Chapter 1

Introduction

1.1 Cytotoxic cells in the immune system

Our immune system protects us from disease and fights infections. It can broadly be separated into two arms, the innate and the adaptive immune system. The innate immune system immediately responds to defend the host, while the adaptive immune system launches a highly specific and long-lasting response that takes several days to develop (Lowry and Zehring, 2017; Pennock et al., 2013). Cytotoxic lymphocytes are found across the innate and adaptive arms of the immune system (Russell and Ley, 2002). Natural killer (NK) cells are part of the innate immune system, while cytotoxic T lymphocytes (CTL) are part of the adaptive immune system (Russell and Ley, 2002; Topham and Hewitt, 2009). Activated T cells that express the cluster of differentiation (CD) 8 co-receptor on their surface will be referred to as CTLs throughout this thesis. However, it is important to note that CD4 T cells, which are usually known for cytokine production, can also show cytotoxic activity (Jellison et al., 2005; Pennock et al., 2013). Additionally, NK cells have been reported to develop memory to antigens, a quality usually attributed to the adaptive immune system (Lowry and Zehring, 2017).

CTLs recognise antigens presented in complex with major histocompatibility complex (MHC) class I molecules on the cell surface (de la Roche et al., 2016). MHC class I surface expression can be decreased in virally infected and tumour cells to prevent CTL mediated cytotoxicity. However, cells lacking MHC class I surface expression can be recognised and eliminated by NK cells (Mandal and Viswanathan, 2015; Topham and Hewitt, 2009). Both NK cells and CTLs kill their target cells through the focused secretion of cytotoxic compounds, which are stored in secretory lysosomes, referred to as lytic granules. Thereby cytotoxic cells can kill infected and cancerous cells, protecting the body from disease (de la

Roche et al., 2016; Topham and Hewitt, 2009). Defects in CTL and NK cell cytotoxicity cause immunodeficiency disorders, which are discussed in detail in section 1.4.3. One of the infections that affected patients frequently present with are Epstein-Barr virus infections, indicating that CTL and NK cells are particularly important for effective responses to this pathogen (Janka and Lehmberg, 2014). Additionally, cytotoxic T cells were shown to be effective in the treatment of metastatic melanoma (Rosenberg et al., 2008).

1.2 The role of CTLs in the immune response

Each T cell expresses numerous copies of a unique T cell receptor (TCR) on its surface, which recognises a specific peptide (antigen) presented by an MHC class I molecule. The TCR is a heterodimer and associates with CD3ε, γ, δ and ζ (Wucherpfennig et al., 2010). Selection in the thymus gives rise to T cells that express TCRs that react to non-self peptides displayed by MHC class I molecules (Harty and Badovinac, 2008). TCR variability is mediated by rearrangement of the genes that encode them. It is estimated that up to 10^{18} different $\alpha\beta$ heterodimer TCRs can be produced (Cantrell, 2015). Due to the large repertoire of naive T cells that are generated, T cells that recognise a particular peptide are rare, and therefore need to undergo vast proliferation when they encounter their antigen in order to destroy all diseased cells (Harty and Badovinac, 2008).

Naive CD8 T cells travel between the blood and secondary lymphoid organs, such as the lymph nodes and spleen, where they interact with antigen presenting cells (APCs), such as dendritic cells (Harty and Badovinac, 2008; Zhang and Bevan, 2011). If, for example, an individual is infected by a virus, APCs acquire viral peptides at the site of infection. The APCs then travel from the infected tissue to the secondary lymphoid organs to present the antigens from the infectious agent to CD8 T cells (Harty and Badovinac, 2008). As each naive CD8 T cell expresses a unique TCR, APCs may have to interact with many T cells until they find one that recognises their antigen (Cooper and Herrin, 2010).

Three signals have to be received for naive T cell activation to occur (Mescher et al., 2006). Firstly, the TCR of the naive CD8 T cell has to recognise the specific peptide presented by the MHC class I molecule on the surface of the APCs (signal 1) (Brownlie and Zamoyska, 2013; Harty and Badovinac, 2008). In response to recognition, signalling molecules are recruited to the TCR complex. The molecular effects of signal 1 will be discussed in more detail in section 1.3. The naive T cell also has to receive co-stimulatory signals in order to be activated (Signal 2). Receptors on the APCs interact with co-stimulatory receptors on the

T cells. The best studied interaction is between CD28 on the T cells and B7.1 or B7.2 on APCs (Williams and Bevan, 2007). This interaction stimulates survival, proliferation and production of cytokines, such as interleukin (IL) 2 (Williams and Bevan, 2007). Cytokines, such as IL-12 and type 1 interferons (IFNs) provide the third signal. They contribute to T cell survival and expression of effector molecules, such as granzyme B (Harty and Badovinac, 2008; Mescher et al., 2006; Williams and Bevan, 2007).

Naive CD8 T cells that receive all three signals are triggered to differentiate into effector CTL and proliferate extensively (Harty and Badovinac, 2008; Williams and Bevan, 2007). Numerous transcription factors are activated downstream of TCR signalling. These induce a transcriptional program that results in immense changes, including a doubling in cell size (from 5 μm to 10 μm) (de la Roche et al., 2016), changes in metabolism (MacIver et al., 2013) and proliferation status (Best et al., 2013; Brownlie and Zamoyska, 2013). In total, the differentiation and clonal expansion processes are estimated to take 4-5 days (de la Roche et al., 2016). The differences between naive and effector cells will be explored in more detail in chapter 4.

Mature effector CTL leave the lymph node and enter peripheral tissues to kill diseased cells at the site of infection (Zhang and Bevan, 2011). In the periphery, intracellular peptides are continuously degraded by the proteasome inside the cell and the resulting peptides are loaded into the peptide binding groove of MHC class I proteins in the endoplasmic reticulum (Neefjes et al., 2011). Peptide-MHC class I complexes are then displayed on the cell surface. This process happens in all nucleated cells of the body (Neefjes et al., 2011). The constant monitoring of intracellular antigens by effector CTLs provides a crucial control mechanism to ensure that the cells of the body are healthy. CTLs that recognise a peptide-MHC complex eliminate the infected cell through the secretion of cytotoxic compounds, described in detail in section 1.4, and also release cytokines such as IFNγ and tumour necrosis factor (TNF) (Harty and Badovinac, 2008; Parish and Kaech, 2009; Williams and Bevan, 2007).

The antigen-specific CTLs proliferate and kill target cells, normally resulting in the elimination of the infection. After 5-8 days, CTL numbers decline in a 'contraction' phase, driven by apoptosis (Harty and Badovinac, 2008; Parish and Kaech, 2009). It is estimated that CTL numbers are reduced by 90-95% (Harty and Badovinac, 2008; Williams and Bevan, 2007). This is important, as the immune response has to be terminated to limit damage to the body (Brownlie and Zamoyska, 2013).

However, some cells survive the contraction phase as interaction with antigen also results in the formation of memory T cells (Harty and Badovinac, 2008; Williams and Bevan, 2007). Memory T cells reside in secondary lymphoid organs and peripheral tissues and provide long term protection against the disease. In response to re-stimulation by encountering the antigen for a second time, memory cells can respond rapidly, producing larger amounts of effector cells than during the first immune response (Parish and Kaech, 2009). This ability of the adaptive immune system to form long-lasting memory cells is crucial for the concept of vaccination (Harty and Badovinac, 2008). Memory T cells were found 75 years post vaccination in humans, demonstrating the longevity of this response (Williams and Bevan, 2007).

It is therefore clear that CTLs are crucial to protect us from diseases. This is also demonstrated by disorders that are caused when these lymphocytes are not functioning properly, as discussed in detail in section 1.4.2 and 1.4.3. The following section outlines the intricate mechanism underlying T cell activation.

1.3 TCR signalling

When naive T cells in the secondary lymphoid organs recognise a peptide-MHC complex on APCs, or an effector CTL encounters a target cell in the periphery, binding of the TCR to the peptide MHC complex triggers a complex signalling response (Brownlie and Zamoyska, 2013).

The main signalling events triggered by TCR engagement are outlined in Figure 1.1. The lymphocyte-specific protein tyrosine kinase (Lck) is activated downstream of the TCR and CD3 (Brownlie and Zamoyska, 2013). The TCR depends on Lck, which binds to the cytoplasmic portion of the CD8 co-receptor, to initiate a complex signalling cascade (Brownlie and Zamoyska, 2013). Lck phosphorylates tyrosine residues in immunoreceptor tyrosine-based activation motifs (ITAMS) of the cytoplasmic portions of CD3 γ, δ, ε and ζ chains (Brownlie and Zamoyska, 2013). ζ-chain-associated protein kinase of 70 kDa (Zap70) binds to phosphorylated ITAMs, and Lck also phosphorylates Zap70 (Smith-Garvin et al., 2009). Zap70 is activated by phosphorylation and undergoes conformational change. Subsequently, Zap70 is able to phosphorylate target molecules, such as the linker for activation of T cells (LAT) (Brownlie and Zamoyska, 2013) and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP76) (Smith-Garvin et al., 2009).

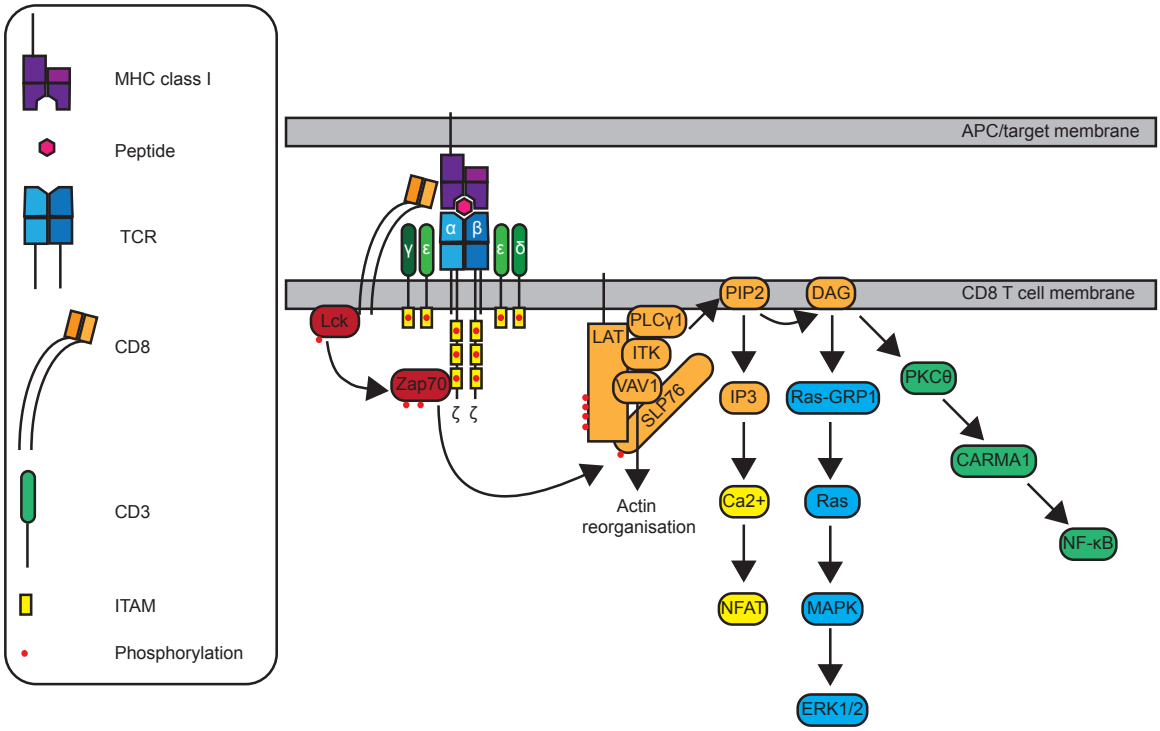

Fig. 1.1 ***TCR signalling.*** *When the TCR recognises a peptide in the context of MHC class I, Lck phosphorylates CD3 chains, as well as Zap70. This triggers the formation of the LAT signalosome, which consists of several molecules, including SLP76, ITK, VAV1 and PLCγ1. The conversion of PI(4,5)P_2 to DAG and IP_3 by PLCγ1 is required for the activation of several signalling molecules and transcription factors, including NFAT, ERK1/2 and NF-κB. This extensive transcription factor activation results in drastic changes in gene expression that are crucial for the differentiation of naive T cells to effector CTL (Brownlie and Zamoyska, 2013; Cantrell, 2015; Navarro and Cantrell, 2014).*

Phosphorylated LAT allows recruitment of further signalling molecules, forming the LAT signalosome, which is crucial for the production of downstream signals (Figure 1.1). ZAP70 phosphorylates SLP76, which subsequently forms a complex with LAT, phospholipase Cγ1 (PLCγ1), interleukin-2-inducible T cell kinase (ITK) and Vav Guanine Nucleotide Exchange Factor 1 (VAV1) (Brownlie and Zamoyska, 2013; Smith-Garvin et al., 2009). This complex is crucial for rearrangement of the actin cytoskeleton. Furthermore, PLCγ1 activation drives conversion of phosphatidylinositol-(4,5)-biphosphate (PI(4,5)P_2) from the plasma membrane to the second messenger molecules inositol-(1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG), which triggers complex downstream events (Brownlie and Zamoyska, 2013; Macian, 2005).

Production of IP_3 results in the release of Ca^{2+} from the endoplasmic reticulum into the cytosol, which in turn leads to the activation of the nuclear factor of activated T cells (NFAT) transcription factor (Hogan et al., 2003; Macian, 2005). Meanwhile, DAG activates Ras and members of the protein kinase D (PKD) and protein kinase C (PKC) families, resulting in the activation of transcription factors (Navarro and Cantrell, 2014).

PKCθ interacts with CARMA1, which leads to activation of the IKK complex, that in turn can induce nuclear factor-κB (NF-κB) transcriptional activity (Matsumoto et al., 2005; Navarro and Cantrell, 2014; Paul and Schaefer, 2013). The NF-κB signalling pathway is outlined in more detail in chapter 5. Additionally, PKCs were found to be important for cell adhesion in effector CTL and centrosome polarisation to the contact site between the CTL and its target (Letschka et al., 2008; Quann et al., 2009). PKD2, which is phosphorylated by PKCs, is needed for transcriptional upregulation of IL-2 and IFNγ (Navarro and Cantrell, 2014). Production of DAG also triggers RAS activation through RAS guanine nucleotide exchange factor RAS guanyl-releasing protein 1 (RAS-GRP1) (Brownlie and Zamoyska, 2013). RAS, in turn, leads to activation of the mitogen-activated protein kinase (MAPK) cascade and subsequent activation of the extracellular signal-regulated kinase (ERK) (Brownlie and Zamoyska, 2013).

In its entirety, this signalling network results in complex intracellular events and changes in gene transcription. In naive T cells this drives differentiation to effectors. In CTL, TCR signalling induces cytoskeletal rearrangements, secretion of lytic granules and cytokine production (Brownlie and Zamoyska, 2013; de la Roche et al., 2016). TCR signalling has furthermore been speculated to be important for re-filling the cytotoxic content of the lytic granules, ensuring that pre-formed granules are available continuously (Isaaz et al., 1995).

1.4 CTL effector function

In response to TCR signalling naive T cells undergo proliferation, metabolic changes and differentiate into effector CTL over several days. In effector CTLs, peptide-MHC complex recognition leads to the rapid polarisation of lytic granules to the contact site between the CTL and the target cell. Within 20 minutes (min) of target cell recognition, the CTL has secreted the cytotoxic proteins contained within lytic granules, resulting in target cell death, and detached from the dying target cell to find its next target (Ritter et al., 2015). CTLs are therefore serial killers that can eliminate one target after another within a short period of time. Some of the intracellular events typically associated with the CTL killing process

are outlined in brief in Figure 1.2. The precise molecular steps underlying this process are covered in detail in the following sections.

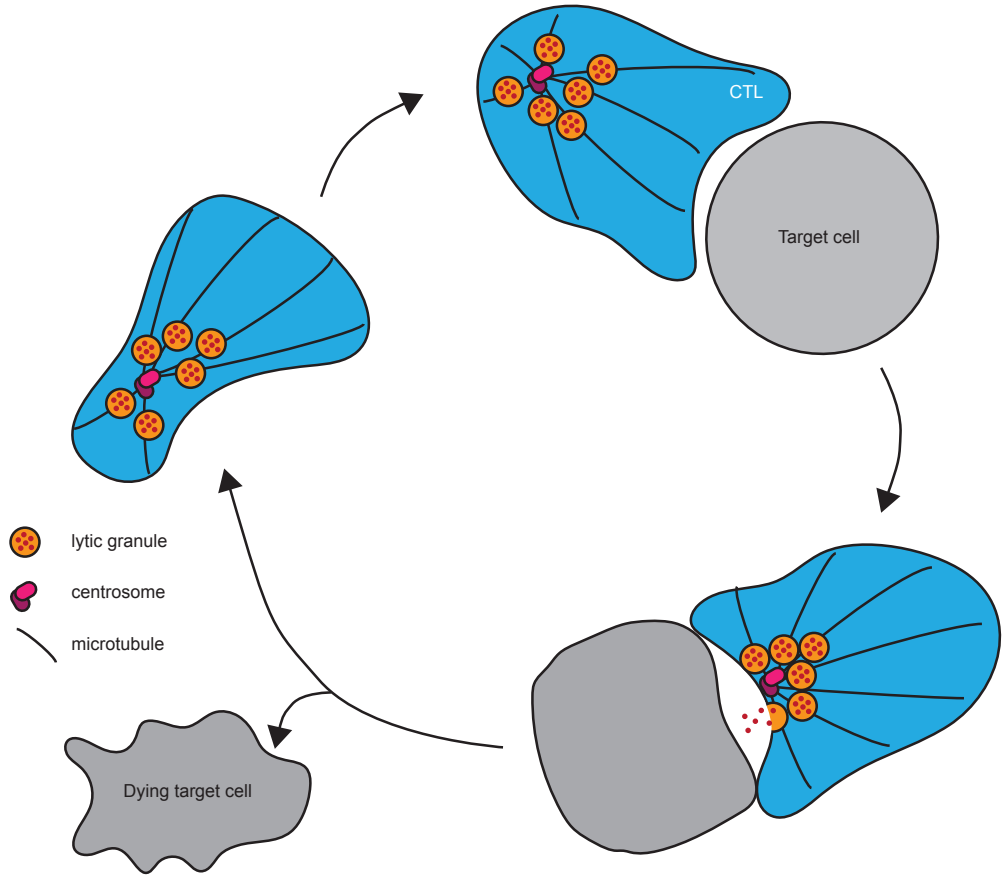

Fig. 1.2 ***CTL killing process.*** *CTL travel around the body, searching for cells that present their specific peptide in complex with MHC class I. As the CTL searches for target cells it adapts a migratory shape with a protruding leading edge and a uropod. Lytic granules and the centrosome are mostly found at the back of the cell. Upon recognition of a target cell, the CTL forms a tight cell-to-cell contact with the target cell. Subsequently, the centrosome polarises towards the contact site, delivering the lytic granules to the immunological synapse. The lytic granule membrane fuses with the plasma membrane, secreting the cytotoxic contents which trigger apoptosis of the target cell. The entire process from target cell recognition to detachment of the CTL takes around 20 min. The CTL can immediately interact with another target, acting as a serial killer (Ritter et al., 2015; Stinchcombe et al., 2001b).*

1.4.1 Formation of the immunological synapse

Both naive T cells and effector CTL form a tight contact with their APCs and target cells, respectively. This contact is known as the immunological synapse (IS) and has a highly organised structure (Figure 1.3). Actin depletes from the centre of the synapse forming an outer ring around the IS, known as the distal supramolecular activation complex (dSMAC) (Carisey et al., 2018; Ritter et al., 2015; Stinchcombe et al., 2001b, 2006). The actin depletion is suggested to be of functional significance as it removes the actin barrier for granule secretion (Ritter et al., 2017). A ring of adhesion molecules, also referred to as the peripheral SMAC (pSMAC), lies within the dSMAC. The pSMAC is reported to contain adhesion molecules such as LFA-1 and talin (Grakoui et al., 1999; Monks et al., 1998; Potter et al., 2001; Stinchcombe et al., 2001b). TCRs and associated signalling molecules, such as Lck and PKCθ can be found at the centre of the IS, also referred to as the central SMAC (cSMAC) (Grakoui et al., 1999; Monks et al., 1998; Stinchcombe et al., 2001b). TCRs are internalised for recycling from the cSMAC (Das et al., 2004). The secretory zone, where the centrosome docks and cytolytic components are released, lies adjacent to the cSMAC (Ritter et al., 2015; Stinchcombe et al., 2006). The polarisation of the centrosome, which is the microtubule organising centre in CTL, is thought to be crucial for the focused delivery of lytic granules to the IS (Stinchcombe and Griffiths, 2007; Stinchcombe et al., 2006). Granule polarisation happens within 6-10 min of target cell recognition (Ritter et al., 2015). The importance of centrosome polarisation during this process was demonstrated by knockdown of Cep83, a distal appendage protein necessary for the centrosome to dock at the plasma membrane (Tanos et al., 2013). Cep83 knockdown decreases lytic granule secretion (Stinchcombe et al., 2015). Additionally, other organelles have been reported to polarise towards the IS, such as the Golgi apparatus (Stinchcombe et al., 2006).

Recently, actin depletion was found to be mediated by changes in the composition of the plasma membrane as the IS forms. As explained in section 1.3, PLCγ1 is recruited to the plasma membrane upon TCR signalling, and hydrolyses PI(4,5)P_2 to DAG and IP_3. It has been observed that loss of PI(4,5)P_2 correlates with actin depletion at the centre of the IS (Gawden-Bone et al., 2018; Ritter et al., 2015, 2017). In the absence of a TCR stimulus, phosphatidylinositol 4-phosphate 5-kinase type I (PIP5K), which controls PI(4,5)P_2 formation, and actin were found at the plasma membrane. Upon TCR engagement, PLCγ1 conversion of PI(4,5)P_2 to DAG leads to a reduction of negative charge in this area of the plasma membrane, resulting in detachment of PIP5K. However, if PIP5K was modified so that it was maintained across the IS membrane, actin depletion did not occur. Furthermore, centrosome docking as well as lytic granule secretion was reduced, and target cell killing

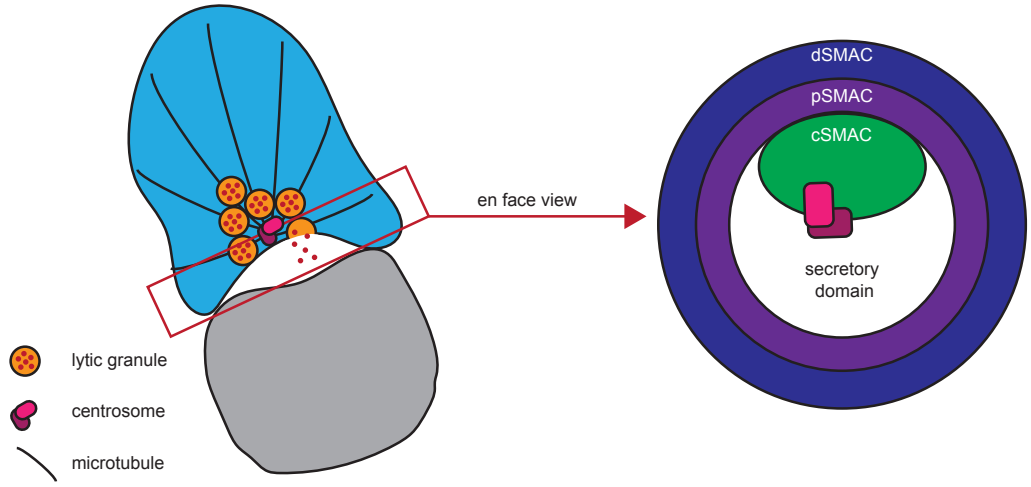

Fig. 1.3 ***Structure of the IS.*** *The en face view of a CTL in contact with its target shows the formation of a 'bulls-eye configuration' at the IS (Stinchcombe and Griffiths, 2007). TCRs and associated signalling molecules form the cSMAC, which lies at the centre of the IS together with the secretory domain, where the centrosome docks and the contents of lytic granules are released (Ritter et al., 2015; Stinchcombe et al., 2006). Centrosome polarisation is thought to be crucial for the delivery of lytic granules precisely to the contact site between CTL and target. A ring of adhesion molecules (pSMAC) surrounds the cSMAC, containing molecules such as LFA-1 and talin. Actin depletes from the centre of the IS and forms the dSMAC (Ritter et al., 2015; Stinchcombe et al., 2006). Once granules are released, actin depletion is reversed, preventing further lytic granule secretion (Ritter et al., 2017).*

was impaired when PIP5K was maintained at the membrane (Gawden-Bone et al., 2018). This demonstrates the importance of membrane composition for actin depletion, although the precise molecular events linking PI(4,5)P_2 to actin still remain to be elucidated.

1.4.2 Lytic granule contents

CTLs kill target cells through the release of lytic granules that contain cytotoxic proteins. Polarisation of the centrosome precisely delivers the lytic granules to the contact site between the CTL and the target cell (Stinchcombe and Griffiths, 2007; Stinchcombe et al., 2006). Lytic granules contain granzymes, granulysin, perforin and Fas ligand (FasL), which are

crucial for inducing apoptosis of target cells (Bossi and Griffiths, 2005). The content of the lytic granules, and how they trigger target cell death, are described in detail below.

Perforin

The lytic granule component perforin multimerises to form pores in the target cell membrane in order to allow cytotoxic proteins to enter target cells (Lopez et al., 2013a,b; Tschopp and Nabholz, 1990). These pores are big enough to allow granzymes to pass into the cytosol of the target cell (Law et al., 2010). Perforin contains a C2 domain that is required for membrane binding via a calcium dependent mechanism (Uellner et al., 1997). While perforin is active at high calcium concentrations and neutral pH in the extracellular environment upon secretion, it was found to be inactive at the low pH within lytic granules (Kuta et al., 1989; Young et al., 1987).

The importance of perforin for cytotoxicity was demonstrated by a study using a knockout (KO) mouse model (Kägi et al., 1994a) and in patients, where perforin deficiency causes an immunodeficiency disorder, familial hemophagocytic lymphohistiocytosis (FHL) type 2 (Kogawa et al., 2002; Stepp et al., 1999). Several other inherited genetic defects can cause FHL, which has 5 subtypes (Janka and Lehmberg, 2014), covered in more detail in section 1.4.3. Patients affected by FHL present with hemophagocytic lymphohistiocytosis (HLH), which is a clinical syndrome characterised by extremely high cytokine levels. This is the result of continuous stimulation of the immune system due to an inability to resolve the infection (Janka and Lehmberg, 2014).

Granzymes

Another component of the lytic granules are granzymes, a family of serine proteases, which were shown to constitute 1-2% of the overall CTL proteome (Hukelmann et al., 2016). Five granzymes have been identified in humans (granzyme A, B, H, K and M) and several additional granzymes have been identified in mice (Bots and Medema, 2006; Masson and Tschopp, 1987). Granzymes are made in an inactive form, and cleavage by cathepsin C in the granules triggers enzymatic activation (Trapani, 2001). As granzymes are inactive at low pH, CTLs likely store these potentially dangerous proteins in granules to avoid autolysis (Masson et al., 1986). To enter the target cell, granzymes rely on perforin to form pores in the plasma membrane (Bots and Medema, 2006; Lopez et al., 2013a,b; Trapani and Sutton, 2003). Interestingly, deficiency in individual granzymes does not cause severe phenotypes in mice, in contrast to the effect observed in response to perforin deficiency, likely due to

redundancy between granzymes (Trapani, 2001).

Granzyme B is the most widely studied granzyme and triggers caspase-dependent apoptosis in target cells (Bots and Medema, 2006). CTLs derived from granzyme B deficient mice showed a defect in their ability to trigger rapid apoptosis in target cells (Heusel et al., 1994). Granzyme B cleaves the protein BID, which together with other proteins permeabilises the outer membrane of mitochondria, triggering the release of pro-apoptotic proteins (Bots and Medema, 2006; Trapani, 2001). Release of cytochrome c activates caspase-9, which cleaves further caspases, triggering a cascade leading to apoptosis (Bots and Medema, 2006). Granzyme B can additionally trigger apoptosis by cleaving members of the anti-apoptotic BCL-2 protein family, as well as cleaving certain caspases, such as caspase 3 and caspase 8 (Bots and Medema, 2006). Granzyme B has been suggested to cleave several other molecular targets, including cytoskeletal proteins and proteins in the nuclear membrane (Bots and Medema, 2006; Trapani and Sutton, 2003). Granzyme A is reported to induce target cell apoptosis by triggering loss of mitochondrial inner membrane potential, which results in release of reactive oxygen species that induce DNA damage and impair DNA repair (Bots and Medema, 2006). Granzyme A does not act via caspases, but is also reported to target histone H1 and nuclear lamins (Bots and Medema, 2006).

Granulysin

Another cytotoxic protein found in lytic granules is granulysin (Peña et al., 1997; Peña and Krensky, 1997). Granulysin triggers membrane damage and mitochondrial damage in target cells, resulting in cytochrome c release and activation of caspases that trigger apoptosis (Krensky and Clayberger, 2009). The acidic pH in the lytic granules is needed for the processing of an inactive 15 kilodalton (kDa) version of granulysin to the lytic 9 kDa version (Hanson et al., 1999; Peña and Krensky, 1997). Additionally, the lower molecular weight (MW) version has reduced cytolytic activity at acidic granule pH (Hanson et al., 1999). This again highlights the importance of lytic granules to store these potentially dangerous proteins so no harm is caused to the CTL (Hanson et al., 1999).

Fas ligand

In addition to the perforin mediated pathway, CTLs can also trigger target cell death through interaction between FasL (also known as CD95L), with Fas (also known as CD95 and APO-1) on the surface of target cells (Krammer, 2000; Strasser et al., 2009). As FasL is also stored in the lytic granules, the expression of FasL on the cell surface is carefully regulated by focused

secretion of lytic granules in response to TCR signals (Bossi and Griffiths, 1999). Upon interaction with FasL, the Fas receptor on target cells induces the formation of a signalling complex containing, among others, caspase 8, which can trigger apoptosis upon activation (Krammer, 2000; Strasser et al., 2009). Fas signalling can additionally trigger cell death through the BCL-2 regulated apoptotic pathway involving release of pro-apoptotic molecules from mitochondria (Krammer, 2000; Strasser et al., 2009). Experiments using CTL deficient in FasL and perforin indicated that these two pathways are the main killing pathways used by CTL (Kägi et al., 1994b).

All lytic granule components discussed here have in common that they need to be released from the lytic granules in order to be exposed to the target cell and trigger apoptosis. Fusion between the lytic granule membrane and the plasma membrane results in localisation of FasL on the CTL plasma membrane and secretion of perforin, granzymes and granulysin. The precise molecular mechanism underlying the fusion of these two membranes will be covered in the next section.

1.4.3 Secretion of lytic granules

The lysosomal membrane fuses with the plasma membrane to release granule content. This process is also referred to as degranulation (Betts et al., 2003). Through precise secretion into the secretory cleft, lytic granules are delivered to the target cell without causing damage to surrounding cells (Stinchcombe and Griffiths, 2003). The machinery required for CTL degranulation was in large part identified through the study of patients suffering from inheritable immunodeficiency conditions, where CTL and NK cells are unable to kill target cells efficiently, such as FHL and Griscelli syndrome (GS) type 2 (de Saint Basile et al., 2010; Janka and Lehmberg, 2014). These immunodeficiency conditions and their genetic causes are summarised in Table 1.1.

FHL is divided into several subtypes according to the underlying genetic defect; mutations in *PRF1*, encoding perforin, cause FHL2 (Stepp et al., 1999), mutations in *MUNC13-4* cause FHL3 (Feldmann et al., 2003), FHL4 is caused by defects in the gene encoding syntaxin-11 (*STX11*) (zur Stadt et al., 2005), FHL5 is caused by mutations in *MUNC18-2* (Côte et al., 2009; zur Stadt et al., 2009) and GS2 was found to be caused by mutations in *RAB27A* (Ménasché et al., 2000). Mutations in these genes were found to result in a degranulation defect in patient-derived CTL and NK cells, with the exception of mutations in *PRF1*, which impairs target cell killing downstream of degranulation (de Saint Basile et al., 2010). Therefore, despite having intact lytic granule content, cytotoxic lymphocytes from FHL3,

FHL4, FHL5 and GS2 patients cannot kill target cells. This indicated that the proteins encoded by the mutated genes are important for the secretion of lytic granule content (de Saint Basile et al., 2010).

Table 1.1 ***List of genes and proteins associated with CTL-related immunodeficiencies.*** *HLH is a hyperinflammatory syndrome that is reported to lead to the following clinical symptoms: fever, cytopenia, hepatosplenomegaly, hypertriglyceridemia and/or hypofibrinogenemia, and hemophagocytosis in bone marrow, lymph nodes or spleen. Additionally, patients can present with further symptoms, such as neurological abnormalities (Henter et al., 2007; Janka and Lehmberg, 2014). HLH = hemophagocytic lymphohistiocytosis, FHL = Familial hemophagocytic lymphohistiocytosis, GS = Griscelli syndrome, CHS = Chediak-Higashi Syndrome and HSP = Hermansky-Pudlak Syndrome.*

Disorder	**Gene/protein affected**	**Symptoms**	**Reference**
FHL1	genetic locus = 9q22, gene/protein not known	HLH	(Ohadi et al., 1999)
FHL2	*PRF1*, PRF1	HLH	(Stepp et al., 1999)
FHL3	*MUNC13-4*, MUNC13-4	HLH	(Feldmann et al., 2003)
FHL4	*STX11*, STX11	HLH	(zur Stadt et al., 2005)
FHL5	*MUNC18-2*, MUNC18-2	HLH	(zur Stadt et al., 2009), (Côte et al., 2009)
GS2	*RAB27A*, RAB27A	HLH, partial albinism	(Griscelli et al., 1978), (Ménasché et al., 2000)
CHS	*LYST*, LYST	HLH, partial albinism, neurological symptoms	(Barbosa et al., 1996), (Perou et al., 1996), (Nagle et al., 1996), (Ward et al., 2000)
HPS2	*AP3B1A*, AP3β3A	HLH, partial albinism, prolonged bleeding time	(Hermansky and Pudlak, 1959), (Dell'Angelica et al., 1999), (Clark et al., 2003)
HPS10	*AP3D1*, AP3δ	HLH, partial albinism, neurological symptoms	(Ammann et al., 2016)

FHL and GS2 tend to have an early onset, usually within the first year of life. Patients present with hyperinflammation caused by a highly activated but unsuccessful immune response. Symptoms include fever, hepatosplenomegaly, cytopenia and these disorders can be fatal in the absence of treatment. Currently, the treatment options are limited to immunosuppressants and the only potential cure is hematopoietic stem cell transplantation (Janka and Lehmberg, 2014). The fact that genetic defects in proteins involved in the lytic granule secretion process, or lytic granule content in the case of perforin, cause immunodeficiency disorders highlights how important the function of cytotoxic cells is to maintain a healthy immune system.

Furthermore, other immunodeficiency disorders, such as Chediak-Higashi Syndrome (CHS) and Hermansky-Pudlak Syndrome (HPS), enabled the identification of proteins important in the maturation of lytic granules and their transport to the IS (de Saint Basile et al., 2010; Janka and Lehmberg, 2014). CHS is caused by defects in LYST, which is reported to cause a lysosome fission defect, resulting in granules that are too big to fuse at the IS (Baetz et al., 1995; Durchfort et al., 2012). HPS type 2 is caused by defects in AP3β (Dell'Angelica et al., 1999) and HPS type 10 is caused by defects in AP3δ (Ammann et al., 2016). Some mutations in AP3 have been shown to result in an inability of lysosomes to move towards the centrosome (Clark and Griffiths, 2003; Clark et al., 2003).

Analysis of the mutations underlying immunodeficiency disorders has therefore been crucial for the identification of the molecular machinery involved in lytic granule movement to the IS, degranulation and target cell killing. Simultaneously, these disorders demonstrate the devastating consequences of defective cytotoxic cells on an organismal level. Below I focus on the key regulators of the final secretion steps at the IS and their point of action in the secretion process is visualised in Figure 1.4.

RAB27A

In RAB27A depleted cells, granules can polarise to the IS but they can not dock at the plasma membrane and fusion between the lytic granule membrane and the plasma membrane is impaired (Stinchcombe et al., 2001a). RAB27A has also been implicated in the secretion of granules from granulocytes (Munafó et al., 2007) and in pigment secretion in melanocytes (Hume et al., 2001; Wu et al., 2001), indicating that RAB27A also regulates fusion between vesicles and the plasma membranes in these biological settings. This explains why GS2 patients often present with albinism as well as immunodeficiency (Stinchcombe et al., 2004).

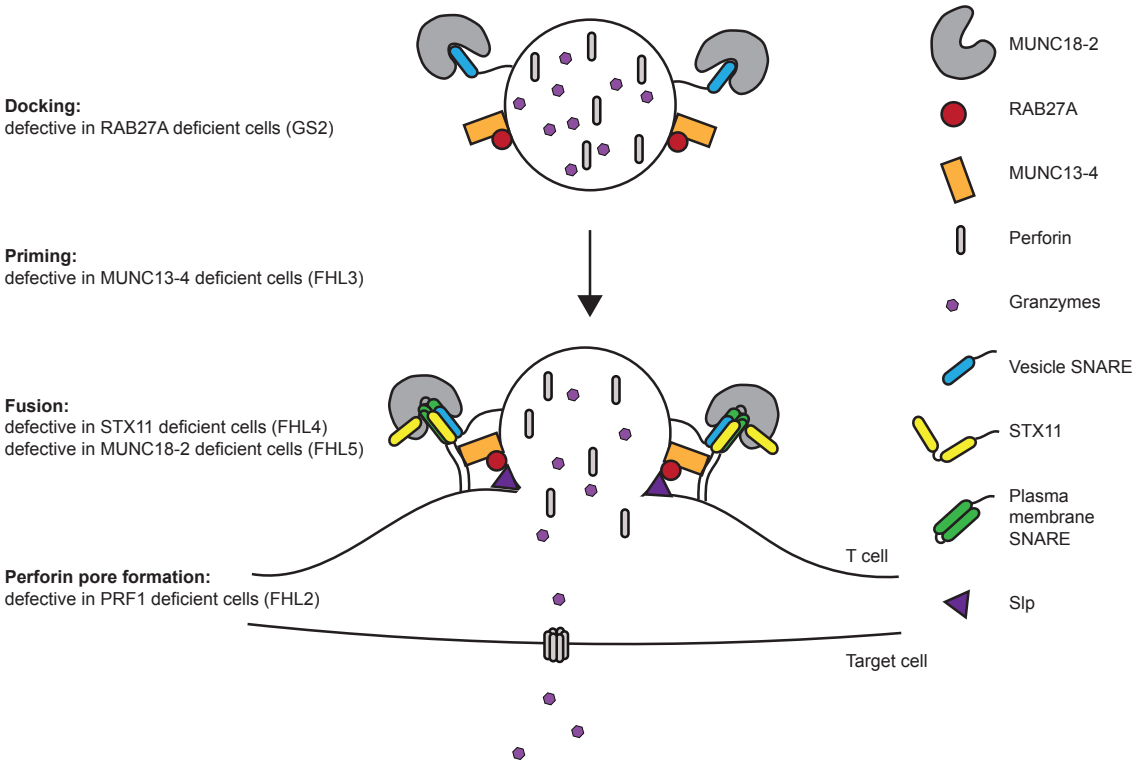

Fig. 1.4 ***Proteins crucial for lytic granule secretion at the IS.*** *Lytic granule secretion is an intricate process that involves fusion between the lytic granule membrane and the plasma membrane. Key regulators of this process are RAB27A, MUNC13-4, MUNC18-2 and several SNARE proteins. Defects in some of the proteins highlighted in this figure cause immunodeficiency diseases, indicated in brackets on the left. RAB27A interacts with MUNC13-4 (Neeft et al., 2005; Shirakawa et al., 2004) and SLPs, which are located on the plasma membrane in CTL (Holt et al., 2008; Kuroda et al., 2002b; Kurowska et al., 2012; Ménasché et al., 2008). MUNC13-4 additionally interacts with STX11 and phospholipids (Boswell et al., 2012; Elstak et al., 2011). MUNC18-2 and STX11 bind to one another (Cetica et al., 2010; Côte et al., 2009; zur Stadt et al., 2009) and STX11 was found to form a SNARE complex with the plasma membrane SNARE SNAP23 and one of the VAMP vesicle SNAREs (Spessott et al., 2017; Ye et al., 2012).*

RAB27A is a member of the Rab protein family, which control numerous cellular processes by acting as molecular switches (Wennerberg et al., 2005). Rab proteins are known to regulate vesicle trafficking, docking and fusion with target membranes by interacting with certain effector molecules. RAB27A switches between its guanosine triphosphate (GTP)-

bound active state and its guanosine diphosphate (GDP)-bound inactive state. The switch is regulated by guanine nucleotide exchange factors (GEFs), which trigger GTP binding, and GTPase-activating proteins (GAPs), which trigger GTP hydrolysis. The structure of the "switch" regions in Rab proteins is altered depending on whether GDP or GTP is bound, thereby affecting binding to effector proteins (Fukuda, 2013). Additionally, membrane attachment, which is mediated by prenylation of Rab cysteine residues, is important for Rab protein function (Pereira-Leal et al., 2001).

RAB27A can bind different effector proteins in different cell types in its active GTP-bound form. Several RAB27A effector molecules have been identified, including Synaptotagmin-like protein lacking C2 domains (SLACs), Synaptotagmin-like proteins (SLPs), and MUNC13-4. SLAC2-a (also known as melanophilin) and SLP1-5 interact with GTP-bound RAB27A via their SLP-homology domain (SHD) (Kuroda et al., 2002a,b). Melanophilin interacts with both RAB27A and MYO5A in melanocytes, thereby mediating melanosome transport and pigment secretion, but melanophilin is not expressed in CTLs and can therefore not mediate lytic granule secretion (Fukuda et al., 2002; Hume et al., 2002; Nagashima et al., 2002; Wu et al., 2002). In contrast, several SLPs were found to be expressed in CTLs, and the expression of a dominant-negative SHD construct, interfering with binding of SHD-domain containing proteins to RAB27A, reduced CTL killing capability (Holt et al., 2008; Ménasché et al., 2008). CTLs derived from *Slp1* and *Slp2-a* KO mice did not show reduced CTL cytolytic activity, indicating that different SLP family members functioned as RAB27A effectors in CTL (Holt et al., 2008). Subsequently, SLP3 was found to be important for lytic granule secretion in CTL (Kurowska et al., 2012). MUNC13-4 has been shown to bind GTP-bound RAB27A in hematopoietic cells, but in contrast to the other RAB27A effector proteins mentioned above, MUNC13-4 does not contain an SHD domain (Neeft et al., 2005; Shirakawa et al., 2004). The role of MUNC13-4 in lytic granule secretion will be covered in more detail in the following subsection.

Missense mutations observed in GS2 patients have led to the identification of functionally important RAB27A residues. For example, the Trp73Gly mutation was found to result in RAB27A protein with reduced GTP hydrolysis and reduced binding to MUNC13-4 and melanophilin, suggesting that residue 73 is important for binding to both of these effector proteins (Menasche et al., 2003; Neeft et al., 2005). A further study investigating the underlying mutations of GS2 patients that presented with immunodeficiency symptoms but not albinism identified that mutations in Arg141, Tyr159 and Ser163 resulted in loss of interaction of RAB27A with MUNC13-4, while interaction with melanophilin was

maintained (Cetica et al., 2015). This demonstrates clearly that RAB27A is able to control secretion in different cell types via interaction with cell-specific effector proteins.

MUNC13-4

In cells carrying mutations in MUNC13-4, granules can dock at the plasma membrane but cannot secrete their contents, suggesting that MUNC13-4 is crucial for lytic granule priming before fusion can occur (Elstak et al., 2011; Feldmann et al., 2003). MUNC13-4 contains two MUNC13-homology domains (MHD1 and MHD2) with a C2 calcium binding domain either side (C2A and C2B) (Feldmann et al., 2003). RAB27A and MUNC13-4 have been shown to interact with one another in platelets, mast cells and CTL (Elstak et al., 2011; Neeft et al., 2005; Shirakawa et al., 2004). MUNC13-4 binds to GTP-bound RAB27A, but not GDP-bound RAB27A, and this binding required the N-terminal region between the C2A and MHD1 domains of MUNC13-4 (Ménager et al., 2007; Neeft et al., 2005). A study using a truncated construct comprised of MUNC13-4 residues 240-290 suggested that this region is sufficient for binding to RAB27A (Elstak et al., 2011), however these findings should be treated with caution as the truncated construct may not be folded correctly.

MUNC13-4 has additionally been shown to interact with soluble NSF attachment protein receptor (SNARE) proteins, such as STX11, as well as syntaxin-1, -2 and -4, which are crucial in membrane fusion (Boswell et al., 2012). While the C2A domain is important for binding to SNAREs in a calcium dependent manner, the C2B domain binds to phosphatidylserine containing membranes (Boswell et al., 2012). MUNC13-4 was furthermore shown to co-immunoprecipitate with MUNC18-2 in platelets (Al Hawas et al., 2012). MUNC13-4 has therefore been linked to both MUNC18-2 and STX11, two components thought to be required for the final fusion between the lytic granule membrane and plasma membrane (Côte et al., 2009; zur Stadt et al., 2009).

MUNC18-2 and STX11

Mutations that cause defects in STX11 and MUNC18-2 cause FHL4 and FHL5, respectively. These two proteins bind to one another and loss of either protein results in a degranulation defect (Bryceson et al., 2007; Cetica et al., 2010; Côte et al., 2009; zur Stadt et al., 2009, 2005).

STX11 belongs to the SNARE protein family but does not contain a C-terminal transmembrane domain, instead it is thought to associate with membranes by palmitoylation of the C-terminus

(Tang et al., 1998; Valdez et al., 1999). Studies in cells derived from FHL4 patients and *Stx11* KO mice showed that STX11 controls CTL and NK cell degranulation (Arneson et al., 2007; Bryceson et al., 2007; D'Orlando et al., 2013; zur Stadt et al., 2005).

MUNC18-2 is part of the Sec1/Munc18-like protein family, members of which function in membrane fusion by interacting with SNAREs (Carr and Rizo, 2010; Südhof and Rothman, 2009). Members of the MUNC18 family of proteins have arch-shaped structures and their cavity binds to syntaxins and SNARE complexes (Misura et al., 2000; Yu et al., 2013). MUNC18-2 binds to the N-terminal peptide of STX11 and MUNC18-2 mutations that cause FHL5 (Arg39Pro, Leu130Ser, Glu132Ala and Pro334Leu) mapped onto the surface of the structure, indicating that these residues may be important for the interaction of MUNC18-2 with SNARE proteins (Hackmann et al., 2013).

In addition to showing that MUNC18-2 and STX11 interact, patient studies have shown that MUNC18-2 deficient cells also show decreased STX11 levels, leading to the conclusion that MUNC18-2 acts as a chaperone, stabilising STX11 (Bin et al., 2013; Cetica et al., 2010; Côte et al., 2009; zur Stadt et al., 2009). Additionally, some mutations in MUNC18-2 (Arg65Gln/Trp) have been identified that do not lead to loss of protein or loss of STX11 binding (Spessott et al., 2015). Instead these mutant proteins seem to stabilise STX11, but interfere with SNARE complex assembly, thereby still blocking secretion and still causing FHL-symptoms in affected individuals (Spessott et al., 2015). In summary, MUNC18-2 is both a chaperone of STX11 and may also be involved in membrane fusion as it can control SNARE complex assembly (Spessott et al., 2017).

STX11 has been described to form a SNARE complex with synaptosome associated protein (SNAP)23 and vesicle-associated membrane protein (VAMP)3 or VAMP8 to regulate fusion between the vesicle membrane and the plasma membrane (Al Hawas et al., 2012; Spessott et al., 2017; Valdez et al., 1999; Ye et al., 2012). Spessott et al. (2017) showed that MUNC18-2 and STX11 interact with VAMP8 and SNAP23 in human CTL, and that knockdown of VAMP8 reduces CTL cytotoxicity. However, different VAMPs may be able to participate in CTL degranulation, and could be important during vesicle maturation, as well as fusion at the IS. In addition to reduced degranulation and cytolytic activity observed in CTL derived from *Vamp8* KO mice, CTL derived from *Vamp2* KO mice showed similar phenotypes (Loo et al., 2009; Matti et al., 2013). Furthermore, VAMP7 knockdown in NK cells decreased degranulation and cytotoxicity (Marcet-Palacios et al., 2008). These results indicate partial redundancy between different VAMPs in the degranulation process.

It is the intricate interaction of the proteins summarised in Figure 1.4, and perhaps further unknown proteins, that leads to the final secretion of cytotoxic granule content into the secretory cleft between the CTL and the target cell, which subsequently leads to target cell death.

1.4.4 Detachment from target and serial killing

Upon degranulation, actin recovers forming a barrier for further lytic granule secretion (Ritter et al., 2015, 2017). Detachment of CTLs from their target cells was shown to depend on the CTL sensing target cell apoptosis (Jenkins et al., 2015). In the absence of perforin, or if the caspase response in the target cell was inhibited, the time that CTLs and targets remained conjugated was extended fivefold, from around 8 min to 40 min (Jenkins et al., 2015). Furthermore, cytokine and chemokine secretion from CTLs was increased in response to this continuing attachment. Sustained secretion of IFNγ caused production of proinflammatory cytokines from other immune cells, such as IL-6 from macrophages (Jenkins et al., 2015). These findings provide a potential molecular mechanism for how the dangerous hyperinflammatory state observed in FHL2 patients is generated. In healthy individuals, the caspase-dependent signal prompts the CTL to detach from its target, so that it can move on to kill the next target cell (Jenkins et al., 2015).

A crucial question that remains largely unanswered is how the secreted proteins damage the membrane of the targets, but not the CTL plasma membrane. The CTL membrane is not generally resistant to perforin, as CTLs could be used as targets and be killed by other CTLs when loaded with appropriate peptide (Lopez et al., 2013a). Degranulation has been suggested to protect from perforin pore formation (Lopez et al., 2013a). Additionally, CTLs were found to strain the surface of their target cell (Basu et al., 2016). Perforin pore formation and target killing were enhanced when target cell tension was increased (Basu et al., 2016). Further studies will be necessary to explain this phenomenon fully.

1.5 Measuring CTL function with the degranulation assay

In addition to the secretion of lytic granule contents, transmembrane proteins of the granules translocate to the cell surface when the lytic granule membrane fuses with the plasma membrane (Betts et al., 2003; Peters et al., 1991). The transmembrane proteins include lysosomal-associated membrane protein 1 (LAMP1, also known as CD107a), as well as

LAMP2 (also known as CD107b) and LAMP3 (also known as CD63) (Peters et al., 1991). These proteins are only found at low levels on the plasma membrane of unstimulated cytotoxic lymphocytes. Upon lytic granule secretion, LAMP levels on the cell surface of CTLs and NK cells are transiently increased and can be used as a marker for degranulation (Alter et al., 2004; Betts et al., 2003). An assay has been developed that measures the increase in cell surface exposure of LAMP1 over time (Betts et al., 2003). The exposure of LAMP1 to the extracellular environment allows binding of fluorescently-conjugated antibodies that recognise LAMP1, which enable the identification of cells that degranulated (Figure 1.5) (Alter et al., 2004). Betts et al. (2003) showed that LAMP1 could be detected on the cell surface within 30 min of T cell stimulation, and that LAMP1 levels peaked after 4-5 hours (h). After degranulation, LAMP1 and the antibody bound to it are internalised from the plasma membrane into an acidic vesicle, as shown by the quenching of some fluorochromes (Figure 1.5) (Betts et al., 2003).

The level of degranulation was found to correlate to cytokine secretion, loss of intracellular perforin and target cell lysis (Alter et al., 2004; Betts et al., 2003). Traditional assays, such as the chromium release assay (Brunner et al., 1968), quantify CTL effector function by measuring the resulting target cell death. In contrast, the degranulation assay does not give a direct readout of CTL killing, but gives a direct readout of effector cell activation. As this was shown to correlate with target cell lysis, it gives an indication of the cytotoxic capability of CTLs (Betts et al., 2003).

The degranulation assay is currently used for the diagnosis of putative FHL patients (Bryceson et al., 2012; Chiang et al., 2013). While it can give an insight into which genes should be sequenced, the assay cannot detect the FHL2 phenotype, as degranulation is not impaired in perforin deficient cells (Bryceson et al., 2012; Janka and Lehmberg, 2014). Furthermore, the LAMP1 degranulation assay has been used to isolate tumour reactive CTLs from patients after vaccination (Rubio et al., 2003). In this thesis, I use the degranulation assay to screen for regulators of CTL function. The concept of the degranulation assay, as well as the different in vitro activation stimuli used in this study, are explained in Figure 1.5.

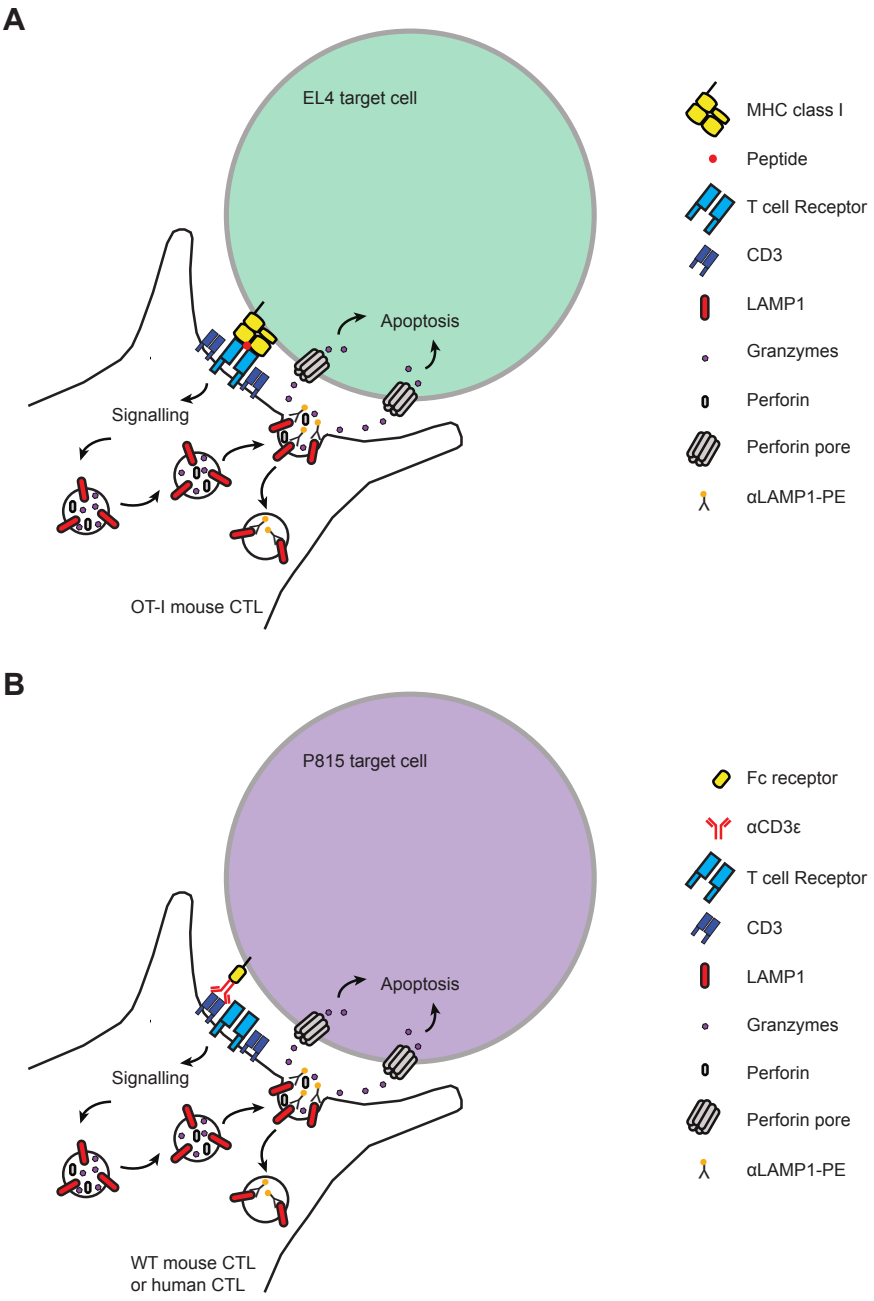

Fig. 1.5 ***Principle of the degranulation assay.***

Fig. 1.5 ***Principle of the degranulation assay.*** *In this thesis I used the degranulation assay to measure functional activation of primary mouse and human CTL. Mouse CTL were either derived from OT-I mice or wild-type (WT) mice.* ***A*** *OT-I CTL express a transgenic TCR that recognises a peptide derived from the ovalbumin (OVA) protein in the context of MHC class I. EL4 lymphoma cells pulsed with OVA peptide were used as target cells for OT-I CTL.* ***B*** *CTL derived from WT mice and human CTL were activated with an antibody recognising mouse or human CD3ε, respectively. The mastocytoma cell line P815 expresses Fc receptors on its surface and can therefore bind to the constant region of antibodies. The variable region of the αCD3ε antibody can then bind to CD3ε on the CTL, triggering TCR signalling. In both experimental set-ups, a fluorescently-conjugated antibody recognising LAMP1 is added to the cell culture supernatant. Upon exposure of LAMP1 to the plasma membrane through lytic granule secretion, the αLAMP1 antibody can bind to LAMP1 and remain bound even upon internalisation of LAMP1. This enables the identification of CTLs that have been activated by the stimulus.*

1.6 Use of CTL for medical purposes

T cells have become of great interest medically due to their successful use for cancer treatment. The treatment options can be broadly separated into cell-based and antibody-based therapies.

1.6.1 Cell-based therapy

Several different cell-based approaches have been used to treat cancer patients. Initially tumour-infiltrating lymphocytes that recognise tumour antigens were isolated from patient samples, expanded in vitro and re-introduced into the patient after lymphodepletion (Rosenberg et al., 2008). This showed promising results in metastatic melanoma. From patients where tumour infiltrating lymphocytes could be isolated, this approach was reported to trigger a response in 50% of the patients. Around 20% of the patients were complete responders, with complete tumour regression over a 3 year follow up period (Rosenberg et al., 2011).

Since tumour infiltrating lymphocytes can be difficult to obtain, two related approaches have been developed that use peripheral T cells of the patients and modify them in vitro to recognise cancer cells. This is achieved through the incorporation of artificial receptors, either genetically-engineered TCRs or chimeric antigen receptors (CARs), which specifically recognise cancer antigens (Sadelain, 2016).

CARs consist of a single-chain variable region of an antibody linked to intracellular signalling components of the TCR to trigger T cell activation. In recent years, CARs have been developed further to also include co-stimulatory domains, such as CD28 (Fesnak et al., 2016; Maher et al., 2002; Sadelain et al., 2013). After genetic modification, the CAR-expressing T cells are transferred back into the patient. Treating patients suffering from acute lymphoblastic leukemia with CARs targeting CD19 expressing cells has shown clinical success, achieving complete remission in 90% of the patients in one study (Maude et al., 2014). Treatment with CTL containing a genetically-modified TCR showed success in melanoma treatment (Morgan et al., 2006).

Both the engineered TCR and the CAR approach have advantages and disadvantages. CARs can recognise any cell surface antigen without the need for it to be presented by an MHC molecule. Additionally, the choice of different TCR signalling components and co-stimulatory domains provides flexibility in CAR design (Sadelain et al., 2013). Genetically-modified TCRs have the advantage of allowing recognition of intracellular peptides and are therefore able to target a broader range of tumour antigens (Corrigan-Curay et al., 2014).

A big challenge that both approaches face is to identify molecular targets for immunotherapy, which should ideally be cancer-specific antigens that are not expressed in healthy tissues (Corrigan-Curay et al., 2014). T cells can identify even very low antigen expression levels in normal tissues, leading to off-target attacks and adverse effects (Coulie et al., 2014; Fesnak et al., 2016). Additionally, successful tumour clearance by cell-based therapies often coincides with other symptoms of hyperactivation of the immune system, such as cytokine release syndrome, and such side effects require additional treatment (Fesnak et al., 2016). However, the effects of the cell-based immunotherapy treatment can be long-lasting, resulting in an improvement of patient survival, likely due to the memory capabilities of the adaptive immune response (Sharma et al., 2017).

1.6.2 Antibody-based therapy

Antibody-based therapies, also known as immune checkpoint blockage, function by counteracting T cell inhibition. Inhibitory molecules, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) that binds to B7 ligands and therefore competes with the co-stimulatory molecule CD28, can inhibit the T cell response (Krummel and Allison, 1995). Blocking CTLA-4 through the use of antibodies results in enhanced T cell effector function and tumour clearance (Leach et al., 1996). The CTLA-4 checkpoint inhibitor, ipilimumab, has been successfully used for treatment of advanced melanoma in patients, and has been shown to

improve survival (Hodi et al., 2010; Robert et al., 2011). Similarly to cell-based therapies, some toxicity is also observed with antibody-based therapies due to the enhancement of the immune response, requiring systemic steroid treatment (Sharma et al., 2017).

Antibodies against another inhibitory receptor, programmed cell death 1 (PD-1), or its ligand PD-L1, can similarly enhance T cell function, and showed success in clinical trials (Okazaki et al., 2013; Zou et al., 2016). Pembrolizumab and nivolumab, two antibodies that recognise PD-1, and atezolimumab, an antibody recognising PD-L1, have been approved for treatment of a variety of cancer types (Sharma et al., 2017).

Combined treatment with PD-1 and CTLA-4 antibodies has shown even more promise than individual treatments in melanoma patients (Larkin et al., 2015; Postow et al., 2015; Wolchok et al., 2013). Additional immune checkpoints, such as lymphocyte activation gene 3 (LAG-3) and T Cell ITIM Domain (TIGIT), are being explored for checkpoint blockage therapy (Topalian et al., 2015). This highlights the need to identify further immune checkpoints that could be potential therapeutic targets. In contrast to cell-based therapies, the antibody-based approach requires recurrent treatment. Studies that are investigating the effect of combining checkpoint blockade and cell-based therapies are currently ongoing (Fesnak et al., 2016).

Interestingly, there are some patients that do not respond to immunotherapy or relapse after an initial response (Sharma et al., 2017; Sucker et al., 2014). Therefore, a better understanding of how CTL killing is mediated at a genetic and molecular level is desperately needed to inform both cell- and antibody-based therapies.

1.7 Techniques used for functional analysis of genes in primary T cells

Genetic modification of primary cells is a powerful way to study the function of genes and their products. One common approach to assess the function of genes is to investigate the phenotypes caused in response to disruption of gene expression. This method of targeting the gene of interest directly to subsequently investigate the phenotype is generally referred to as reverse genetics. This is in contrast to forward genetic approaches, which screen for interesting phenotypes in response to random mutagenesis and only subsequently try to

identify the particular genetic modification that has been introduced (Doyle et al., 2012).

There are several different ways to disrupt expression of a gene in primary cells. For all approaches, reagents that modify gene expression have to be delivered to cells in culture. The delivery methods can be broadly separated into viral and non-viral methods. Viral delivery, also referred to as transduction, results in stable expression of the delivered material, due to integration into the host genome. Non-viral methods include lipid-based reagents, such as lipofectamine, and electroporation, also referred to as nucleofection (Kim and Eberwine, 2010). Depending on the nature of the transfected material, expression after non-viral transfer can be stable or transient. For example, transposons encoding the gene of interest can result in stable genetic modification by integration into the host genome. Meanwhile nucleofected DNA plasmids, synthetic RNA or protein are generally only expressed transiently (Hackett et al., 2010; Kim and Eberwine, 2010).

With regards to primary T cells, it has been found that both lipid-based reagents and nucleofection can be toxic (Chicaybam et al., 2013; Ebert et al., 1997). Meanwhile, viral transduction may trigger an inflammatory reaction and carries the risk of causing detrimental mutations in the host genome due to random insertion. Additionally, there are restrictions on the size of a construct that a virus can contain while maintaining infectivity (Kim and Eberwine, 2010; Ramezani and Hawley, 2002). It is therefore clear that genetic modification of primary T cells is technically challenging.

One way to circumvent these challenges is by using mouse models, where gene expression can be deleted permanently, or conditionally using the Cre/loxP or FLP/FRT recombination systems. Recombination systems allow for conditional KO of genes in certain cell or tissue types, or at certain time points in the mouse's life (Bouabe and Okkenhaug, 2013). KO mice can be generated by incorporating the desired genetic change into the genome of an embryonic stem cell by homologous recombination. This involves introduction of DNA, e.g. encoding a selection marker, flanked by sequences homologous to the desired insertion site, thereby disrupting the sequence of the gene of interest upon incorporation. Modified embryonic stem cells can be selected for using the selection marker and are subsequently injected into early-stage mouse embryos, resulting in mice that have the gene of interest knocked out in tissues derived from the modified embryonic stem cells (Bouabe and Okkenhaug, 2013). Subsequent breeding generates homozygous KO mice, which can be used for in vivo experiments, or primary cells can be harvested from the mice for in vitro studies.

While mouse models are a powerful tool to study gene function, manipulation of gene expression directly in cells can be advantageous as it can yield results more quickly. Subsequently, I describe two methods that are commonly used to disrupt protein expression in cells, one technique that functions at the messenger RNA (mRNA) level, small interfering RNAs (siRNA), and another that functions at the DNA level, clustered regularly interspaced short palindromic repeats (CRISPR).

siRNAs have been widely used to silence gene expression in mammalian cells, including primary T cells (Stinchcombe et al., 2015). In this approach, siRNAs form a complex with the RNA-induced silencing complex (RISC). Functional RISC particles contain a single stranded siRNA that binds to its complementary mRNA. The RISC complex also contains an RNase which cleaves the mRNA, leading to post transcriptional gene silencing and therefore a reduction in protein expression (Meister and Tuschl, 2004). This mechanism of gene silencing has been used to affect the expression of individual genes as well as gene expression on a genome-wide scale. However, often only partial protein knockdown is achieved, and the knockdown will only be maintained while the siRNA is present (Joung et al., 2017).

In recent years, the discovery of the CRISPR system and its use for gene editing has revolutionised this field by enabling the creation of KOs in mammalian cells where protein expression is permanently lost (Hsu et al., 2014). CRISPR has been used to modify the genome of many species, including human and mouse (Cho et al., 2013; Cong et al., 2013; Doudna and Charpentier, 2014; Jinek et al., 2013; Li et al., 2013; Mali et al., 2013). The system uses CRISPR-associated proteins (Cas), such as Cas9, an endonuclease that cuts DNA and causes double-stranded breaks. Cas9 is guided to a specific region in the genome by RNAs. In bacteria, the system requires two RNAs to function. Firstly, it requires the CRISPR RNA (crRNA) which is the reverse complement of the target site in the genome and binds to the target site by Watson-Crick base pairing. The second RNA that is required is the trans-activating crRNA (tracrRNA), which forms a duplex with the crRNA and is important for complex formation with Cas9 (Doudna and Charpentier, 2014). For genome-editing approaches, the crRNA and tracrRNA duplex has been combined into one molecule to give a single guide RNA (sgRNA) (Jinek et al., 2012). The target sequence in the genome must contain a protospacer adjacent motif (PAM) in order for Cas9 to bind and a cut to occur (Jinek et al., 2012; Sternberg et al., 2014). The PAM sequence therefore limits which sequences can be cut in the genome. However, the PAM sequence that is recognised varies between different Cas nucleases (Cong et al., 2013).

After the Cas9-mediated cut occurred, cell intrinsic DNA repair pathways are triggered and it is the inaccuracy of the DNA repair mechanisms that result in the desired editing of the genome (Hsu et al., 2014). Endonuclease cutting leaves behind a double-stranded break that is repaired by the non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways (Hsu et al., 2014). The error-prone NHEJ is induced in the absence of a repair template and can introduce insertions or deletions (indels) during repair (Hsu et al., 2014). Indels may cause frameshift mutations, resulting in gene disruption. Precise genome editing using HDR is also possible, and provides the opportunity to incorporate a specific DNA template containing homology sequences to the cut site. However, HDR occurs less frequently and is therefore more challenging (Hsu et al., 2014; Komor et al., 2017; Ran et al., 2013).

CRISPR allows to KO individual genes, and therefore allows to examine gene function in a clean experimental system. Several different technical approaches to use CRISPR for genome-editing purposes have been developed. Transient expression of CRISPR components is achieved through the transfection of plasmids or ribonucleoprotein complexes (RNPs), which contain recombinant Cas9 protein and synthetic crRNA and tracrRNA (Kim et al., 2014). Stable expression of CRISPR components has been achieved through lentiviral delivery (Hsu et al., 2014; Shalem et al., 2014; Wang et al., 2014a). The CRISPR approach has furthermore been scaled up to a genome-wide level using lentiviral sgRNA libraries, which has given powerful insights into genes that regulate cell viability and resistance to drugs (Shalem et al., 2014; Wang et al., 2014a).

It is clear that using CRISPR in primary CTL has immense clinical potential, and could be used to further enhance the immunotherapy approaches outlined in section 1.6. Clinical trials using CAR T cells in combination with CRISPR-mediated disruption of PD-1 are currently ongoing (Cornu et al., 2017). Related gene-editing technologies, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have already been used clinically (Fesnak et al., 2016). ZFNs have been used to KO C-C motif chemokine receptor 5 (CCR5), a crucial co-receptor for the human immunodeficiency virus (HIV), in autologous CD4 T cells. Gene-edited CD4 T cells were infused back into patients and this treatment was found to decrease the abundance of HIV RNA in the peripheral blood (Tebas et al., 2014). Allogeneic CAR T cells, where TALENs had been used to KO the endogenous TCR to prevent graft-versus-host disease, were used to treat patients with acute lymphocytic leukemia (Qasim et al., 2017). In addition to TCR KO, TALENs were used to KO CD52 in the

therapeutic CAR T cells. This enabled targeting of the patients' CD52 expressing cancerous cells using an antibody against CD52, without affecting the transferred CAR T cells. This use of genetically-modified T cells resulted in a remarkable clinical outcome (Qasim et al., 2017).

Together, these studies demonstrate the great potential of genome editing in primary human T cells for clinical purposes. Optimisation of the techniques used to deliver the CRISPR components to primary T cells, as well as a more thorough understanding of the fundamental biology underlying CTL function, will contribute to the advancement of this field.

1.8 Aims of this thesis

The work in this thesis aimed to identify regulators of CTL killing through screening approaches. Engineering the genome of CTLs was explored in order to provide a clean experimental system to investigate the involvement of individual genes in the CTL killing process. The currently known regulators of cytotoxic function have largely been identified using cells derived from patients suffering from immunodeficiency disorders or KO mouse strains. The hypothesis is that in addition to the proteins outlined in section 1.4.2 and 1.4.3 there are further proteins important for CTL cytotoxic function, perhaps functioning at the secretion step or further upstream, that remain to be discovered. This thesis aimed to establish the CRISPR Cas9 technique in primary T cells and to discover regulators of CTL function through screening using the CRISPR-Cas9 technique and chemical compounds. Furthermore, the work in this thesis contributes to the goal to perform large-scale screens in primary T cells in order to understand the molecular mechanisms underlying CTL function at large.

- **Aim 1: Develop strategies to use CRISPR-Cas9 in primary mouse CD8 T cells and establish a robust protocol that generates KO at high efficiency and disrupts gene expression reproducibly.**

  In chapter 3, I focus on establishing the CRISPR technology in primary mouse CTL (mCTL) using RNP complexes, which contained recombinant Cas9 protein, synthetic crRNAs and tracrRNA. CRISPR-mediated gene editing had not been performed in primary mouse T cells at the start of this project. I targeted genes whose protein products are known to be important for CTL killing (*Rab27a*, *Prf1* and *Munc13-4*) to confirm that CRISPR generated KOs at an efficiency that allowed detection of a phenotype in functional assays, such as the degranulation assay.

- **Aim 2: Develop an assay to measure cytotoxic capabilities of CTL at scale. Test the newly developed method in combination with a CRISPR screen and a compound library screen to identify regulators of CTL function.**

  Since one of the aims of this project was to identify regulators of CTL function, an assay that measures killing as well as degranulation was desirable. The set up of such an assay is described in chapter 3.

  To inform a targeted CRISPR screen, I compared gene expression between naive and effector CD8 T cells using RNA-sequencing (RNA-seq), described in chapter 4. The underlying hypothesis was that genes that are highly expressed in activated but not naive CD8 T cells are likely to be important for effector CTL functions, including killing capability. I used the readout of this transcriptomic dataset to select a set of genes for a targeted CRISPR screen. Samples were screened using the combined degranulation and killing assay, which was set up in chapter 3, in order to identify if any of these genes regulated CTL function.

  In chapter 5 I investigated whether the combined degranulation and killing assay can also be used in human CTL (hCTL) using samples derived from FHL2 and FHL3 patients. I furthermore tested the scalability of the assay using a compound library that targeted the NF-κB signalling pathway.

- **Aim 3: Establish protocols to use the CRISPR-Cas9 technique in primary human CD8 T cells.**

  An alternative CRISPR screening approach to using RNP complexes would be to stably transduce CTL with a lentivirus encoding Cas9 and sgRNAs. In contrast to the RNP approach, lentiviral transduction would allow for the incorporation of a fluorescent marker or an antibiotic resistance gene, which could be used to select edited cells. As the lifespan of mCTLs is limited to around 10 days in tissue culture, they are not suitable for a long selection procedure. However, this approach could be applied to hCTLs, which can be grown for months in tissue culture. This approach was explored in chapter 6.