in syntaxin-11 can impact protein expression and interaction with Munc18-2
- Explain how an N-terminal mutation in syntaxin-11 can cause disease (syntaxin-11 p.L58P)

Paper III

- Understand the role of VAMP8 in cytotoxic granule exocytosis
- Gain insigth into the trafficking route of VAMP8
- Study if there is a relationship between VAMP8 trafficking and synatxin-11 trafficking

3 RESULTS AND DISCUSSION

3.1 PAPER I

The study contains clinical and functional follow-up data on an FHL5 patient, who had previously suffered from active HLH, and had at the time been reported with the clinical parameters [129]. That HLH flare had initiated with prolonged fever, abdominal pain and sore throat. The patient reported decreased ability to breathe and adenoids as well as tonsils were removed or abraded. This however yielded severe bleeding which was accompanied by reduced platelets. The patient was thus given platelet infusions, coagulation factors, fibrinogen concentrate and, due to hypogammaglobulinemia, intravenous immunoglobulin (IVIG). The patient subsequently progressed towards renal failure, and had to undergo renal replacement therapy. Besides, the patient was tested EBV+. Heterophile antibodies as well as a high EBV DNA copy numbers were detected. Hence, rituximab was administered which helped to improve the patient condition. There was no hemophagocytosis detected in a bone marrow aspirate of the patient. When the authors tested for NK cell function, they found a profound degranulation and target cell killing defect. However, the patient partially regained function after 3 days of IL-2 incubation. As the lack of NK cell function had been the final criterion for being diagnosed with HLH, treatment according to the HLH-94 guidelines was started [258]. The patient received etoposide (a chemotherapeutic drug) and betamethasone (a corticosteroid). The therapy stabilized the patient substantially, and the protocol was reduced to administering corticosteroids and IVIG, as antibody titers remained low. However, the patient kept suffering from recurrent flares of abdominal pain, vomiting and diarrhea. Another virus infection was detected (Calicivirus) which however cleared rapidly. The patient had also developed a colitis which could be successfully treated. The hypogammaglobulinemia persisted though, and thus IVIG was administered throughout the following symptom-free time. About two years later, the patient presented to the clinics again, and we wanted to follow up on this particular case [259]. This time the patient presented with a fast-growing lateral swelling on one side of the neck (**Paper I, figure 2**). A localized lymphadenopathy was found and the affected lymph nodes were removed. When analyzing that tissue, the markers EBER and PAX5 were detected, reconfirming the presence of EBV. The tissue was also tested regarding the possibility that the swelling was induced by a rapidly progressing lymphoma. The hematoxylin-eosin staining revealed a high lymphocyte infiltrate and some cells morphologically resembled Reed-Sternberg Cells (RSC, see also Figure 2). Further analysis confirmed this, as these enlarged multinuclear cells stained positively for PAX5, CD30 and CD15, which indicate EBV+ RSC. Specific stainings for CD3, CD8, perforin, granzyme B and CD68 confirmed high numbers of infiltrating cytotoxic T cells and macrophages. These findings supported the diagnosis of classical Hodgkin lymphoma.

Because of this second clinical episode, we decided to look at the protein expression of Munc18-2, since it was not clear how stable Munc18-2 would be, as the patient carries a biallelic Munc18-2 mutation (p.Q432X and p.S545L, see [129], table 1). Therefore, a Western blot was performed on patient PBMC (**Paper I, figure 1**).

Protein levels were dramatically reduced, almost to the threshold of detection. Interestingly, this did not change when the PBMC had been incubated in high amounts of IL-2 overnight, which is sufficient to achieve partial recovery of cytotoxicity. The protein level of syntaxin-11 had not been assessed at the time. Syntaxin-11 and Munc18-2 interact with each other ([169], **Paper II, figure 4e** and Figure 7), and both proteins are important for cytotoxic granule exocytosis, similarly to the syntaxin-1 – Munc18-1 interaction determining neurotransmitter release.

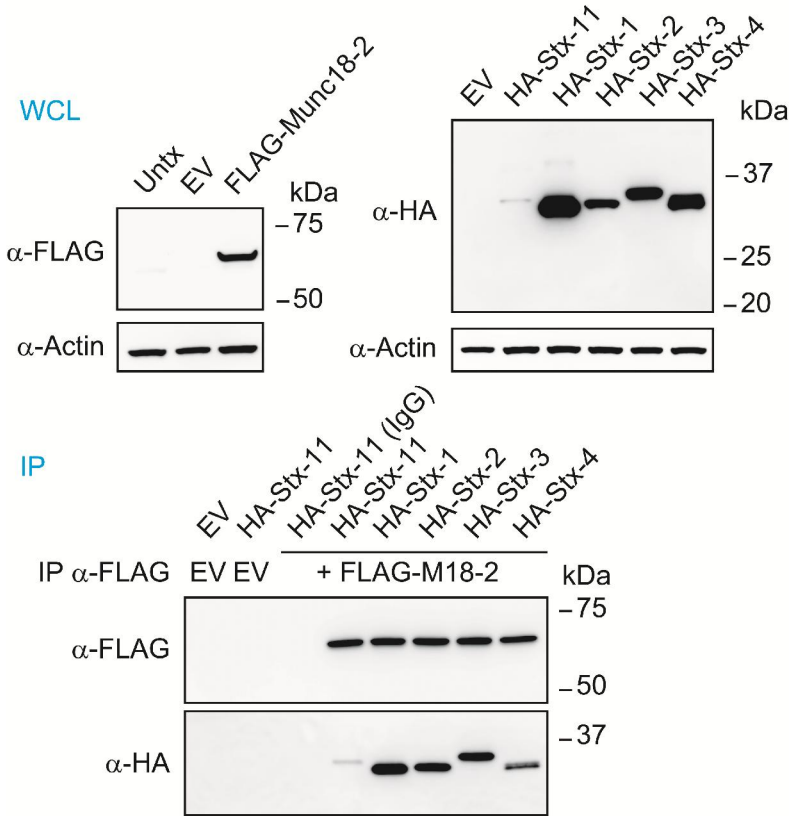

Figure 7. Co-immunoprecipitation experiment addressing the promiscuity of Munc18-2 – syntaxin interaction. HA-tagged syntaxin-1 to -4, all of which are closely related to syntaxin-11, were overexpressed in HEK293 cells. FLAG-tagged Munc18-2 was overexpressed in another batch of HEK293 cells. The FLAG transfectants were lysed, and the IP was performed with a monoclonal α-FLAG antibody. The bead-bound α-FLAG IP was washed and equally distributed for the Co-IP reactions. Subsequently, the whole cell lysates of the syntaxin transfectants were added. The upper Western blots show the whole cell lysates, the Co-IP is shown in the lower panel. WCL, whole cell lysate; IP, immunoprecipitation; Untx, untransfected; EV, empty vector.

It has been observed in numerous FHL5 patients that loss of Munc18-2 results in reduced levels of syntaxin-11 [23, 169, 171]. This is somehow reminiscent of Munc18-1 stabilizing syntaxin-1 [260]. It could be that FHL5 patients, who lose both proteins, suffer from the degranulation defect mostly because they lose syntaxin-11, which could be degraded due to the lack of protection from its chaperone. A constitutively open version of syntaxin-1 produced greatly reduced interaction with Munc18-1 and the syntaxin was more susceptible to trypsin-mediated cleavage [261]. However, this has not been addressed in molecular detail. Analysis of intrinsic FHL protein stability is complicated by the fact that for example variants of syntaxin-11 which are dramatically destabilized in patient cells (**see Paper II, figures 3, 4**) can be perfectly transiently overexpressed in cell lines as well as primary cytotoxic

lymphocytes. Such overexpression systems might thus not mirror physiological conditions very well. An optimal system copying physiological conditions is currently not available. Ideally, such a test system would be based on 1) primary cells and expression levels, 2) inducible (to gain insights into expression kinetics and maybe turnover times, and compare physiological parameters before and after expression), and c) subcellularly traceable to simultaneously understand how they traffic, and if they ever reach the membrane upon conjugate formation with a target cell. The impact of Munc18-2 binding on syntaxin-11 stability has to be addressed in more detail in the future.

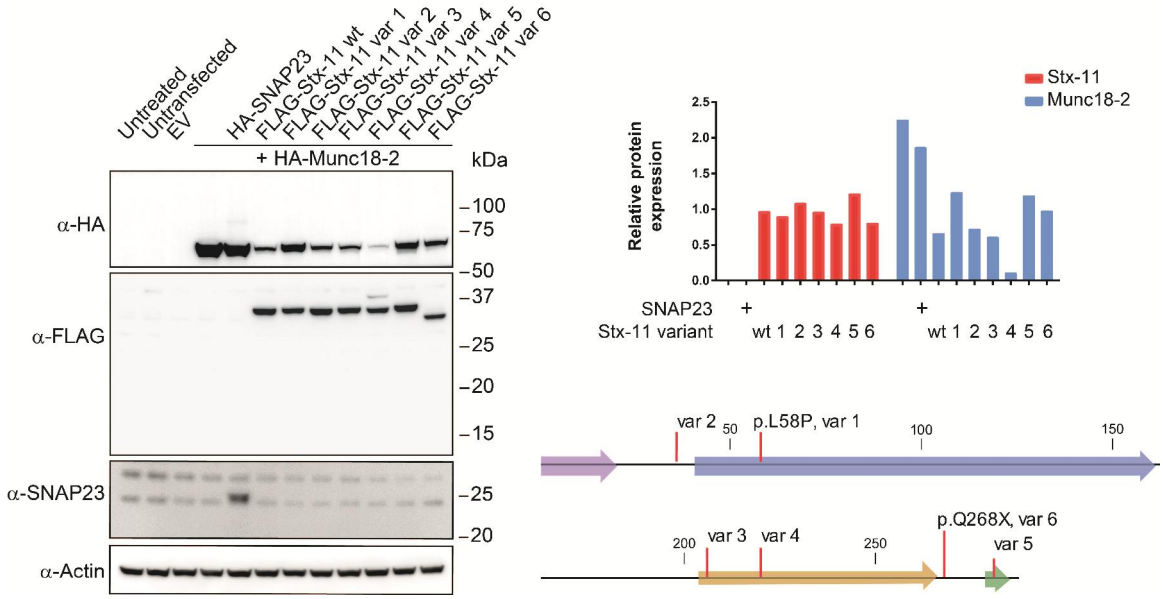

Figure 8. HEK293 cells were co-transfected with HA- or FLAG-tagged Munc18-2, SNAP23, syntaxin-11 *wt* or variant constructs, as indicated. The cells were lysed and corresponding protein amounts were loaded. The membrane was blotted for the tags, HA or FLAG, as well as actin. The corresponding densiometry is shown on the upper right. Protein levels were normalized to actin. In the lower right corner, a schematic syntaxin-11 overview is given, with the variant positions indicated. In raspberry, the N peptide is indicated, the purple stretch refers to the Habc domain, orange indicates the SNARE domain and green marks the palmytoylation sites for membrane anchorage. The two mutations which are later on described in PAPER II are given with the amino acid exchange. Untreated: No lipofectamine. Untransfected: Cells had been treated with lipofectamine. EV, empty vector; var, variant.

Also, an important side observation highlights that the interaction between syntaxin-11 and Munc18-2 may not only impact stability unidirectional. Not only does Munc18-2 determine syntaxin-11 levels, but we saw that depending on the version of syntaxin-11 being co-expressed with *wt* Munc18-2 in a cell line, the levels of Munc18-2 can also vary greatly (Figure 8). In summary, we can currently interpret that the quality of interaction, which is to some extent determined by both proteins, could dictate protein stability, availability for SNARE complex formation and efficiency of exocytosis. The other protein assessed in the Western blot, Rab27a, is linked to a HLH-like disease, Griscelli syndrome type 2. Rab27a is not known to interact with syntaxin-11 or Munc18-2. But it plays a role in cytotoxic granule tethering and exocytosis at the immunological synapse; and for these functions it needs to interact for example with Munc13-4 or Slp2a, wherein Slp2a might impact Rab27a recruitment to cytotoxic granules and the immunological synapse [262-265]. Though this may not be surprising, it is helpful to confirm that interruption of the Munc18-2 - syntaxin-11 interaction and the subsequent lack of exocytic activity likely does not downregulate protein levels of other exocytosis-related genes [266].

An intriguing question is whether cytotoxic granule exocytosis is perceived by the cell and accordingly if granule or exocytotic protein availability is controlled and adjusted. This exciting question remains to be addressed in detail, but from the current and other FHL patients, we can at least deduce that biogenesis of cytotoxic granules cannot be coupled to FHL protein function or expression, which in turn of course determines exocytosis capability. Hence, even if the lymphocytes sense the lack in exocytic activity, one interesting difference to neurons is that lymphocytes apparently do not need Munc18-2 expression or exocytosis for long-term survival, as opposed to neuronal cells being dependent on Munc18-1 expression [218]. Of course, a decrease in degranulation and killing might also stem from a further possible complication which might influence other proteins (expression levels and / or distribution). That is, for example, if the syntaxin-11 / Munc18-2 interaction would have additional roles in intracellular fusion processes affecting organelle maturation or sorting of lysosomal cargo. We briefly looked into this question in **Paper II, figure 2**, where we did not observe abnormalities in granule constituents upon syntaxin-11-related degranulation deficiency [267].

A new assessment of patient cell degranulation and cytotoxicity displayed similar results as in the first patient report. This time, not only K562 were used as targets, but also stimulation via monoclonal antibodies activating CD16, or CD3, respectively, in a redirected lysis assay (**Paper I, figure 3 B-E**). This is possible because P815 cells are not killed by NK cells, but they express Fc receptors and can thus bind the monoclonal antibodies directed against lymphocyte activating receptors (CD16, and CD3), cluster them and induce degranulation and target killing. The patient cells gained cytotoxicity upon IL-2 pre-incubation, against K562 and following CD16 stimulation. The same held true for cytotoxic T cells. This corroborated and widened the previous findings. It also shows that the compromised peripheral blood lymphocytes do not alter functionality on the long run, due to for example medical treatment or the state of inflammation in the surrounding tissues, as it has been postulated for some FHL patients [127].

In general, a few FHL5 patients, but even more so, literally all FHL4 patients have been observed to partially gain back degranulation and killing ability after pro-inflammatory cytokine incubation. The same effect has been well established in healthy cytotoxic lymphocytes, and can be achieved by several pro-inflammatory cytokines (Figure 5). Nevertheless, as the FHL patient records clearly show, *per se*, cytotoxic granule exocytosis is not redundant and cannot be compensated for during the initial phase, the trigger, of disease *in vivo*. Otherwise the patients should be able control the pathologic stimulus and a hyperinflammation would never be established. Actually, the non-redundancy comprises cytotoxic granule exocytosis as well as proper detachment from the target, as this was shown to play a key role in the high production of pro-inflammatory cytokines [133]. The pro-inflammatory cytokines at some point might reach sufficient local tissue concentrations to aid the lymphocytes to partially regain function, but at that point the hyperinflammation might already be independent of the initial trigger, where other mechanisms such as self-maintaining inflammation with limiting IL-2 availability and shift of immune populations might dominate. Hence, *in vivo*, as opposed to the initial phase of the disease, once the HLH is fully ongoing, it may not matter anymore whether the lymphocytes gain some functionality back due to elevated cytokines (like, for example, the cells of the patient of this study possibly could, in highly inflamed tissues), and eventually could even limit the initial infectious trigger.

From a cell biological point of view, the observed cytokine effect is a very interesting phenomenon, because it highlights a) adjustability of the process, and b) the elevation of lymphocyte function must be independent of the respective FHL protein. Regarding this compensatory capability, it should be noted that there is a grading over different FHL genes, with syntaxin-11 being most easily compensated for, but also for a number of FHL5 patients this has been observed [32, 129]. The ease of such compensation in FHL4 cells as opposed to other FHL genes could also explain why FHL4 patients develop the hyperinflammation later than other FHL patients [32]. One group suggested that the compensation in FHL4 and FHL5 may be ascribed to upregulation of other syntaxins, or other Munc18 proteins, respectively. This has been observed in lymphocytes stimulated with high doses of cytokines for several days [268], and we and others have observed that Munc18-2 can indeed interact with several syntaxins closely related to syntaxin-11 (Figure 7). Moreover, a study on Munc18-1 and -2 double knockdown neurosecretory cells showed that loss of both Munc18 proteins affected a range of syntaxins, as syntaxins-1a, b, -2 and -3 were all found to be reduced significantly [206]. Syntaxin-11 had not been addressed in that study. Of note, as shown in Figure 5, the increase in cytotoxicity starts already after very few hours. We assume in general that the gain in function is based on equivalent mechanisms in healthy and FHL cells, as it is not known that FHL lymphocytes would suffer from aberrant cytokine signaling or changes in cytokine receptor levels. Careful assessment of early time points after cytokine stimulation (4-8h) would thus greatly contribute important cell biological knowledge on lymphocyte exocytosis, because the early compensation phase is most likely based on another mechanism than upregulation of compensatory protein levels (which certainly can occur at later stages).

The tumorous tissue of the swollen neck had revealed high numbers of infiltrating T cells. It was thus suspected that in this case, cytotoxic T cells might be involved in the lymphoma control, rather than other immune cells (such as NK cells for example). Indeed, EBV specific T cells were identified in the patient PBMC, when they had been mixed with an EBV specific peptide (BZLF-1), as they produced TNF, whereas heathy control PBMC did not. In many cancers, the anti-apoptotic protein Bcl-2 is overexpressed and presented in an MHC class I context. It was shown that it has great immunogenic potential, as Bcl-2 reactive cytotoxic T cells have been found in patients suffering from several unrelated tumor types [269]. When the current patient tissue was analyzed, the RSC indeed were positive for Bcl-2. In summary, the patient in this study had developed a classical Hodgkin lymphoma characterized by highly immunogenic EBV+ RSC. The reduced degranulation capacity due to the genetic defect produces a continuously reduced immunosurveillance in such individuals. An interesting question would be why the patient did not undergo a second full-blown HLH flare instead of lymphoma development. The RSC were immunogenic for a subpopulation of the CD8T cells, which were recruited to the tissue. There, they were highly activated, and maybe because they could not eradicate the RSC, they were retained in the tissue, producing more cytokines, recruiting more lymphocytes, and increasing the tumor mass. This however may only represent another wave in the overall disease course. If there had been no medical interference at that point, the patient possibly might have progressed into another full-blown HLH flare, on top of the lymphoma development.

In summary, this is an interesting case report potentially constituting a link between lowered immunosurveillance, a challenging immunological history and the development of malignancy.

3.2 PAPER II

In this study, we were informed about three patients admitted to a hospital in Pakistan, which were suffering from hyperinflammation, and due to the clinical values, FHL was suspected. Indeed, the patient cells displayed almost absent degranulation and target cell killing in resting state (**Paper II, figure 1**) whilst the healthy controls degranulated readily. The degranulation and killing defect in these patients was thus confirmed. Overnight incubation in IL-2 led to partial regain in function in the patient cells, and it enhanced function also in the healthy donors, as expected. The patients were screened for mutations in the FHL genes *UNC13D*, *STX11* and *STXBP2*. Due to the deficiency in both degranulation and killing, perforin was not sequenced. All patients carried a homozygous missense mutation in syntaxin-11, p.L58P. None of them displayed mutations in Munc18-2. Patient A additionally carried variants in Munc13-4. All patients displayed a dramatic degranulation and killing defect, but within the patients, interestingly, the cells of patient A were the least capable in resting state. This could further support the notion that FHL variants / mutations can add up to each other and consequently increase morbidity, lower the age of onset and possibly worsen the disease course.

To evaluate the possibility of syntaxin-11 impacting intracellular fusion events needed for cytotoxic granule integrity, an intracellular staining on some key cytotoxic granule proteins was performed (**Paper II, figure 2**). If this would have shown abnormal CD107a or granzyme levels for example, that could have been part of the mechanism abrogating target cell killing. Additionally, it would have been reminiscent of syntaxin-11 fulfilling a role in endosome-lysosome fusion processes in macrophages [239], even if in the macrophages, syntaxin-11 had a negative (regulatory) role. However, normal levels of perforin, granzyme B and CD107a suggested that syntaxin-11 does not couple to cytotoxic granule integrity.

The p.L58P mutation in syntaxin-11 is particularly intriguing from a mechanistic point of view. Mutations residing in the SNARE domain can affect the formation of the SNARE complex, or for example interfere at a later step, such as dissolving the complex and re-sorting of components. Mutations which truncate the protein can yield loss of important features such as membrane-binding capacity. This could in turn lead to disturbed trafficking. The p.L58P mutation is a point mutation residing in the presumable Ha domain of syntaxin-11. We were curious whether the protein was expressed and whether it could still interact with Munc18-2, since both, syntaxin N peptides and Habc domains, harbor interaction sites for Munc18 proteins. We employed cells from patient C, which harbors the syntaxin-11 mutation solely. The amount of patient blood available was not sufficient for isolation and Western blot analysis of primary, resting NK cells. Thus, we decided to start with analysis of PBMC, being aware of the fact that this is a mixture of cell types. However, in these lysates we noted that syntaxin-11 was almost entirely absent, whilst Munc18-2 was not affected in the patient (**Paper II, figure 3**). Some PBMC were then used to isolate very low numbers of NK cells and grow LAK cells (Lymphokine-activated killer cells). LAK cells are grown on irradiated feeder cells, and maintained amongst others in IL-2 and the mitogen PHA-L. That way, the NK cells are continuously activated and at some point overgrow the irradiated feeders. The result is a highly activated, primary NK cell-derived culture. Keeping this in mind, we analyzed the lysates for protein expression, and found no syntaxin-11 expression.

In healthy cytotoxic lymphocytes, even overnight stimulation with pro-inflammatory cytokines can suffice to pronouncedly enhance syntaxin-11 levels, and even more so, syntaxin-11 is upregulated in LAK cells compared to freshly isolated NK cells (Figure 9). Obviously, the genetic defect in the patient LAK cells is still inevitable, but one possibility could have been that the substantial increase in syntaxin-11 expression seen in healthy lymphocytes could occur in patient cells, too, and a Western blot might allow for diminutive residual protein detection. That is, for example, if the mutant protein turnover would not be as fast as its cytokine-driven increased production, or not as stringently regulated (like in healthy cells allowing for syntaxin-11 upregulation and maintenance under pro-inflammatory cytokines). However, we could not detect syntaxin-11, whereas Munc18-2 was comparable to controls, similar to the PBMC results (**Paper II, figure 3**). This clearly indicates that the protein is unstable in the patient cells, and massive cell activation due to cytokines and other factors cannot even partially stabilize syntaxin-11 p.L58P.

As observed for other FHL4 patients, Munc18-2 is perfectly expressed in these cells. This is interesting because it could indicate several important things. First, the mutant syntaxin-11 protein likely cannot be stabilized by Munc18-2 anymore, which may indicate reduced binding quality. Second, we know that syntaxin-3 is greatly upregulated in LAK cells whilst barely detectable in freshly isolated NK cells (Figure 9), and Munc18-2 can bind syntaxin-3 (Figure 7), via its Habc and its N peptide domains [270]. On top of that, syntaxin-3 might be maintained in Munc18-2 deficient cells, which makes it a valid candidate to be tested for the pro-inflammatory cytokine-induced compensatory mechanism [23]. It was published that syntaxin-11 N peptide has a much higher binding affinity for Munc18-2 than that of syntaxin-3 [268], but given the high levels of syntaxin-3 and the likely impaired interaction between syntaxin-11 and Munc18-2, syntaxin-3 might easily outcompete syntaxin-11 in such LAK cells, which can further promote the degradation of the mutant protein. The same paper [268] also suggested that Munc18-1 could be able to compensate for Munc18-2 in FHL5 patients, when the cells are stimulated with IL-2 *ex vivo*. If this was true and pro-inflammatory cytokines would trigger Munc18-1 expression, it is expressed in LAK cells, and available for syntaxin interactions. That in turn could implicate that syntaxin-11 has similar binding requirements for both Munc18 proteins, because otherwise it could bind to Munc18-1 instead in these LAK cells, and get stabilized through that interaction.

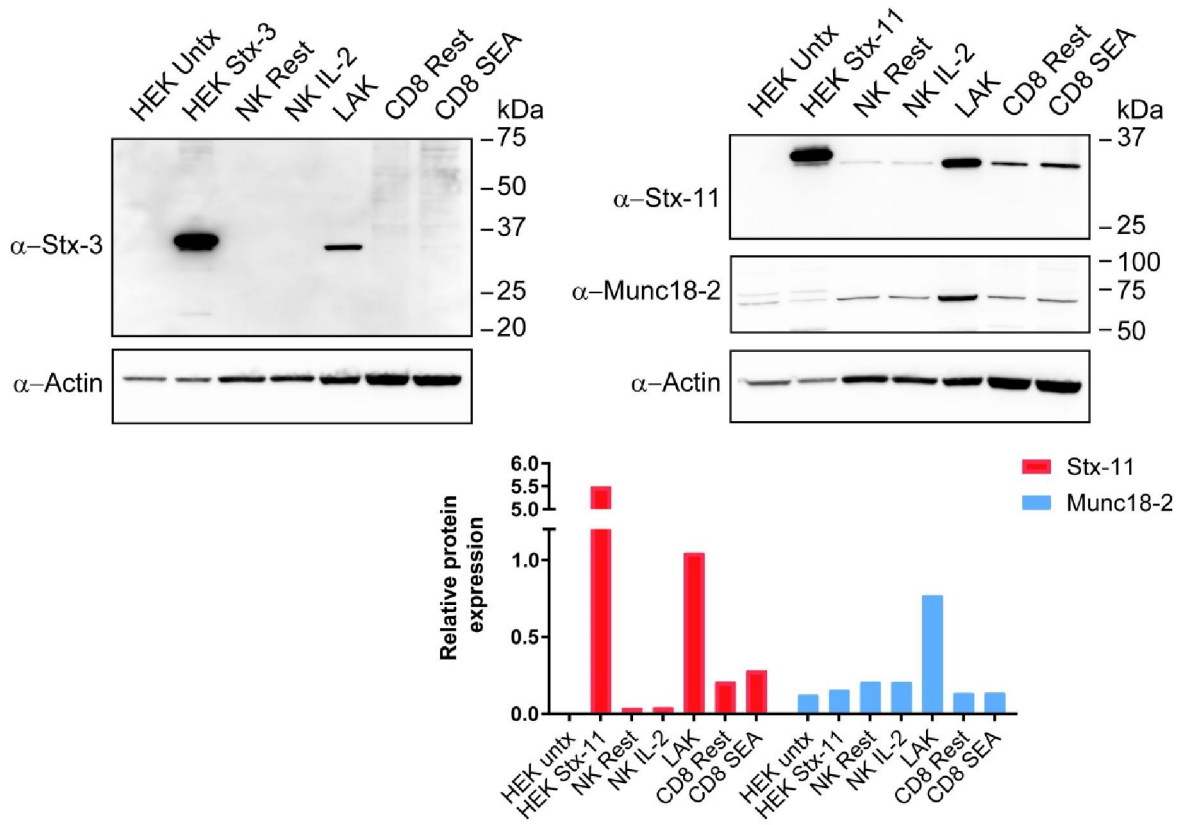

Figure 9. (Left) Untransfected or syntaxin-3 transfected HEK293 cells, resting (Rest) or IL-2 stimulated NK cells, LAK cells or resting vs. SEA-stimulated bulk CD8T cells were lysed and blotted with an antibody against syntaxin-3. Even though protein loading was not even in this experiment (see actin), none of the primary cell lysates displayed a detectable signal for syntaxin-3. (Right) Similarly, the same lysates (exchange of the syntaxin-3 to a syntaxin-11 transfected HEK293 control) were blotted for syntaxin-11 and Munc18-2. A densiometric analysis of the right blot, with protein levels normalized to actin, is shown below. Untx, Untransfected; Rest, resting; LAK, lymphokine-activated killer cells; SEA, *Staphylococcus aureus* enterotoxin A.

The mutation might thus destroy an important interaction site for Munc18-2, hence we wanted to test its interaction with Munc18-2. Given the number of syntaxin family members, and their Habc domain being important for one of the binding modes with Munc18 proteins, we first compared the amino acid environment corresponding to the L58 position in different syntaxins. Aligning the entire N terminal domains of syntaxins revealed that the closest relatives to syntaxin-11 are syntaxin-19, -1, -2, -3, and -4 (**Paper II, figure 4a**). When we matched the amino acid sequences surrounding the L58 position, we saw that the leucine is conserved on syntaxin-19, -1B and -4, whereas syntaxin-1A, -2 and -3 carry an isoleucine at this position (**Paper II, figure 4c**), which is structurally very similar to leucine. Both amino acids are uncharged and nonpolar. Aligning only the very N termini corresponding to the N peptide in syntaxin-1, we saw that there are three positions which display high but not complete conservation, over all syntaxins: D3, R4 and L8. In the syntaxins being most similar to syntaxin-11 these are almost entirely conserved. We were thus curious to simultaneously study Munc18-2 interaction with another N-terminal mutant, this one residing in the N peptide, which might possibly also affect the binding with Munc18-2 [189]. Syntaxin-11 p.R4A was cloned onto the same backbone as syntaxin-11 *wt* and p.L58P. Similarly, as a C-terminal mutant control, we mimicked the syntaxin-11 mutant which is truncated after the SNARE domain (see Figure 8), and has been linked to FHL4 [32]. The protein encodes a nonsense mutation yielding syntaxin-11 p.Q268X. For this mutant, we would not

expect a defect in binding to Munc18-2, unless the interaction could only take place in context of a physiological membrane environment. As a side remark, interestingly, this mutant was also seen to be highly unstable in patient PBMCs [32]. Thus, correct interaction with Munc18-2 may not be the only determinant of syntaxin-11 stability, despite possibly being important.

Regarding protein stabilities, we observed that simultaneous overexpression of Munc18-2 and different versions of syntaxin-11 can induce different Munc18-2 expression levels (see Figure 8). Since we observed this repeatedly, in order to equalize the protein input levels for the Co-IP, we decided to express the proteins in different batches of HEK293 cells, followed by precipitation of the syntaxins, and subsequent exposure of the IP to the lysates containing Munc18-2 (**Paper II, figure 4d, e**). Thus, each reaction is offered the same amount of Munc18-2 and, due to pulling down syntaxins based on the N-terminally fused tags, there is no bias in how well the antibody would bind to the different syntaxin-11 variants. All variants expressed well. The Co-IP revealed that indeed, syntaxin-11 *wt* can bind Munc18-2 under these conditions. Similarly, the truncation mutant pulled down Munc18-2. However, both N terminal mutants could not bind to Munc18-2. Thus, in fact, the position L58 is crucial for the interaction with Munc18-2, and equally, the interaction is based on an integer N peptide. One report showed that total abrogation of the first 12 amino acids of syntaxin-11 did neither impair the interaction of recombinant syntaxin-11 / Munc18-2, nor when the proteins were overexpressed in HEK293 cells [268]. That is interesting, since, regarding our results; it could mean that disturbing the N peptide interaction is “worse” than not possessing an N peptide at all. It does however not imply that the N peptide would be negligible in cytotoxic lymphocytes. It specifically shows that an interaction without N peptide is possible *in vitro*. When the first reports appeared about the structure of syntaxin-1 it became clear that the N terminal part harbors three alpha-helical regions which can fold to a helical bundle, with a highly conserved outer long groove spiraling around the bundle [271]. That groove was suggested to play a role in either SNARE domain binding within the same molecule, or allowing for interaction with Munc13 or Munc18 proteins. Within and between these three helices (Ha, Hb and Hc), many amino acids are highly conserved between species and several syntaxin members. It was found that isolated syntaxin-1 adopts a closed conformation wherein the SNARE domain folds back onto the N-terminal helix bundle [178]. It might be that the syntaxin-11 p.L58P mutation impairs binding of Munc18-2 to the closed conformation of syntaxin-11. That was based on the idea that syntaxin-11 L.58P potentially may never adopt a completely closed conformation, because the proline substitution could interfere with helicity of the domain and introduce a sterical block to the tight binary interaction [272]. The results clearly show that sole interaction with the N peptide is not sufficient for maintaining interaction with Munc18-2. But it could for example initiate it, additionally or synergistically stabilize it, or in another way add up to the binding strength. Disrupting the syntaxin-1 closed conformation with the mutation in the linker domain (LE mutation) between Habc and SNARE domain led to dramatic reduction of Munc18-1 binding, but not SNAP25, complexins or synaptobrevin-2 [178, 261]. Unexpectedly, in the first study, the mutant protein supported stimulated exocytosis in transfected PC12 cells, and the second study added that the mutant slowed down the kinetics of release whilst increasing the quantal size. In total, constitutively open syntaxin-1, which mostly loses interaction with Munc18, could thus still support exocytosis, even with aberrant features, including an aberrant subcellular distribution [273]. However, all three studies observed residual interaction

between the proteins. In our case of the syntaxin-11 patient mutation, the interaction was entirely lost.

Of note, many studies regarding syntaxin-SM protein interactions and analysis of mutant and variant proteins have been conducted using transient overexpression systems, cell-free recombinant proteins, *in vitro* fusion assays or similar, but barely primary patient material. If there was a patient carrying a homozygous constitutively open syntaxin-1 (LE), the protein would likely be considerably instable *in vivo*, based on the substantial loss of interaction with Munc18, similar to what we observe for the syntaxin-11 p.L58P mutant (which, too, overexpresses decently in cell lines (see Figure 8) and activated primary CTL, unpublished obs.). One study analyzed syntaxin-1a *wt* or KO mice, which additionally carried the LE mutation on syntaxin-1b. The mice were viable but after some months succumbed to seizures [274]. As a side remark, referring to the discussion of **Paper I** and Figure 8, it was interesting to see that in these mice, Munc18-1 levels were found to be decreased upon expression of the LE mutant, no matter whether syntaxin-1a was present. All other tested exocytosis proteins besides syntaxin-1 itself, were normal. As expected, the open syntaxin-1b revealed a dramatically reduced interaction with Munc18-1, but not SNAP25, VAMP2 or synaptophysin-1. Thus, interfering with the sterical freedom of the Habc domain folding greatly affects vesicle fusion behavior. Along those lines, some data indicates that integrity of the entire Habc domain of syntaxin-1 is important for trafficking syntaxin-1 correctly to the plasma membrane [275].

It would be very insightful to generate a crystal-structure –based model of syntaxin-11 p.L58P to gain a better understanding of its folding and its capability also to interact with other proteins. In experiments overexpressing syntaxin-11 variants or *wt* in HEK293 cells, when co-expressing SNAP23, we did not detect an interaction defect of that mutant. Thus, the lack of Munc18-2 interaction likely does not impact the binding of SNARE partners for this mutant. Another curious protein to test syntaxin-11 p.L58P interaction with would be Munc13-4. It is, like Munc18, not a SNARE protein, but an important regulator of fusion. It binds to calcium which couples it to cell activation, and it can bind to the syntaxin-11 SNARE domain [232]. In fact, it might bind even stronger to syntaxin-11 p.L58P in a co-expression system, because the closed conformation might be impaired making the SNARE domain more accessible. However, this remains to be shown. A mutant corresponding to the syntaxin-11 p.L58P mutation has not been reported on syntaxin-1, but the site is conserved, and that could also mean that it represents an essential binding site in general for Munc18 interaction. A less disruptive artificial mutation should be generated on this position, for example syntaxin-11 (and syntaxin-1) p.L58A (p.L47A), and the interaction with Munc18-1 and -2 should be tested, for both. That way, one could address if abrogation in binding is due to sterical hindrance due to reduced helix packing, or due to an amino acid-specific mechanism. It might also be worth testing syntaxin-11 p.L58P in a liposome fusion assay. One study showed that even if syntaxin-1 is only consisting of N peptide, SNARE domain and transmembrane domain, it is capable of mediating fusion [207]. The syntaxin-11 Habc domain might thus possibly not be involved in fusion, and rather play a role for stability, trafficking or other protein features. Alternatively, similar to the discussed N peptide necessity, also for the Habc domain, a curious idea could be that having no Habc domain at all is still allowing for fusion *in vitro*, whereas bearing a sterically interfering Habc domain may not allow for fusion. That remains to be addressed, and both, mediating and not mediating fusion, are potential outcomes.

Since the p.L58P protein is unstable and possibly never reaches the plasma membrane before it is degraded, it cannot mediate fusion in a physiological setting. Besides that, it cannot interact properly with Munc18-2, and if Munc18-2 would fulfil a docking role for cytotoxic granules in the lymphocytes, that function, too, could be abolished by this mutant. Even if it would be "fusion-competent" *in vitro*, syntaxin-11 p.L58P is still fusion-deficient *in vivo*. The syntaxin-11 p.R4A mutation *in vivo* might also not be able to mediate the terminal fusion reaction, and that would be consistent with the previous study, as well as the observation that the probability of vesicle fusion in transfected PC12 cells was coupled to the quality of the N peptide interaction with Munc18 [276]. Similarly to what has been found for the syntaxin-11 p.Q268X patients [32], truncated syntaxin-1, which lacks the transmembrane domain, could also not mediate exocytosis [178], presumably because it can sequester Munc18-1 from the plasma membrane, and the localization of both proteins is aberrant. A FRET-based study showed that syntaxin-1 localization to the plasma membrane is dependent on Munc18-1 when overexpressing the proteins in HEK293 or adrenal chromaffin cells [273]. Similarly, another study showed that truncated syntaxin-11 p.Q268X loses membrane association when overexpressed in an NK cell line (YTS cells) [237]. Of note though, when we overexpressed that truncation mutant in stimulated, primary CD8T cells, it was observed in several donors that some cells present with a vesicular phenotype of that mutant, additionally to the free cytoplasmic distribution (unpublished obs, and [237]). There might thus be other protein-protein interactions which determine syntaxin-11 localization in primary cells, at least partially independent of its palmytoylation sites.
If syntaxin-11 trafficking in primary cytotoxic lymphocytes is dependent on Munc18-2, and more detailed, which domains mediate its trafficking, need to be addressed in more detail.

On a final note regarding protein stability, there was an interesting article which studied the overall maintenance of *wt* syntaxin-11 in the context of proteasomal integrity [247]. They found that, in NKL, syntaxin-11 maintenance is dependent on a functional proteasome, while Munc18-2 levels were not affected by the inhibitors. This was not due to decreased syntaxin-11 mRNA expression. Syntaxin-11 remained stable when the cells were treated with lysosome acidification or tripeptidyl peptidase II inhibitors. But incubation with pan caspase inhibitors increased syntaxin-11. Thus, a functional proteasome supports syntaxin-11 levels, whilst syntaxin-11 levels can be limited by caspases. As an explanation for this, it could be that proteasome activity normally keeps the expression level of caspases in check, which secures syntaxin-11 expression in healthy cells. Also, proteasome inhibitors could stress the cells to an extent which activates caspases, leading to degradation of syntaxin-11. Of note, the effect of the proteasome inhibitor was stronger on NK cells than on CTL. This could imply an additional level of regulation which is different between lymphocyte subsets.

In summary, we have shown that N terminal syntaxin-11 point mutations in N peptide and Habc domain both can completely disrupt the binding to Munc-18-2. The loss of this interaction causes syntaxin-11 p.L58P instability and a profound defect in cytotoxic granule exocytosis. Further N terminal mutations will help to elucidate the exact interaction surfaces on syntaxin-11 and hopefully provide more mechanistic knowledge on the similarities and peculiarities of the syntraxin-11 - Munc18-2 interaction compared to other syntaxin - SM protein pairs.

3.3 PAPER III

There are several studies on VAMP proteins in cytotoxic cells and cell lines but it is still not clear which VAMP protein mediates the exocytosis of cytotoxic granules in human cytotoxic lymphocytes. In this study we addressed whether VAMP8 could be the v-SNARE for this process in human cytotoxic cells. To that end, we first set out to confirm if the protein is expressed in human CTL. VAMP8 was readily expressed in resting and TCR-stimulated cells, with transcript as well as protein levels increasing upon TCR stimulation. Here, the CTL had been stimulated with magnetic beads covered in anti-CD3 and anti-CD28 antibodies, to achieve massive cell activation ≥ 2-3 days. Alternatively, SEA was used as potent stimulant for CTL, also increasing the level of VAMP8 expression (**Paper III, figure 1**). When we then looked at endogenous or overexpressed VAMP8 (side remark: all experiments in this study were conducted with *wt* proteins) in these cells, VAMP8 was observed in a punctate pattern under the plasma membrane and in the cytoplasm all over the cell when the cells were not conjugated to target cells. In conjugates, VAMP8 localized to the contact site, which supported the notion that it might be implicated in cytotoxic granule release. We tested whether VAMP8 would co-localize with cytotoxic granule proteins such as granzymes or perforin. However, VAMP8 did not localize on cytotoxic granules, neither in non-conjugated cells, nor in conjugates with target cells. Thus, surprisingly, VAMP8 is likely not the R-SNARE serving for cytotoxic granule fusion in human CTL. Subsequently, we co-transfected CTL with markers of early, late and recycling endosomes (Rab5a, -7a and -11a, respectively) together with VAMP8, to gain insight into which compartment VAMP8 localizes to. The protein was found mostly on recycling endosomes (marked by Rab11a) with minor fractions residing on early and late endosomes (**Paper III, figure 3**). These experiments were conducted on fixed cells in SIM microscopy. However, we were curious whether VAMP8 and Rab11a would co-localize throughout synapse formation, if the distribution and dynamics of the two proteins would be identical and if these vesicles fused with the membrane in the immunological synapse. Therefore, CTL co-transfected with VAMP8 and Rab11a were allowed to form synapses on anti-CD3 / CD28 coated glass surfaces, and imaged live in TIRF microscopy. This allowed us to gain insights on the molecular dynamics in the area within and immediately above the plasma membrane. Directly after the cells sedimented and spread out to form immunological synapses, VAMP8+Rab11a+ vesicles of variable sizes became visible within the TIRF plane. As seen in **Paper III, figure 4a**, most of these vesicles revealed a striking co-localization between the proteins, and individually measuring vesicle dwell times and numbers accumulating at the synapse, both proteins showed very similar characteristics (**Paper III, figure 4b, c**). These movies also revealed that a number of rapid fusion events of these vesicles occurred soon after the cells had sedimented and spread out (**Paper III, figure 4d, e**). By cloning fusion proteins consisting of VAMP8 with its transmembrane domain fused to monomeric teal fluorescent protein (TFP), with or without a 3x FLAG tag in between, we were able to analyze this type of VAMP8+Rab11a+ vesicle exocytosis in multicolor flow cytometry (**Paper III, figure 4f, g**). Furthermore, constructs of VAMP8 fused to lumenal mCherry and pHlourin were cloned which allow for sensing the fusion events. This is because the pHlourin is not fluorescent in acidic environments, but starts to fluoresce in neutral pH. Once exocytosis of the Rab11a+VAMP8+ vesicles would occur, the pHlourin would start to fluoresce. In both, the flow cytometry assay and the microscopic analysis of the constructs, and in line with the previous data, stimulated exocytosis of these vesicles was observed.

For the VAMP8+pHlourin+ vesicles which fused after entering the plasma membrane proximity (TIRF plane), a sharp peak in pHlourin fluorescence simultaneously to the peak in mCherry was seen upon exocytosis (**Paper III, figure 4h, left**). However, some vesicles had different fusion kinetics, wherein fusion was not immediately followed by dispersion of pHlourin fluorescence. This could indicate that the types of fusion which such vesicles can undergo are heterogeneous, with some resembling full collapse whilst others might resemble kiss-and-run type of exocytosis (**Paper III, figure 4h, right**). That would be in line with results gained with pHlourin-based exocytosis experiments in an NK cell line, NKL [277]. However, this needs further exploration.

The data so far showed that VAMP8 occupies a preponderant portion of the recycling endosomes, and TCR stimulation can trigger recruitment and exocytosis of such vesicles. To gain further insights into the dynamics of VAMP8+Rab11a+ versus cytotoxic granules, CTL were co-transfected with VAMP8-TFP and perforin-mCherry, and imaged in the TCR-stimulating TIRF system, as before. Interestingly, we observed that VAMP8 reached the plasma membrane first; followed by perforin a few seconds later (**Paper III, figure 5a-c**). The VAMP8+ vesicles outnumbered perforin+ vesicles in this system. However, one should keep in mind that the proteins might have different turnover times and they might be differently tolerated by the lymphocytes. The overexpression system might thus not exactly mirror the physiological protein amounts. The VAMP8+ vesicles fused within the first few seconds after entering the TIRF plane, whilst perforin vesicles displayed a significantly increased dwell time, before they either fused or withdrew out of the TIRF plane (**Paper III, figure 5b**). On top of that, on average only very few perforin vesicles fused (<10) whilst many VAMP8+ vesicles fused (>40) per imaged cell. These data further confirm that VAMP8+ and perforin+ vesicles follow different kinetics and are at no point during synapse formation, recycling endosome or cytotoxic granule exocytosis equivalent. The intriguing question was now, as apparently VAMP8 did not mediate cytotoxic granule fusion - what other fusion events could it mediate at the synapse, occurring right upstream of cytotoxic granule exocytosis, imparted via recycling endosomes? And would these events be functionally coupled at all?

To first test if recycling endosome or cytotoxic granule release is dependent on VAMP8, the protein was knocked down with siRNA against VAMP8 in the CTL which simultaneously were overexpressing either Rab11a-mCherry or granzyme B–mCherry, respectively, and imaged in TIRF microscopy (**Paper III, figure 6c-g**). This time, granzyme B was chosen over perforin because the cells tolerate granzyme B transfection better than perforin transfection. Interestingly, we found that the knockdown of VAMP8 impaired recycling endosome fusion with the plasma membrane (**Paper III, figure 6d**) but the vesicle dwell times were comparable. This can mean that recycling endosomes which enter the TIRF plane either undergo exocytosis within a given time frame (approximately 20 s), or they withdraw again from the synapse, moving back into less proximal cytoplasmic areas (**Paper III, figure 6c**). As a note on the side, of course it cannot be excluded that such vesicles re-enter the TIRF plane after some time, which would then be recorded as a “new” vesicle.

Looking at VAMP8 knockdown in cells overexpressing granzyme B, we again found that the granzyme B+ vesicle dwell time was not affected significantly, but there was a sharp, significant decrease in granzyme B+ vesicle fusion events (**Paper III, figure**

6f, g). CTL with control or VAMP8 siRNA were also tested in a standard functional flow cytometry assay (degranulation assay) based on TCR stimulation, where CTL normally release cytotoxic granules and display the granule-associated protein CD107a on their surface. CD107a can then be stained and serves as a surrogate marker for exocytosis. This is the same procedure as the degranulation assays conducted on the lymphocytes of the FHL patients discussed in the previous articles (**Paper I and II**). Lack of VAMP8 significantly reduced cytotoxic granule release (**Paper III, figure 6h, i**). VAMP8 is thus required for both, recycling endosome and cytotoxic granule exocytoses, and the latter effect is due to the previous effect. To exclude the possibility that VAMP8 would also play a role in upstream signaling events such as calcium flux or TCR signaling-induced phosphorylation events, the VAMP8 knockdown CTL were assayed for phosphorylation of ERK, an early TCR signaling event, and calcium influx from the medium with a calcium-sensitive dye (**Paper III, figure 7a, b**). The VAMP8 knockdown CTL showed no aberrant ERK phosphorylation or calcium mobilization, indicating that VAMP8 plays no role in early TCR signaling. Another parameter that we tested was the spreading characteristics of VAMP8 knockdown cells, because it was possible that lack of VAMP8 and lack of recycling endosome fusion would impact synapse integrity, size or for example actin mesh dynamics. However, we could not detect obvious changes in actin dynamics or synapse morphology (**Paper III, figure 7d-f**).

One possible function of the VAMP8-mediated recycling endosome fusion events could be that VAMP8+Rab11a+ vesicles would deliver proteins to the plasma membrane which are subsequently needed for cytotoxic granule release. One key protein for this delivery could for example be syntaxin-11. A previous study showed that syntaxin-11 localizes to recycling endosomes and is transported to the immunological synapse before the cytotoxic granules arrive [245]. Therefore, VAMP8+ recycling endosomes might transport syntaxin-11 to the immunological synapse on demand, so it can mediate cytotoxic granule exocytosis once the cytotoxic granules arrive. Indeed, we observed that VAMP8 knockdown produced a significantly increased syntaxin-11+ vesicle dwell time at the synapse, accompanied by accumulation of syntaxin-11 vesicles over time, and the fluorescence dispersion events indicative of fusion were markedly reduced (**figure 7g-k**). VAMP8 and syntaxin-11 fusion mostly occurred in the center of the immunological synapse, likely corresponding to the central synapse region which is the site of cytotoxic granule exocytosis (see chapter **1.2.4**). However, this was only a trend, and a considerable number of dispersion events occurred in peripheral synapse regions (approximately 20-40 %, **Paper III, figure 7l-n**). When analyzing syntaxin-11 distribution in VAMP8 knockdown cells, surprisingly we found syntaxin-11 to cluster even more in the central region of the synapse than in corresponding control cells (**Paper III, figure 7o**). It is thus possible that in healthy cells syntaxin-11 adopts a specific distribution inside and outside of the central synapse region, and this balance may have functional consequences for cytotoxic granule exocytosis.

In summary, the hypothesis is that syntaxin-11 is trafficked by VAMP8-dependent fusion events on a population of recycling endosomes, and deposited at the immunological synapse, ready for mediating exocytosis of cytotoxic granules (**Paper III, figure 8**). Syntaxin-11 is only one of the proteins needed for cytotoxic granule secretion in CTL and NK cells. It is thus possible that VAMP8+Rab11a+ vesicles deliver other exocytosis mediators, too. One interesting candidate to test would be Munc18-2, given that syntaxin-11 and Munc18-2 might co-traffic, similarly to the neuronal pendants, syntaxin-1 and Munc18-1. Munc13-4 and Rab27a coordinate

and / or traffic on recycling endosomes, too [262, 263], cytokines have also been linked to recycling endosome-mediated transport [250]. It remains to be shown if all of these are separate recycling endosome populations, and how these pools are coordinated.

The observation that VAMP8 mediates syntaxin-11 deposition at the synapse instead of being the v-SNARE for cytotoxic granule fusion was surprising in some ways. Syntaxin-11 has been shown to interact with VAMP8 and SNAP23 in platelets [238]. However, we could not detect VAMP8 co-immunoprecipitating with syntaxin-11 in lysates of primary cytotoxic lymphocytes (unpublished obs.). The observation that VAMP8 did not reside on cytotoxic granules but instead on recycling endosomes outruled this possibility. It remains to be shown which other VAMPs, for example VAMP3, -4 or -7, play roles in human cytotoxic granule fusion in primary lymphocytes, and if so, which exact fusion event they participate in. Given that there is no VAMP deficiency causally linked to cytotoxic granule exocytosis, it is also possible that, either, several VAMP proteins act in concert in this process and can functionally compensate for the loss of one VAMP, or, a specific cytotoxic granule exocytosis VAMP exists, but it has crucial roles in other cell types, too, and thus deficiencies could be embryonically lethal. Combinations of VAMP knockdowns or knockouts in primary human lymphocytes are needed to evaluate this question in more detail.

Regarding the considerable recycling endosome recruitment, an interesting idea is whether, additionally to delivering syntaxin-11 (and maybe other exocytosis proteins), there could be other roles for the VAMP8+Rab11a+ exocytosis exactly prior to cytotoxic granule fusion, meaning exactly prior to destructive protein release into a “non-directional” (diffusion-based) synaptic cleft. Once perforin is released and adopts its functionally active form in the synaptic cleft, there must be mechanisms to ensure membrane protection in the cytotoxic lymphocytes. CD107a has been suggested to play a role in lymphocyte protection from perforin [278], and another article implicated Rab11 and Rab5 in membrane repair mechanisms upon treatment of intestinal epithelial cells with pore-forming bacterial toxins [279]. Producing substantial recycling endosome trafficking at the immunological synapse might thus possibly also serve as a highly dynamic membrane repair system to ensure membrane integrity. However, this idea needs further exploration. Additionally, of course, coupled to the outward membrane flux that occurs upon exocytosis of VAMP8+ vesicles and cytotoxic granules, the recycling endosomes could also aid in retrieving membrane and membrane constituents during and after exocytosis (see also [280]). If recycling endosomes also organize the retrieval of syntaxin-11 (and maybe Munc18-2) after killing is completed, remains to be addressed.

Another very interesting question remaining unresolved is why syntaxin-11 would stay associated with VAMP8+ recycling endosomes and not be deposited at the plasma membrane constitutively in primary lymphocytes. Other syntaxins such as syntaxin-1 are transported to and maintained at the plasma membrane where they can serve their function in exocytosis without delay. In contrast, syntaxin-11 and also Munc18-2 are mostly found intracellularly, even in activated, pro-inflammatory cytokine-stimulated cells or LAK cells (unpublished obs.). The VAMP8-Rab11a-dependent syntaxin-11 transport exactly into the immunological synapse might for example be a mechanism preventing any standby exocytosis events at non-conjugated plasma membrane sites. In this context, it could be informative to generate a syntaxin-11 construct which bears the membrane anchor of syntaxin-1 in

transfected cytotoxic lymphocytes, or a hybrid construct consisting of syntaxin-11 fused to the transmembrane part of an unrelated plasma membrane-resident protein. It remains to be shown whether in such cells, cytotoxic granule exocytosis would still occur to the same extent and in a targeted fashion.

In this context, it would also be very informative to follow VAMP8 and syntaxin-11 in anti-CD16 stimulated NK cells. Even though unlikely to occur as an isolated stimulus in a physiological setting, CD16 stimulation alone lead to non-polarized degranulation of cytotoxic granules [107]. However, the amount of syntaxin-11 approximately doubles when resting NK cells are seeded onto IgG-coated glass surfaces and imaged in TIRF microscopy, and the gain in fluorescence intensity over the synapse area was significant (unpublished obs.). Syntaxin-11 is thus responsive to CD16 stimulation and is recruited towards the immunological synapse, but the recruitment may not be limited to the synapse area. This would mean that certain signaling pathways might promote VAMP8+syntaxin-11+ vesicle exocytosis in a non-polarized fashion, whereas others might promote polarized recycling endosome trafficking, which most likely couples to cytoskeletal elements such as microtubules and MTOC. Another intriguing idea could of course be that non-polarized secretion of cytotoxic granules is also microtubule transport-based, but for some reason in an anterograde (plus-end) -directed fashion, away from the immunological synapse. If this is a transport mechanism resembling the non-polarized exocytosis which can occur for cytokines [250], remains to be addressed.

Looking at our findings from another angle, we have established that VAMP8 delivers syntaxin-11 to the plasma membrane on demand. However, besides the question which VAMP serves as v-SNARE for cytotoxic granule release, we similarly do not know yet which SNARE complex mediates fusion of the syntaxin-11+ recycling endosomes, or if syntaxin-11 itself is implicated in this. Syntaxin-11 is also not exclusively found on recycling endosomes. It should be noted that, additionally to VAMP8 and Rab11, we have observed a high co-localization of syntaxin-11 with Rab5 and Rab7 as well as Munc18-2 (unpublished obs.), and this pattern could be aberrant for some of the mutants. Further studies need to be performed on the trafficking of syntaxin-11 and Munc18-2 to understand their complicated relationship and how some variants and mutations can interfere with functionality of cytotoxic lymphocytes. All these exciting questionings remain to be addressed carefully. Such data will hopefully provide essential knowledge about subcellular transport and exocytosis processes in cytotoxic lymphocytes, and help us to better understand how these processes are coordinated before, throughout and after target cell killing, in order to prepare for a new round of killing, or even understand why some lymphocytes in a given (healthy) population can kill several target cells serially whilst others do not [281-283].

4 CONCLUSIONS AND FUTURE DIRECTIONS

"After climbing a great hill, one only finds that there are many more hills to climb."
(Nelson Mandela)

Understanding how variants and mutations affect cellular functions and can cause disease is a tremendous challenge in medical research. Particularly, it is important to elucidate even very subtle details about protein-protein interactions and their implications for protein stability, localization and protein activity. The more variants and mutations are described, the better we can focus on detailed genotype-phenotype correlations representing mutations mapped to their individual protein domains and interactomes, in relation to patient cell behavior, morphology and function. Simultaneously, the more we can learn about the variant and mutant proteins, the more knowledge can accumulate about the *wt* proteins, too.

It has been long understood that a systemic, comprehensive disease like FHL requests information on several different layers. Studies on the single cell level can amongst others provide insights into protein interactions and trafficking. Studies on a cell population level will help us to understand more collective processes determining disease outcome and intercellular communication, including protein expression or cell degranulation and cytotoxicity against susceptible targets. Studies looking at tissue morphology, immune cell infiltration and production of inflammatory mediators as well as pathogen-derived mediators (for example in an EBV+ background) help to elucidate cell communication in an acute inflammatory tissue environment setting. From these data, we can learn more about overall tissue pathology determining organ function and leading to very aggressive or more slow tissue destruction, which in some cases can even lead to cell transformation. And furthermore, looking at medical records of patients from different geographic and ethnic origins can for example help to identify founder mutation effects and at some point possibly demonstrate if also more peripheral or external factors such as non-FHL related immunological history, lifestyle or other factors could play a role during disease progression.

The three articles introduced here include several important patient cases suffering from loss of functional Munc18-2 or syntaxin-11, respectively. For both proteins, there are still numerous variants and mutations which are poorly understood mechanistically. Some syntaxin-11 variants for example do not impair interactions with Munc18-2, Vti1b or SNAP23, and they may traffic similarly to the *wt* protein in healthy human CTL (unpublished obs.). This indicates that we are still missing out on some protein aspects. There is for example little proteomic, comprehensive data on FHL protein interactomes which could suggest novel candidates impacting FHL protein interactions, localization and stability. Generally, supporting genetic analyses much more with mechanistic data in the future will certainly widen our understanding of the FHL cell biology. This addresses for example a few variants

which have been found in a heterozygous or compound heterozygous state, but for syntaxin-11 it has not been shown how exactly a variant or mutation could act in a dominant-negative fashion.

Considering the body of literature from other exocytosis fields opposing the tenuous knowledge in the human lymphocyte exocytosis field, there are many concrete open questionings for syntaxin-11 and Munc18-2 which deserve full attention. The trafficking of both proteins needs to be understood in detail. We still do not know if they traffic together, on which compartment they meet, or if they can for example form a binary complex co-translationally. From our own observations we know that overexpressed syntaxin-11 co-localizes bulky with VAMP8, Munc18-2, Rab11, Rab5 and Rab7. How dependent this distribution is on Munc18-2, if this distribution is physiological in healthy cells or how it differs in patient cells is not known. Similarly, which interaction surface between syntaxin-11 and Munc18-2 is responsible for which trafficking step is completely unknown, and requires more research. Possibly coupled to this, an intriguing question is how the recycling from the plasma membrane, as well as the endogenous turnover of syntaxin-11 is organized and how the kinetics of these processes is. To that end, the LE mutation could be introduced onto syntaxin-11. The positions are almost completely conserved, with an I (Ile) instead of a L (Leu), and a conserved E (Glu) at the corresponding residues on syntaxin-11. Further, replacing syntaxin-11 palmytoylation sites systemically (for example in an alanine scan approach), or introducing a transmembrane anchor could yield interesting insights on its membrane attachment requirements and help to understand why syntaxin-11, unlike almost all other syntaxins, does not possess a transmembrane domain.

The curious observation of partial compensation in FHL4 and some FHL5 patients urges to gain more knowledge on such a FHL protein-independent compensatory mechanism. Further supplementation and imaging experiments are needed to understand which proteins can mediate this function, and if the mechanism is the same as what increases functionality in healthy cells. It is possible or even likely that besides the suggested candidates (syntaxin-3 and Munc18-1), there are other syntaxins and Munc18 proteins which aid, particularly in the initial phase after pro-inflammatory cytokine stimulation. It is also important to clarify if such compensation is at all based on SNARE protein / complex substitution only, or if other factors such as for example enhanced cell adhesion or cytoskeletal rearrangements (which also occur upon pro-inflammatory cytokine stimulation) play a role in this increased / rescued degranulation process.

The third article describes that VAMP8 traffics on recycling endosomes and serves a crucial, preparatory role for cytotoxic granule exocytosis, as it delivers syntaxin-11 to the plasma membrane. In the future, the role of other VAMPs in cytotoxic lymphocytes needs to be addressed carefully, as it is still not understood which VAMP protein can mediate cytotoxic granule fusion. For VAMP8 itself it could be informative to create hybrid proteins where VAMP8 transmembrane domain is replaced by other VAMP transmembrane domains, so that it would be forced onto cytotoxic granules, for example. Such experiments could amongst others show if the cytotoxic granule fusion is specific for one / a few certain VAMP / VAMPs, or if this process is governed by stringent VAMP protein sorting to specific organelle surfaces.

5 METHODOLOGICAL APPROACHES AND LIMITATIONS

"There were no holds so I had to use skill."
(Anonymyous climber)

5.1.1.1 Cell isolation techniques

For all studies, freshly isolated healthy or patient peripheral blood lymphocytes were employed for different assays. Cytotoxic lymphocytes are isolated from whole blood via differential density centrifugation (for example Ficoll™ gradient) which yields layers of polymorphnuclear cells (red blood cells, thrombocytes, granulocytes) and a layer of PBMC. The PBMC comprise the cytotoxic lymphocytes. NK cells constitute about 5 - 15 % of PBMC. T cells are the majority of PBMC, with approximately 70 %. Out of these, approximately 60 % are CD4+ and 40 % are CD8+. For some experiments, when pure populations rather than whole PBMC were needed (for example for transfection experiments or some Western blots), we isolated cytotoxic lymphocyte populations from the PBMC, using negative selection. This is based on magnetic beads covered with antibodies which deplete other cell types because these remain on the columns. Purity of negatively isolated NK or CD8T cells is never 100 %, there are typically few other cells contained (< 3 %). However, the negative isolation is still suitable for our experiments because, despite lower purity than positive selection, we circumvent the possibility that the isolated cells are activated by antibody binding to the surface antigen (for example CD3 or CD8). Besides that, several other cell types can be distinguished easily by cell size and morphology in imaging experiments or flow cytometry, and given their small percentage we could still interpret results from Western blots, for example.

5.1.1.2 Homogeneity and cytotoxicity of CTL and NK cells

A frequently used, simple definition of NK and CTL are NK cells defined as CD56+(+)CD3- and CTL defined as CD3+CD8+CD57+. For our studies on cytotoxic granule exocytosis and trafficking of proteins, we focused on cells expressing perforin, as it served as our marker for cytotoxic granules. Our rationale was that perforin is needed to perform target membrane perforation; hence granules not containing perforin can certainly add up to the cytotoxic protein pool in the synaptic cleft, but by themselves cannot trigger apoptosis in the target. However, as a compromise for some transfection experiments, we employed a construct for granzyme B expression rather than perforin because perforin transfection is sometimes not tolerated well by the CTL. For the imaging experiments with CTL, we isolated bulk CD8T cells which were subjected to magnetic bead-based antibody stimulation for 48 h (anti-CD3 / CD28 beads) in enriched cell culture medium. Through the intense stimulation, the cells are continuously activated and cytotoxic

granule protein expression is induced or enhanced, as many cells within the CD8T cell pool do not express abundant levels of perforin *per se*. This protocol produces heavily activated cells but these are still more physiological than any cell line. These CTL are much more prone to survive transfection procedures and besides that, the bead stimulation increases cell size, adhesion capability and overall vividness, and the synapses they form represent preeminently mature cytotoxic synapses. All of these traits simplify lymphocyte imaging experiments substantially. Furthermore, some differences in syntaxin-11 and Munc13-4 expression were observed in naïve versus effector CD8T cells [284]. The intense TCR stimulation reconciles such expression differences and drives the entire population to upregulate perforin and to mature into a more effector-like population which was suited for our purposes. NK cells undergo different developmental stages and accordingly express different phenotypical markers, and within the bulk negative NK cell isolation, a number of developmental stages are contained. However, for NK cells in healthy donors there is data (unpublished obs.) hinting towards a homogenous mRNA expression of syntaxin-11, Munc18-2 and Munc13-4 over different developmental stages and also adaptive NK cell subsets in resting states. For that reason it seemed redundant to separate specific NK cell subsets for the studies conducted in this thesis. We did also not observe that cell activation would affect FHL protein localizations (particularly syntaxin-11 or perforin), even though in cytokine-activated cells it could have been an interesting strategy of cells to enhance cytotoxic granule release by speeding up the FHL protein organization at the plasma membrane, and allowing for granules to fuse in a less restricted fashion, by providing more potential granule docking spots at the plasma membrane. However, we did not see any syntaxin-11 or Munc18-2 associated with the plasma membrane, even in heavily cytokine-stimulated cytotoxic lymphocytes. Of course, we cannot conclude that syntaxin-11 displays the exact same endosomal compartment distribution in resting cytotoxic lymphocytes as compared to our model CTL system, but this model still allows us to understand how syntaxin-11 trafficking is regulated by VAMP8 in CTL.

5.1.1.3 Protein expression inhomogeneity

A surprising inhomogeneity within total PBMC regarding syntaxin-11 stability was displayed in **paper II**, when we compared Western blots performed on patient PBMC versus LAK cells. As it turns out, there must be non-NK cell populations in which the mutated protein is maintained longer than in other cell types, because there was a weak band visible in the PBMC Western blot whilst there was absolutely no protein in the NK-based LAK population. We could not determine which cell type was responsible for the residual syntaxin-11 signal, because the number of PBMC typically isolated from such patients is very small, and it is barely possible to sort such PBMC into its major components and then perform a Western blot on all of these subsets. Identifying the cell type being able to maintain the mutant syntaxin-11 (p.L58P) might be informative for FHL protein stability studies, for example in respect to possible differences in activity of degradative systems, such as the proteasome or caspases. It would have been great to compare protein expression in the other patients with the same mutation (mostly patient B), to confirm this finding in the other patients with the same, or even other syntaxin-11 mutations.

5.1.1.4 Genetic modification and protein expression systems

Overall, a major bottleneck when genetically modifying primary human cytotoxic lymphocytes is that transfection protocols can be optimized to some extent, but all electroporation-based methods are very detrimental to freshly isolated cytotoxic lymphocytes. Transduction protocols can provide better viability and transfection efficacy, but the virus stock preparations can be time-consuming and batch variations can occur. For overexpression as well as knockdown experiments, a major difficulty is that neither of them can completely eradicate the effect of the endogenous *wt* proteins. Knockdowns are rarely absolute, and overexpression *per se* does not affect the intrinsic protein expression, should the protein (as in our case) be endogenously expressed by the transfected host cell. Along these lines, of course one issue could be that endogenous protein (which is not labeled or tagged) could dilute some effects applying to the overexpressed protein. In the case of mutant protein overexpression, the mutant protein could for example be outcompeted by endogenous healthy protein and thus not represent the patient cell situation well (unless the patient is for example heterozygous). However, in our trafficking study (**paper III**) we only applied *wt* proteins; hence we would possibly expect some level of dilution but it would be unlikely that the overexpressed proteins would mislocalize because they cannot compete against the endogenous proteins. However, we did not address what the ratio of overexpressed to endogenous protein is, thus we cannot comment on such effects of overexpression. For research purposes, novel genetic manipulation strategies could be helpful to transfer our findings to freshly isolated resting cytotoxic lymphocytes. With such technologies, human genes could be edited permanently and specifically, ideally without off-target effects. New technologies like CRISPR/Cas-based genetic modifications can possibly provide this at some point in the future, but until resting, human cytotoxic lymphocytes are applicable for such a technique we still need alternative model systems to test the behavior of *wt* and mutant proteins. A frequently used system to study protein interactions and possible distribution in mammalian cells are cell lines such as HEK293 or HeLa cells. In our studies, we have employed HEK293 cells for protein interaction studies. They can be easily genetically modified and can produce a lot of protein for testing protein-protein interactions. Producing bacterial, recombinant protein might have been beneficial for large scale interaction studies, for example when addressing the global syntaxin-11 interactome in cytotoxic cells. For our purposes however, the HEK293 system was sufficient. Also, HEK293 are mammalian cells, and in case syntaxin-11 would be post-translationally modified this is not represented on bacterially produced recombinant proteins, whilst possibly maintained in mammalian cells. However, one should keep in mind that recombinant protein can also be produced on a large scale in mammalian cells meanwhile, for example in trypanosomes.

5.1.1.5 Co-immunoprecipitation system

After testing syntaxin-11 and Munc18-2 co-expression followed by co-immunoprecipitation, we realized that syntaxin-11 and Munc18-2 expression is relative to each other and we cannot normalize each reaction input in such a system very well. Hence, we switched to a single-construct expression system, still in HEK293 cells. This allowed us to lyse batches of transfected cells individually, for example one batch expressing Munc18-2, and some others expressing each one or

another version of syntaxin-11. That way we were able to provide the exact same amount of lysed Munc18-2 to each syntaxin-11, which greatly helps to make such Co-IP reactions more comparable. Of course, one has to keep in mind that this system favors interactions which can form rather quickly and without a proper spatio-temporal physiological environment, as the proteins meet in solution, and the time the lysates were allowed to form complexes were 3-4 h under soft rotation, at 4 degrees. Slow interactions, interactions enabled or supported by other proteins not expressed by HEK293 cells, or highly transient interactions might be missed in such an approach. However, we could see strong association of *wt* syntaxin-11 with Munc18-2, hence we were confident to test the mutants in this system, too. Of course, in this system we consequently may miss out on some variants forming very slow or very weak interactions in physiological environments.

5.1.1.6 Concluding statement on methodological considerations

In summary, we are aware that our experimental systems can be optimized further in the future. However, given the technological state of the art when we started and worked on these projects, we employed well-established methods throughout the studies.

"You can grunt and heave, sweat and strain, wear yourself out, and unless you simply forget about it and step up, you won't even get off the ground."
(Mike Borghoff)

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"The best view comes after the hardest climb."
(Anonymous climber)

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“It is not the rock we conquer but ourselves.”
(Sr Edmund Hillary)

Take care!

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