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# New perspectives on T cell function: dissecting the roles of TSP-1 in cytotoxicity and CCDC28B in lysosome biogenesis

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# INDEX

# ABSTRACT

Cytotoxic T cells (CTLs) eliminate tumorigenic and virally infected cells through the release of cytotoxic granules and the Fas/FasL signalling pathway. Recently a novel killing mechanism has been identified, involving the release of cytotoxic particles known as SMAPs (Supramolecular Attack Particles). They consist of a core of granzymes, perforin and serglycin, surrounded by a glycoprotein shell of which thrombospondin-1 (TSP-1) is a predominant component. TSP-1 is a secreted matricellular glycoprotein mainly expressed by vascular cells. The expression of TSP-1 in CTLs, as well as its association within SMAPs, has revealed a novel function for this protein in lymphocytes, however the underlying mechanism remains to be elucidated. Preliminary studies on TSP-1 have highlighted in effector CTLs the presence of a 60 kDa form of TSP-1 corresponding to the C-terminal portion of the protein, which may be generated by cleavage of the full-length protein. We have shown that the 60 kDa TSP-1 portion is able to localize to lytic granules. The main goal of this project is to engineer the 60 kDa C-terminal portion of TSP-1 because of its presence in the outer shell of SMAPs and its expression in mature differentiated CTLs. TSP-1 engineering consists in the deletion or mutagenesis of the natural adhesive determinants of TSP-1 and the addition of a domain to confer SMAPs specificity for a selected cell type, such as a cancer cell. In this thesis I have worked on the first part of engineering by generating three constructs encoding mutants of 60 kDa TSP-1 lacking the adhesive sites. These include a 60-kDa mutant lacking the 8 amino acids corresponding to the CD47 receptor binding site, a 60-kDa variant with a mutated RGD motif that mediates integrin binding, and a double mutant. Immunofluorescence analysis on primary CTLs transfected with all the described TSP-1 mutants have shown that they correctly localise to lytic granules. The next step of this project is to determine if the SMAPs containing the engineered TSP-1 can be released by CTLs and kill target cells as efficiently as the ones containing the wild-type protein. The data are expected to pave the way for the development of a new, SMAPs-based immunotherapy treatment for cancer patients.

In the second part of this thesis, we examined the role of a ciliogenesis protein in fundamental processes within the non-ciliated T lymphocytes. Over the past few years different ciliogenesis proteins have been documented in non-ciliated cells, such as T lymphocytes, where they play a fundamental role in immunological synapse (IS) assembly and function. In particular, the intraflagellar transport (IFT) system component IFT20 has been the first to be associated to several mechanisms beyond its crucial role in the assembly and maintenance of the primary cilium. Indeed, IFT20 is considered a key player not only in ciliogenesis but also in IS assembly and in the vesicular trafficking of membrane receptors and signaling proteins to IS. These findings opened a new field in the characterization of a large panel of ciliogenesis proteins involved in IS assembly such as other IFT proteins, Rab GTPases, SNARE proteins and others. Accordingly, recent findings have demonstrated that another ciliary protein, known as Coiled-Coil Domain Containing 28B (CCDC28B), participates in IS assembly by regulating polarized T cell receptor (TCR) recycling. Lately cellular degradation pathways have emerged as new extraciliary functions of the IFT system, with a key role of IFT20 in a central degradation step controlling lysosome function, by regulating the mannose-6-phosphate receptor (M6PR)-dependent lysosomal targeting of acid hydrolases. Indeed, IFT20 acts as an adaptor molecule coupling the cation-independent mannose-6-phosphate receptor (CI-MPR) to dynein for retrograde transport to the trans-Golgi network (TGN). Since the ciliary protein CCDC28B has a role in regulating the actin cytoskeleton by recruiting the actin regulator WASH and retromer complex components, CCDC28B could be implicated, similarly to IFT20, in the regulation of CI-MPR retrograde trafficking in T cells and contribute to lysosome biogenesis and functions. To assess our hypothesis, experiments were performed on Jurkat cells knockout (KO) for the gene encoding the CCDC28B protein. Colocalization analyses of the M6PR with the TGN were carried out, and the retrograde transport of the MPR was also evaluated by confocal microscopy. These analyses have shown a decrease in the colocalization of CI-MPR with the TGN in CCDC28B KO cells, associated with a failure of antibody-tagged recycling CI-MPR to reach the TGN. These preliminary results suggest a defect of retrograde transport in the absence of CCDC28B. Considering its interaction with the actin regulator WASH and the retromer complex component, CCDC28B could be involved in retrograde transport of CI-MPR. The results could provide new knowledge of ciliary proteins in important cellular processes in the non-ciliated T cells.

# INTRODUCTION

## The immune system

The immune system consists in a network of cells, chemicals and processes that enables the organism to fight infections and to defend the host from foreign antigens, cancer cells and toxins (Chaplin, 2010). Beyond host defence, the immune system is a fundamental part of physiological processes such as development, reproduction and wound healing (Sattler, 2017).

There are two branches that allow the immune system to detect and eliminate pathogenic microbes: innate immunity and adaptive immunity. These two lines of defense function together, and dysfunction in either may result in illness or disease, such as inappropriate inflammation or autoimmunity (Marshall et al., 2018). Innate immunity represents the first line of the immune response. Traditionally, it has been considered devoid of immunological memory. Nonetheless, recent research has demonstrated that innate myeloid and lymphoid cells are capable of retaining memory of prior pathogen encounters and mounting an enhanced response upon reinfection. This process is referred to as trained immunity (Sherwood et al., 2022). Germline-encoded pattern recognition receptors (PRRs) are important for the initiation of the innate immune response. They are able to recognize structures belonging to microbial species, called pathogen associated molecular patterns (PAMPs), and endogenous molecules released from damaged cells, called damage associated molecular patterns (DAMPs). The stimulation of PRRs is crucial for the production of proinflammatory cytokines, type I interferon (IFNs) and chemokines. In addition, the sensing of microbes by PRRs expressed on antigen-presenting cells (APCs), dendritic cells (DCs), leads to the activation of the adaptive immune response (Takeuchi & Akira, 2010).

Adaptive immunity is based mainly on the antigen-specific receptors that are custom tailored and selected through a process of somatic recombination of a large array of gene segments. Cells of the adaptive immune system are T lymphocytes and B lymphocytes. Contrary to innate immunity, this response takes several days or weeks to develop and it is specific, resulting in an immunological memory against the antigen (Parkin & Cohen, 2001).

# T lymphocytes

T lymphocytes are the effectors of the cell-mediated immune response. T cell maturation takes place in the thymus, where T cells are subjected to a developmental checkpoint with the involvement of T cell receptor (TCR) signalling. Specifically, after TCR rearrangement thymocytes undergo strong proliferation, then TCRs can be tested for their ability to recognize self-peptide on a major histocompatibility complex (MHC) also called human leukocyte-associated (HLA) antigen. Positive selection begins when thymocytes with low-affinity TCRs are allowed to survive and differentiate into mature T cells, while thymocytes with high-affinity TCRs undergo negative selection, resulting in apoptosis (Quang et al., 2017). This process ensures that only T cells that are self-tolerant survive while eliminating the self-reactive T cells (Zúñiga-Pflücker, 2004). At this stage, naïve mature T cells leave the thymus and enter the secondary lymphoid organs, where they get exposed to foreign peptides presented by the MHC molecules of APCs or other nucleated cells during a pathogenic event (Sallusto et al., 2004). Upon TCR engagement with the antigenic peptide, T cells get activated, undergoing clonal expansion and differentiation to perform their effector functions (Samelson, 2002). T lymphocyte activation requires the recognition of a processed small peptide antigen which is stably bound to an MHC expressed on the surface of APCs. MHCs are cell surface glycoproteins that bind oligopeptide fragments of proteins that have been either synthesized within the cell (class I MHC molecules) or that have been ingested by the cell and proteolytically processed (class II MHC molecules) (Wieczorek et al., 2017).

The primary mediator of T cell activation is the TCR, generated by somatic recombination of genomic DNA sequences during T cell development in the thymus (Pennock et al., 2013). The TCR complex consists of TCR α/β chains and CD3 γ/δ/ε/ζ subunits, which associate through hydrophobic interactions (Rossjohn et al., 2015). Somatic VDJ recombination enables the generation of distinct TCRα and TCRβ chains, and TCRα/β heterodimers are responsible for antigen recognition by binding to peptide–MHC (pMHC) complexes (Hwang et al., 2020). Successful recombination of a functional TCR and egress from the thymus results in a resting, naïve T cell with the ability to reach secondary lymphoid tissue. There, naïve T cells are activated through their TCRs by pMHC I or II complex on the surface of APCs (Pennock et al., 2013). Naïve T cells need a second, co-stimulatory signal, as ligation of the TCR with its cognate antigen is not sufficient for the cells to proliferate and differentiate into armed effector T cells. The second signal is delivered by the same APC that presents the antigen to the T cell. One of the best characterized costimulatory molecule is the glycoprotein B7.1 (CD80) (Wang et al., 2000). The receptor for B7 molecules on the T cell is CD28, and their interaction results in the clonal expansion of naïve T cells (Burke et al. 2024). Once activated, a naïve

T cell expresses several proteins contributing to sustain or modify the signal necessary for the clonal expansion and differentiation. One of the most relevant proteins is CD40 ligand. The binding of CD40 ligand to CD40 – expressed primarily by B cells, dendritic cells and macrophages – transmits activation signals to the T cell and also causes the APC to express B7 molecules, thus stimulating further T cell proliferation (Elgueta et al., 2009). The presence of a co-stimulatory signal represents a crucial step in the recognition of self-antigens by autoreactive T cells that may escape negative selection in the thymus. Therefore, the necessity for the same cell to present both the specific antigen and the co-stimulatory signal is crucial in preventing a harmful immune response against self-tissues (Jin et al., 2012). When a TCR engages a relevant pMHC ligand TCR signalling is initiated: early events in TCR signalling are characterized by the involvement and activation of protein tyrosine kinases (PKTs), like LCK and ZAP70 (Courtney et al., 2018), followed by the generation of a multimolecular signalosome that mediates downstream events crucial for T-cell activation, including calcium mobilization, the activation of transcription factors such as the activator protein 1 (AP-1) the nuclear factor-κB (NF-κB), and the nuclear factor of activated T cells (NFAT) (Gaud et al., 2018). The TCR signalling response, together with external influences such as the presence of inflammatory mediators or cytokines, drives the differentiation of T lymphocytes into distinct functional subsets. The strength and duration of TCR signalling have been linked to the development of effector cells versus memory T cells (Daniels & Teixeiro, 2015). Effector T cells are divided into two main groups: T helper (Th) cells, which express CD4 molecules on their surface, and cytotoxic T lymphocytes (CTLs), which express CD8 molecules. Detailed TCR signalling pathways are illustrated in Figure 1.

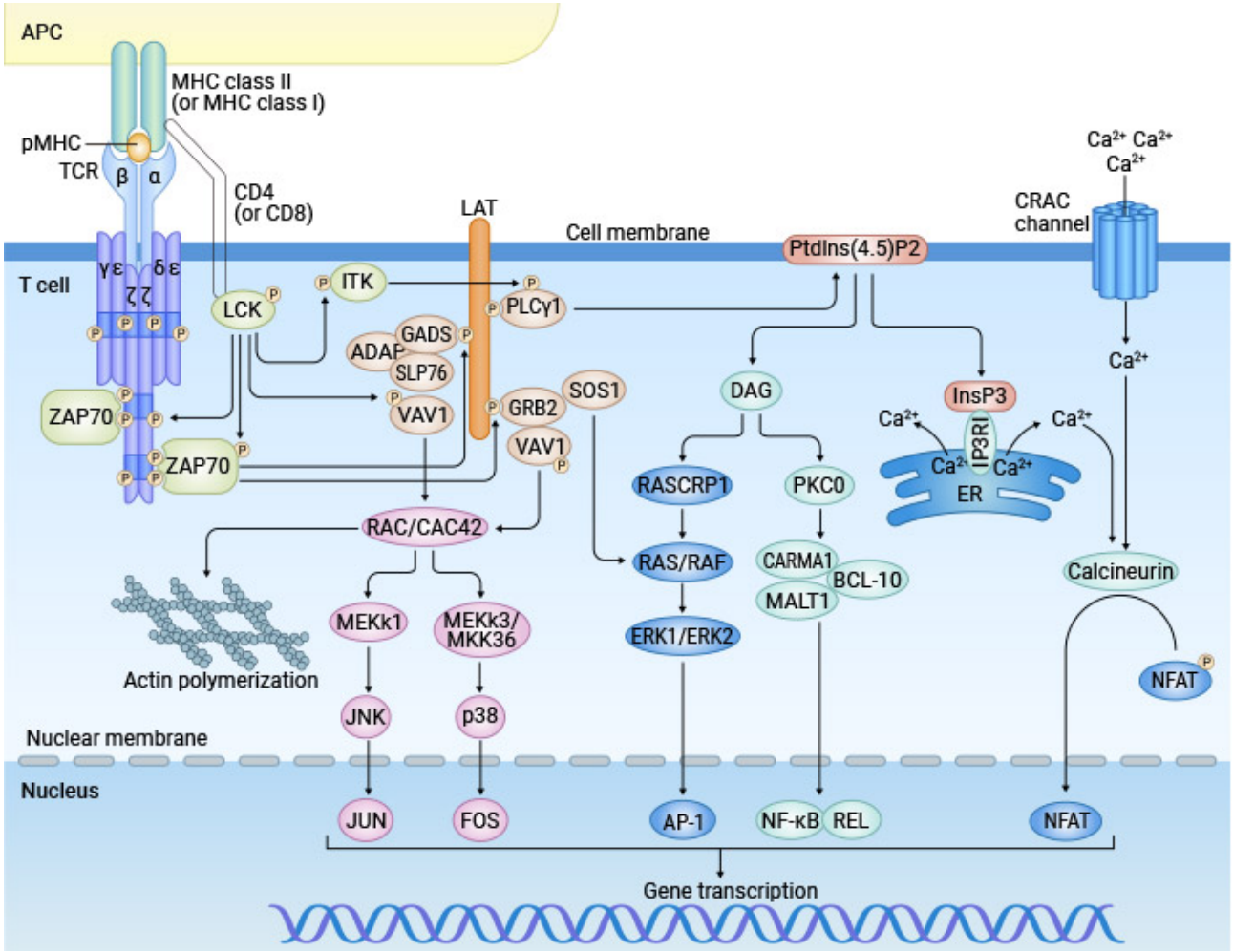


**Figure 1 | Overview of TCR signaling pathways**

The recognition of the pMHC on the APC triggers TCR signal transduction, a complex pathway that involves several kinases and activates multiple transcription factors resulting in T cell proliferation, migration, cytokine production and effector functions (Gaud et al., 2018)

# $CD4^+$ T lymphocytes

$CD4^+$ T cells play a pivotal role in the immune defence. They accomplish this by helping B cells in antibody production, stimulating macrophages to enhance their microbicidal capabilities, recruiting neutrophils, eosinophils, and basophils to sites of infection and inflammation, and coordinating the range of immune responses via their secretion of cytokines and chemokines (Zhu & Paul, 2008). Depending on the cytokines in the microenvironment, naïve $CD4^+$ T cells can differentiate into distinct subsets, each with its own functional profile and cytokine secretion patterns. These subsets include Th1, Th2, Th17, Tfh and regulatory T cells (Raphael et al., 2014).

Th1 cells mediate immune responses against intracellular pathogens. They play a critical role in resistance to mycobacterial infections, such as tuberculosis, by secreting cytokines that activate macrophages to kill intracellular bacteria (Lyadova & Panteleev, 2015). There are different transcription factors that are responsible for Th1 differentiation, among these T-box transcription factor (T-bet) is the master regulator. In particular, T-bet increases the production of IFN$\gamma$ and participates in suppressing the development of Th2 and Th17 cells (Luckheeram et al., 2012).

Th2 cells are responsible for facilitating the immune response against extracellular parasitic organisms, such as helminths. The differentiation of Th2 cells is primarily driven by IL4 and IL2. STAT6, activated by IL4 signalling, is a key transcription factor that drives Th2 cell differentiation by upregulating the master regulator GATA3. Additionally, GATA3 inhibits Th1 cell development by suppressing STAT4 (Zhu, 2015).

Th17 cells are important for the clearance of extracellular bacteria and fungi, particularly at mucosal surfaces. The master transcriptional regulator of Th17 cell development is the transcription factor retinoic acid receptor-related orphan receptor gamma (RORγt), which drives the expression of Th17-associated genes (Ivanov et al., 2007). The signalling cytokines involved in the differentiation of Th17 cells are IL6, IL21, IL23 and TGFβ (Veldhoen et al., 2006).

Tregs play an important role in the maintenance of self-tolerance and regulation of immune responses. Cytokines such as IL2, TGFβ, and IL6 have been found to be crucial for the development and function of Tregs (Tang et al., 2022). IL2 is essential for the survival and proliferation of Tregs, while TGF-β drives the differentiation of naïve T cells into peripherally induced Tregs (Plitas & Rudensky, 2016).

Follicular Helper (Tfh) T cells participate in the development of antigen-specific B-cell immunity. These specialized T cells reside within the germinal centres of secondary lymphoid organs and provide crucial support and signals to B cells, enabling them to undergo affinity maturation, class switching, and differentiation into memory B cells and long-lived plasma cells (Wu et al., 2018).

# $CD8^+$ T lymphocytes

$CD8^+$ T cells are a subset of T lymphocytes important for the recognition of foreign antigens presented by MHC class I molecules (Dranoff, 2004). Together with $CD4^+$ T cells, $CD8^+$ T cells have a central role in adaptive immunity. To initiate their response $CD8^+$ T cell must undergo a process called T cell priming, which occurs in secondary lymphoid tissues. The encounter of $CD8^+$ T cell with an antigen presented on MHC class I on an APC cell induces the generation and proliferation of effector $CD8^+$ T cells with cytotoxic ability (CTLs) (Farhood et al., 2019). Interactions between APCs and $CD8^+$ T cells is mediated by CXC-chemokine receptor 3 (CXCR3) expressed in APCs and CXC-chemokine ligand 9 (CXCL9), and CXCL10 produced by APCs. Thus, APCs express ligands CD70 and CD80-CD86 for binding to their respective CD27 and CD28 receptors on $CD8^+$ T cells. These interactions are considered the first step for $CD8^+$ T cell priming (Borst et al., 2018). This priming represents the fate-determining event of naïve $CD8^+$ T cells, resulting in changes in their chemotaxis, gene expression and function (Zhang & Bevan, 2011).

As effector cells, CTLs are able to kill and eradicate infected or tumor cells, providing valuable help in protection against infections or cancer.

In order to exert their cytotoxic activity, CTLs need to establish a direct cellular connection with the target cell, with the consequent formation of a highly organised interface called immunological synapse (IS) (Dustin & Long, 2010). IS formation is a characteristic feature of T cell mediated cytotoxicity. IS assembly and function is achieved by the acquisition of cell polarity characterised by the translocation of the microtubule organizing centre (MTOC) toward the synaptic interface (Huse, 2012) (Ritter et al., 2015), an event coordinated by the cytoskeleton along with motor proteins (Angus & Griffiths, 2013). CTLs polarity is critical for the directional release of their killing content towards target cells such as infected or cancerous cells, leading to their apoptosis (Capitani et al., 2021).

CTLs exert their killing activity by two different mechanisms to successfully remove infected or tumoral cells (Figure 2). One mechanism consist in the release of lytic granules (LGs), which are composed mostly of perforin (Prf) and granzyme (Gzm) (Voskoboinik et al., 2015). The second killing mechanism is achieved through the death receptor mediated apoptosis (Fas/FasL interaction) (Keckler, 2007). FasL is expressed on CTLs and NK cells, whereas Fas receptor is expressed on the surface of several cell types. The interaction of Fas, containing a death domain, with FasL on CTLs leads to the activation of the caspase cascade resulting in the apoptosis of the target cell (Yamada et al., 2017).

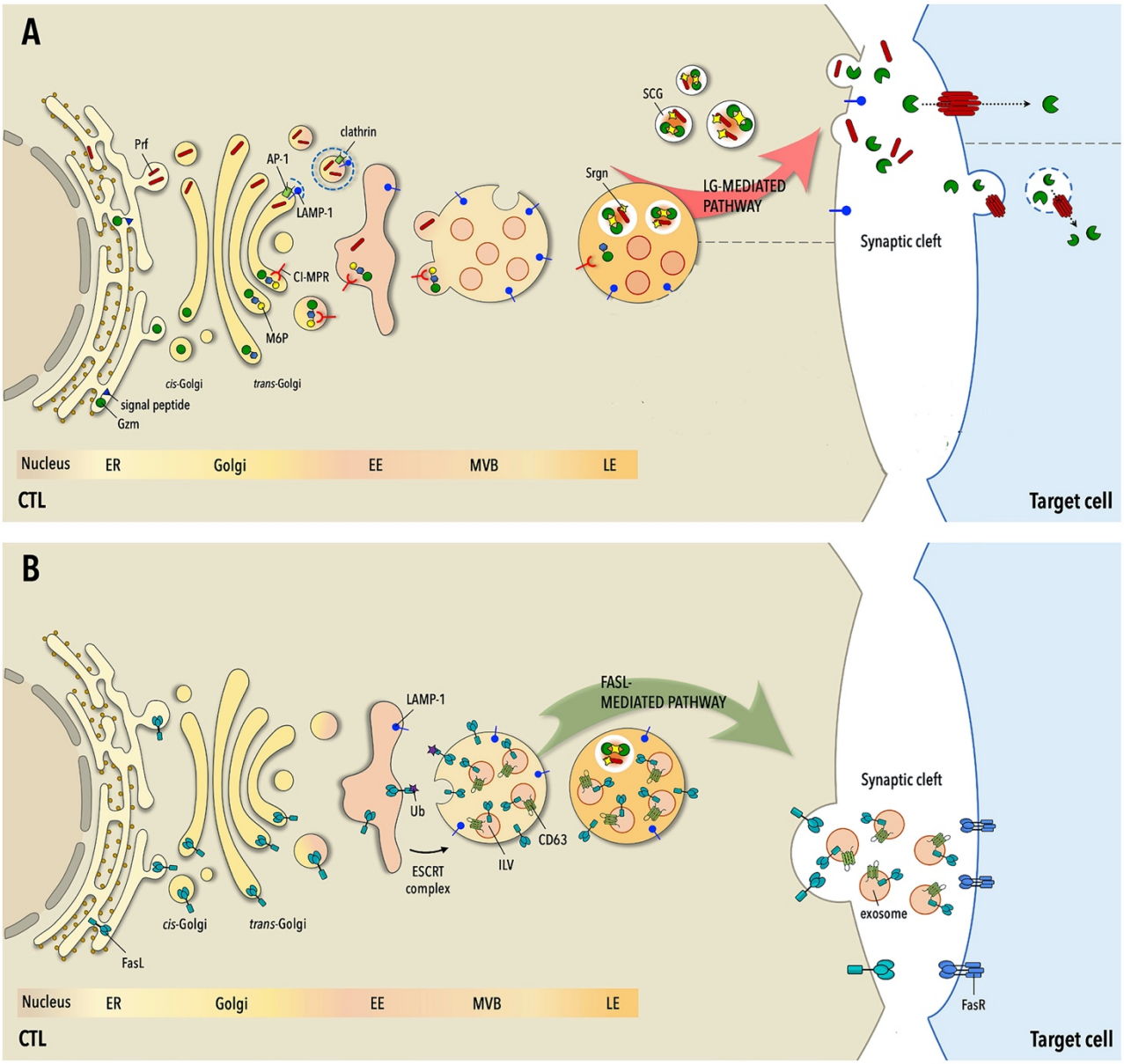


**Figure 2 | Pathways of cell killing by CTLs**

**A.** Components of LGs, Gzms, Prf and serglycin (Srgn), are sorted at the Golgi apparatus in order to be transported to the early endosomes (EE). From here they transit through multivesicular bodies (MVB) and late endosomes (LE) into mature LGs. At LEs Gzms and Prf become activated and ready to be release. Upon formation of the IS with the cognate cell target, soluble Gzm-Prf complexes are released and subsequently enter into the target cell through the pore-forming activity of Prf. **B.** FasL is transported through the ER, Golgi apparatus, and early endosomes to MVBs, where it associates with both the limiting membrane and intraluminal vesicles that mature into exosomes. When CTLs encounter their target cells, MVBs are mobilized to the IS, and FasL is released both at the synaptic membrane and into the synaptic cleft as FasL-containing exosomes. Both plasma membrane-associated and exosome-associated FasL can interact with FasR on the target cell membrane, triggering the Fas- and caspase-dependent cell death pathway (Cassioli & Baldari, 2022)

## Lytic granules

LGs are particular granules specialized in the secretion of cytotoxic effector molecules. They are also referred to as secretory lysosomes or lysosome-related organelles, since they function like conventional lysosomes, while also storing cytotoxic proteins prior their secretion (Blott & Griffiths, 2002). Thus, several evidences indicate that LGs have a lysosomal origin, such as the low pH and the presence of lysosomal hydrolases and lysosomal membrane glycoproteins (Cassioli & Baldari, 2022). The multivesicular cortex of LGs is also enriched in the cation-independent mannose-6-phosphate receptor (CI-MPR), which transports the acid hydrolases to lysosomes (Burkhardt et al., 1990). The cytotoxic content of LGs mainly consists of serine proteases like Gzm (Chowdhury & Lieberman, 2008), and Prf, a protein similar to bacterial pore forming toxins (Spicer et al., 2017), packed together on a scaffold of the proteoglycan serglycin (Srgn) (Kolset & Tveit, 2008). In addition, they contain a 9 kDa isoform of granulysin, a saponin-like membrane-disrupting protein (Sparrow & Bodman-Smith, 2020). LGs-mediated cytotoxicity is triggered by TCR engagement by pMHCI expressed on the target cell, which promotes the polarized secretion of LGs into the synaptic cleft. Prior their release LGs are transported along the microtubular network of the CTLs towards the IS where they fuse with the plasma membrane, release their content and kill target cells (Ming et al., 2015). This fusion step is controlled by different SNARE proteins, together with SNARE-associated proteins like Rab27a and Munc13-4, required for LGs pre-fusion steps such as docking and priming, respectively (Elstak et al., 2011).LGs are subjected to a multi-step process to develop into mature granules capable to dock the plasma membrane and deliver their cytotoxic content into the synaptic cleft. In this process Munc13-4 is present on Rab11-positive recycling endosomes and Rab27a on late endosomes. The subsequent fusion of these two organelles results in the generation of an intermediate precursor organelle in a Munc13-4 dependent fusion step. This intermediate precursor exocytic vesicle now may fuse or tether with Prf/Gzm containing immature LGs to become mature LGs (Ménager et al., 2007) (van der Sluijs et al., 2013).

Once released into the synaptic cleft, Prf and Gzms work together to promote the apoptosis of the target cell with Gzms diffusing through perforin pores on the plasma membrane of the target cell (Voskoboinik et al., 2015). Another proposed mechanism consists in the internalisation of Gzms as a membrane repair response triggered by Prf-induced membrane damage (Keefe et al., 2005).

Once in the cytosol of target cells, Gzms induce cell apoptosis by activating caspase-dependent and caspase-independent pathways (Martinvalet et al., 2008).. Granulysin also contributes to the cytotoxic activity of LGs by interacting with the target cell membrane through its positive charges and inducing the influx of $Ca^{2+}$, leading to mitochondrial damage and caspase-3 activation (Sparrow & Bodman-Smith, 2020).

## The Fas-Fas Ligand pathway

The interaction of Fas to FasL is another pathway by which CTLs exert their cytotoxic activity. The Fas molecule is a cell surface protein of 45 kDa belonging to the tumor necrosis factor receptor (TNFR) type I family, expressed on the surface of target cells. It has one extracellular domain rich in cysteines that binds FasL and a cytoplasmic domain involved in death signal transduction (Chávez-Galán et al., 2009).

FasL is a 40 kDa type II transmembrane protein belonging to the TNF family, and is expressed in activated T cells (Ivanova et al., 2017). Indeed, FasL is not constitutively expressed on CTLs and its expression is influenced by various factors and signalling pathways such as the activation of PCK, NFAT, NF-κB and calcium signalling (Kavurma & Khachigian, 2003). CTLs contain an intracellular pool of FasL which is stored in vesicles distinct from cytolytic granules, and mobilization of this stored FasL to the cell surface occurs independently of degranulation (He & Ostergaard, 2007).

FasL is directed to MVBs, where it co-localises with APO2L/ TRAIL both at the limiting membrane and in $CD63^+$ intraluminal vesicles (ILV) that are released into the synaptic cleft as EVs (Monleón et al., 2001). The targeting of FasL into MVBs is regulated by phosphorylation by Src kinases that interact with its proline-rich domain, as well as by mono-ubiquitylation (Zuccato et al., 2007) (Blott et al., 2001). Both modifications facilitate FasL targeting to the ESCRT pathway (Hurley, 2008).

Upon target cell recognition, MVBs polarize towards the centrosome and undergo fusion with the synaptic membrane. In T cells this process is regulated by diacylglycerol kinase α and the tetraspanin MAL (Alonso et al., 2011) (Ventimiglia et al., 2015). Depending on its localization within the MVB, FasL is either redistributed to the plasma membrane or released in association with EVs.

The interaction of Fas with its ligand, FasL, triggers the aggregation of Fas trimers. This enables the death domain (DD) in the cytoplasmic tail of Fas to recruit the death-inducing signalling complex (DISC) (Ramaswamy et al., 2009). The DISC complex is composed of Fas, an accessory protein called Fas-associated death domain (FADD) and pro-caspase 8 (Scott et al., 2009). As a consequence of receptor aggregation, Fas recruits FADD. This interaction allows the recruitment of pro-caspase 8 to the Fas signalling complex. Formation of the DISC triggers the self-cleavage of pro-caspase 8 into active caspase 8. Activated caspase 8 then cleaves the downstream pro-caspases 3, 6, and 7. Activated caspase 3 cleaves a variety of cellular substrates, including DNA repair enzymes, cellular and nuclear structural proteins, and endonuclease inhibitors. Moreover, caspase 3 has the ability to activate other caspases, such as caspases 6 and 7, thereby amplifying the apoptotic cascade to ensure full execution of the Fas-induced cell death program (Xu & Shi, 2007).

## The immunological synapse

The IS consists of a specialized supramolecular structure that forms after the interaction of the TCR with cognate pMHC, forming an interface between the T cell and the APC (Alarcón et al., 2011). Within this structure, several components such as TCRs, integrins and co-stimulatory receptors accumulate, and the respective signals are integrated to initiate actin and tubulin cytoskeleton reorganization, and also to activate a gene expression cascade resulting in the generation of effector and memory T cells (Dustin & Choudhuri, 2016).

The IS in its mature conformation shows a particular "bull's eye" architecture consisting in concentric domains, known as supramolecular activation cluster (SMAC), that differ in molecular composition and function (Monks et al., 1998). Specifically, the central SMAC (cSMAC) is the region where the TCRs and TCR associated proteins are located. Externally, there is the peripheral SMAC (pSMAC), mainly enriched in integrins such as lymphocyte function-associated antigen (LFA-1), and cytoskeleton-associated proteins. Finally, the pSMAC is surrounded by the distal SMAC (dSMAC), where the presence of F-actin is predominant. Here, we find also large and bulky molecules such as CD43 and CD45, with CD45 acting as a negative regulator of TCR signalling (Huppa & Davis, 2003). Moreover, the dSMAC is the region where signalling begin with the formation of TCR-CD28 microclusters that translocate centripetally towards the cSMAC, where exhausted TCRs are internalized to make space to new TCR microclusters that repeatedly form at the periphery (Figure 3) (Saito & Yokosuka, 2006). In this initial step of IS formation the actin cytoskeleton plays a critical role. In fact, the formation of TCR microclusters occurs thanks to F-actin acting as the driving (Ritter et al., 2013). Immediately after TCR activation, the centrosome translocates towards the IS in a process that is partially controlled by centrosomal F-actin dynamics. Centrosome polarization is coordinated with F-actin clearance to generate a central F-actin-free area that facilitates the polarized release of effector molecules, such as the cytotoxic contents of the CTLs LGs (Calvo & Izquierdo, 2021). TCRs are associated with the plasma membrane as well as recycling endosomes, since these are responsible for the transport of the TCRs to the IS. The intracellular pool of TCRs has an essential role in replenishing the plasma membrane pool, which eventually will become exhausted, through polarized recycling to the IS membrane (Onnis & Baldari, 2019). Moreover, other molecules that participate in IS architecture and function are transported to the area of contact through polarized recycling. These include surface receptors, like CTLA-4, and signalling molecules, such as CD45, Lck and LAT (Das et al., 2004).

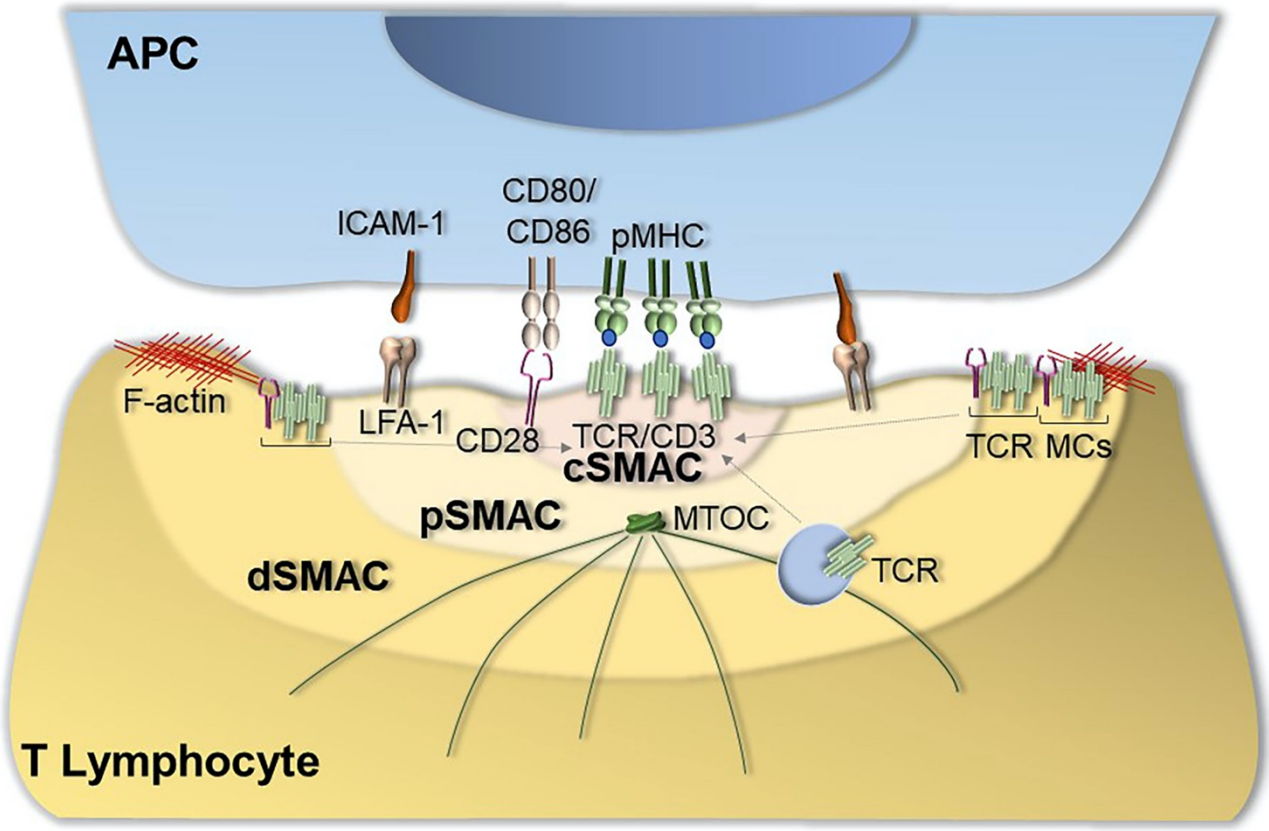


**Figure 3 | Structure and assembly of the immunological synapse.**

The IS is characterized by a well-organized concentric architecture. The central supramolecular activation cluster (cSMAC) is enriched with TCRs and associated co-stimulatory molecules like CD28. The peripheral SMAC (pSMAC) is enriched in the integrin LFA-1, while the distal SMAC (dSMAC) contains TCR-CD28 microclusters that move centripetally towards the cSMAC, driven by actin dynamics. IS assembly is coordinated by cytoskeletal rearrangements, which facilitate centrosome translocation towards the T:APC contact and directional vesicular trafficking of receptors and signalling mediators to sustain signalling at the IS (Capitani & Baldari, 2022)

Part I

# Characterization and engineering of the SMAP component TSP-1 in Cytotoxic T lymphocytes

# INTRODUCTION

## Supramolecular Attack Particles

A recent study has revealed that CTLs can perform their killing activity through a novel mechanism, based on the release of cytotoxic molecular aggregates, in addition to the known LGs and Fas/FasL pathway (Bálint et al., 2020). These particles, called Supra Molecular Attack Particles (SMAPs), are released at the IS and consist of a core of Gzm, Prf and Srgn surrounded by a glycoprotein shell. SMAPs are released by CTLs following contact with a target cell and are able to kill cells autonomously, without the presence of CTLs (Figure 4). Specifically, live imaging of CTLs plated on a supported lipid bilayer (SLB) platform coated with anti CD3 antibody and ICAM-1 (intracellular adhesion molecule1), used to resemble the surface of a target cell, revealed that the SMAPs are stable for hours after release. Thus, SMAPs remain attached to the SLB, while CTLs are washed out, and are capable to kill target cells independently. After acknowledging this peculiar characteristic of SMAPs, mass spectrometry analysis was carried out to evaluate their structural features. They have a diameter of 120 nm and contain a glycoproteic shell of which thrombospondin-1 (TSP-1) is one of the main components. The presence of TSP-1 within SMAPs is crucial for CTLs-mediated killing (Cassioli et al., 2025).

The presence of the classic cytotoxic core components Gzms, Prf and Srgn within SMAPs is a feature shared with LGs. However, unlike LGs, which are enclosed by a phospholipidic membrane, SMAPs are surrounded by the glycoprotein shell. Also, the biogenesis seems to differ: a recent study has identified two different classes of granules, with one characterized by the presence of a single core and uniform diameter (single core granule, SCG), and the other class containing multiple cores and differing in diameter (multi core granule, MCG). A proteomic analysis has revealed more in detail how different is the composition of these two classes of granules. SCGs are enriched in lysosomal proteins and release soluble GzmB, while MCGs are enriched in endosomal trafficking regulators and release particulate GzmB. Interestingly MCGs could be labelled with TSP-1, confirming them as the source of SMAPs (Chang et al., 2022). According to these findings, SMAPs represent a new class of cytotoxic particles and could be a promising future target for modulating T cell killing efficiency.

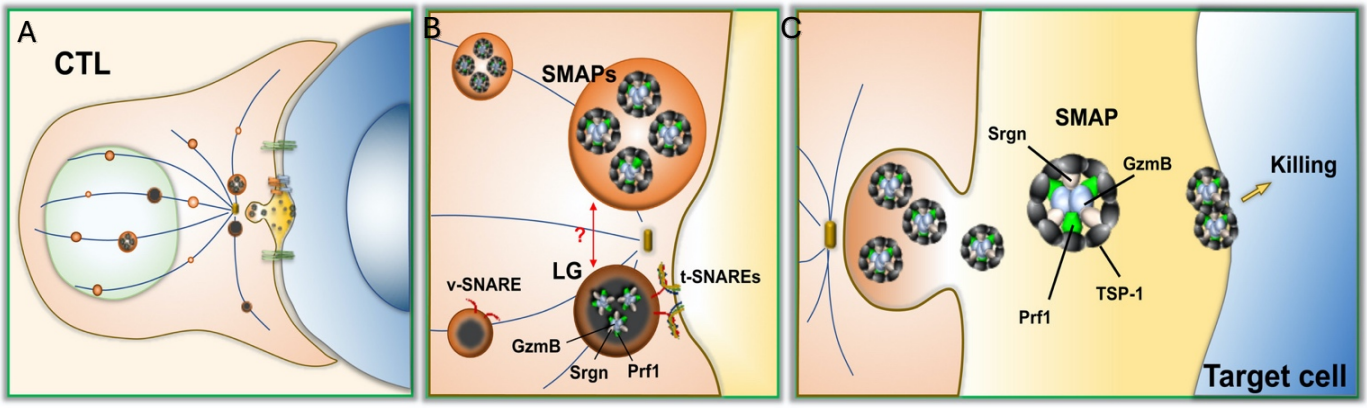


**Figure 4 | Role of SMAPs in CTLs-mediated killing.**

**A.** Following IS formation, CTLs release their lytic content into the synaptic cleft. **B.** CTLs release of cytotoxic component is promoted by the SNARE-dependent fusion of dense-core LGs, packed with Gzms-Prf1-Srgn complexes, with the synaptic membrane. CTLs contain also multicore acidic compartments enriched in SMAPs. **C.** SMAPs consist of glycoprotein shell, of which TSP-1 is a major component, surrounding a dense core of Gzms, Prf1 and Srgn, and they are released and induce killing of target cell through unknown mechanisms (Rettig & Baldari, 2020).

## Thrombospondin-1

Thrombospondins are secreted extracellular matrix (ECM) proteins forming the TSP family, comprising five homologous members TSP-1, TSP-2, TSP-3, TSP-4, TSP-5 (Stenina-Adognravi, 2013). Thrombospondins are able to mediate ECM organisation, and have a role also in cell-to-cell communication since they interact with several growth factor and cytokines (Adams & Lawler, 2011) (Tan & Lawler, 2009). Moreover, thrombospondins have different roles in both physiological and pathophysiological conditions such as in angiogenesis, platelet activation, inflammation, wound healing and tumorigenesis (Kyriakides & MacLauchlan, 2009) (Zhao et al., 2018). TSP-1 was discovered in 1971 as a glycoprotein secreted by activated platelets, suggesting that its major function could be associated with haemostasis (Baenziger et al., 1971).

The structure of TSP-1 consists of an N-terminal (NH2) domain, three properdin type-I repeats, three epidermal growth factor-like type-II repeats, a calcium-binding type-III motif, and a C-terminal (COOH) domain that enables cell attachment (Adams et al., 2008). The multiple binding domains present in the structure of TSP-1 allow it to interact with several other proteins such as receptors, integrins, ECM, and growth factors. Through the N-terminal domain, TSP-1 can bind to heparin, beta1 integrins, and sulphated glycolipids (Resovi et al., 2014). The type I repeats bind to collagen V

and VII, fibrinogen, CD36 and von Willebrand factor, the type II repeats bind to calcium channel subunit, while the type III repeats bind to β3 integrins and fibroblast growth factor 2. At the C-terminal domain TSP-1 binds to CD47, and important receptor for this protein (Figure 5) (Resovi et al., 2014). CD47, also called integrin-associated protein (IAP) is a cell surface receptor expressed on various cell types, including red blood cells (Brown, 2001). CD47 regulates the effects of nitric oxide (NO) in metabolic diseases and has critical functions in immunity and haemostasis. This domain also interacts with integrins modulating cell adhesion and spreading (Gutierrez & Gutierrez, 2021).

Mass spectrometry analysis of SMAPs have confirmed the presence of Prf and GzmB, while revealing the absence of membrane-associated proteins, like the lysosomal marker LAMP-1 that marks LGs, and the presence of TSP-1.

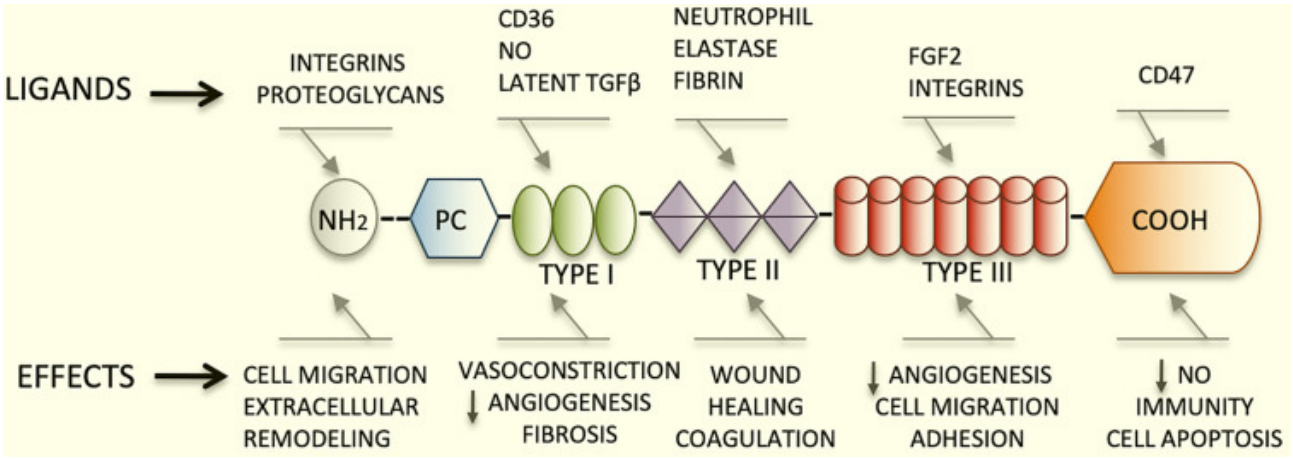


**Figure 5 | Structure of TSP-1 and its more representative ligands and effects.** TSP-1 consists in an amino terminus ($NH_2$) domain that interacts with integrins and proteoglycans. The remaining portions of the protein are organized in the type I, II, III repeats and the carboxyl-terminal (COOH). The type I and type II repeats contain the binding domain for CD36 and fibrin, respectively, responsible for endothelial apoptosis, wound healing and coagulation. The type III repeats of TSP-1 contain domains that interact with integrins. Finally, the carboxyl-terminal portion of TSP-1 binds to CD47, accounting for the modulation of cell adhesion, spreading and migration (Gutierrez & Gutierrez, 2021).

# AIM OF THE THESIS

CTLs exert their killing activity both by the release of lytic granules containing cytotoxic enzymes, and through the Fas/FasL pathway, leading to target cell death. A novel mechanism of cell mediated cytotoxicity involving the release of cytotoxic particles known as SMAPs has been recently identified. SMAPs consist of a core of Gzms, Prf and Srgn, surrounded by a glycoprotein shell of which TSP-1 is a predominant component. The expression of TSP-1 within SMAPs has revealed a novel function for this protein in lymphocytes. Preliminary studies on TSP-1 have highlighted in effector CTLs the prevalence of a 60 kDa form of the protein, corresponding to the C-terminal portion, which may be generated by the cleavage of the full-length species.

The aim of this thesis is to engineer TSP-1 as the outermost component of SMAPs, in order to set the foundation to generate SMAPs that selectively kill neoplastic B lymphocytes from patients with Chronic Lymphocytic Leukaemia (CLL). TSP-1 engineering involves two steps: first, the removal and the mutagenesis of sites involved in generic interactions with other cell types, such as the RGD motif for integrin binding present on type III repeats, and the CD47 binding domain found in the carboxy-terminal globular domain of the protein; the second step consists in engineering TSP-1 protein by adding the scFv (single chain Fragment variable) portion of an anti-CD19 antibody, to confer specificity toward cells expressing CD19 on their surface, like B cells. In this thesis we have focused on the first step, where the TSP-1 adhesion mutants have been generated and tested for expression, intracellular localisation and SMAPs incorporation in CTLs activated and differentiated in vitro.

# MATERIAL AND METHODS

## Generation of TSP-1 full length GFP Spark and TSP-1 60 kDa GFP Spark constructs.

The construct p-Max-TSP-1 full length-GFP Spark (FL) was generated from a commercial vector pCMV-TSP1-GFP Spark (~10.000 bp) containing the protein of interest, namely TSP-1 FL, already fused with the fluorescent tag GFP Spark. TSP-1 FL-GFP Spark was removed from the pCMV vector and cloned into the pMax vector (~3.600 bp) using the restriction enzymes HindIII/KpnI (FastDigest, Thermo Fisher Scientific).

To generate TSP-1 60kDa-GFP Spark, two PCR reactions were performed using primers containing restriction enzyme sites on the previously generated pMax TSP-1 FL-GFP Spark construct. The first PCR reaction was carried out to amplify the signal peptide (SP) region, which comprises the first 54 nucleotides of TSP-1 FL and is responsible for the correct intracellular localization of the protein. For the amplification of the SP, primer pair A (Table 1) was used, and the resulting insert was cloned into the pMax plasmid using the restriction enzymes KpnI/HindIII (Thermo Fisher Scientific). This resulted in the pMax-SP construct.

The second PCR was then performed, using pMax TSP-1 FL-GFP Spark as the template, to amplify the 60kDa portion of TSP-1 fused to the GFP Spark tag, using primer pair B (Table 2). The amplified product was subsequently cloned into pMax-SP using the restriction enzymes HindIII/XbaI (Thermo Fisher Scientific), yielding the final construct pMax-SP-TSP 1 60kDa-GFP Spark.

## CD47 binding site deletion

CD47 binding site is represented by 8 amino acids and is located in the C-terminal globular domain of TSP-1. Deletion of CD47 binding site was performed adapting a protocol from Stoynova and colleagues (Stoynova et al., 2004). We performed a PCR reaction on pMax- 60kDa-GFP Spark, using phosphorothioate modified PCR primers and t7 gene 6 exonucleases. PCR primers were designed to incorporate four consecutive phosphorothioate residues, located 12 nucleotides from 5’ ends of the primers (see primer C in table 1). Soon after PCR the product was treated with DpnI endonuclease

(10U/ µl) for 1 hour at 37°C to hydrolyse the original template. Then the product was digested with T7 gene 6 exonuclease (Thermo Scientific) for 10 min at 37°C. The enzyme was heat-inactivated for 10 min at 80°C and the product allowed to circularize through its single-stranded ends. Next, the resulting mixture was transformed in competent X-L1 *E.coli*. The purified plasmid DNA was sequenced to confirm the presence of the desired deletion.

## Site directed mutagenesis of RGD motif integrins binding site

Site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies), following the manufacturer's instructions. Briefly, the procedure consists in three steps: as a first step a synthesis of the mutant strand is performed (see primers D in table 1); the second step consists in the digestion of the template with DpnI; as a final step, the transformation of the mutated molecule into competent cells will ensure nick repair. This method was utilized to introduce a specific amino acid substitution. In particular, the aspartic acid (D) residue within the RGD motif at position 928 was replaced with glutamic acid (E). To achieve this, the codon GAT (encoding D) was modified to GAG (encoding E). The mutagenesis was carried out on the constructs pMax-60kDa-GFP Spark and pMax-60kDa ΔCD47BS-GFP Spark, generating the new constructs pMax-60kDa D928E-GFP Spark and pMax-60kDa D928E+ΔCD47BS-GFP Spark. The plasmidic DNA obtained from the bacterial colonies was subjected to sequencing analysis to verify the correct incorporation of the desired mutation.

| Primer | Forward 5'-3' | Reverse 5'-3' |
|---|---|---|
| **A** | CGG**GGTACC**CCATGGGGCTGGCCTG | CCC**AAGCTT**GGTGCCACACACATGCATCA |
| **B** | CCC**AAGCTT**ACAGACCTGGATGGCTG | TGC**TCTAGA**GCCTCGAGTTTACTTGTACAGC |
| **C** | AGCAGCCAAGTC**ACCC**AGTCCTAC | GACTTGGCTGTC**GGAC**TGGTAGGCC |
| **D** | CGATGGTCGAGGT**GAG**GCCTGCAAAGAT G | CATCTTTGCAGGC**CTC**ACCTCGACCATCG |

**Table 1 | List of primes used in this thesis.**

Nucleotides recognized by restriction enzymes or modification as phosphorothioates nucleotides are indicated in bold

## Cell culture and transfection of CTLs for confocal imaging

Primary human $CD8^+$ T lymphocytes were isolated from peripheral blood of anonymous healthy donors recruited at Siena University Hospital. Blood collection was approved by local ethics committee following standard ethical procedures outlined in the Declaration of Helsinki. $CD8^+$ T cells were isolated by negative selection through the RosetteSepTM Human $CD8^+$ T Cell Enrichment Cocktail (StemCell technologies) following manufacturer's instructions. Immediately after isolation, $CD8^+$ T cells were resuspended in the culture medium: RPMI 1640 with 25 mM Hepes (Sigma-Aldrich), supplemented with 10% BCS (Bovine Calf Serum, Hyclone), 20 U/mL Penicillin (Sigma-Adrich) and 1X non-essential amino acids (MEM non-essential amino acids solution 100X, Gibco). Cell cultures were maintained at 37 °C and 5% $CO_2$. $CD8^+$ T cells were differentiated *in vitro* to CTLs by incubation of 48 h with anti-CD3/CD28 coated magnetic beads (Dynabeads Human T-activator CD3/CD28, Gibco) for cell expansion and activation and 50 U/mL of IL2 (human Interleukin-2, Miltenyi Biotech) for cell proliferation and differentiation. Afterwards, CTLs were transiently transfected with the constructs p-Max-TSP-1 FL-GFP Spark, p-Max-TSP-1 60kDa-GFP Spark, pMax-60kDa ΔCD47BS-GFP Spark, pMax-60kDa D928E-GFP Spark and pMax-60kDa D928E+ΔCD47BS-GFP Spark, by using the Human T Cell Nucleofector Kit (Amaxa Biosystem) and the Amaxa Nucleofector II system (Lonza), using the program T-023. Cells were analysed 24h post transfection.

## Immunofluorescence and colocalization analysis

Transfected CTLs were seeded on to poly-L-lysine (Merck)-coated slides (ThermoFisher Scientific) and fixed for 20 min with 4% paraformaldehyde and then permeabilized with 1% BSA, 0.1% Triton-X100 at RT for 20 min. After washing with PBS, samples were stained with primary antibodies (Table 2) at 4°C overnight and then incubated at RT for 45 min with Alexa fluor 488- and 555-labeled secondary antibodies (Table 2) and mounted with 90% glycerol/PBS. Confocal microscopy was carried out on a Zeiss LSM700 microscope (Carl Zeiss, Jena, Germany) using a 63x/1.40 oil immersion objective. Co-localization analyses were performed on a medial optical section of single cells using ImageJ and the JACoP plugin to calculate Mander's coefficient M1 and M2. M1 indicates the proportion of the green signal (Alexa fluor 488) overlapping a signal in the red channel (Alexa fluor 555) over its total intensity, and M2 is defined conversely for red (Manders et al., 1992) . Mander's coefficients range from 0 to 1, corresponding to non-overlapping images and 100% co-localized images, respectively.

| Antibody | Host Species | Catalogue number | Source | Dilution IF |
|---|---|---|---|---|
| Anti-GFP | Rabbit | A11122 | Invitrogen | 1:200 |
| Anti-LAMP-1 | Mouse | 328602 | Biolegend | 1:400 |
| Anti-GzmB 647 | Mouse | 515406 | Biolegend | 1:20 |
| Anti-Mouse 555 | Goat | A32727 | Invitrogen | 1:80 |
| Anti-Mouse 647 | Goat | A21238 | Invitrogen | 1:80 |
| Anti-Rabbit 488 | Goat | A32731 | Invitrogen | 1:80 |

**Table 2 | List of antibodies used in this thesis**

# RESULTS

## TSP-1 intracellular localization

Consistent with the proteomic analysis of SMAPs, a recent study on TSP-1 expression during CD8 differentiation to CTLs has revealed the presence, together with the canonical 150 kDa form of TSP-1, of a 60 kDa form of the protein corresponding the C-terminal portion, mainly expressed in mature CTLs and probably resulting from the processing of the full-length TSP-1 (Cassioli et al., 2025).

To investigate the intracellular localization of TSP-1 in CTLs, we generated two constructs: one encoding the GFP-tagged full-length TSP-1 (TSP-1 FL) and the other encoding the GFP-tagged 60 kDa C-terminal portion of the protein. The latter construct was designed based on previous findings indicating that the 60 kDa isoform represents the predominant form of TSP-1 expressed in mature CTLs (Bálint et al., 2020) (Figure 6).

Confocal immunofluorescence analysis were performed on CTLs transiently transfected with these constructs to determine the intracellular localization of TSP-1. The transfected cells were co-stained for GzmB and LAMP-1, markers of lytic granules and lysosomes, respectively.

As shown in Figure 7, both GFP-tagged TSP-1 FL and GFP-tagged TSP-1 60 kDa colocalize with lytic granules and lysosomes. These findings demonstrate that the 60 kDa C-terminal portion of TSP-1 contains all the molecular determinants for proper localization to lytic granules.

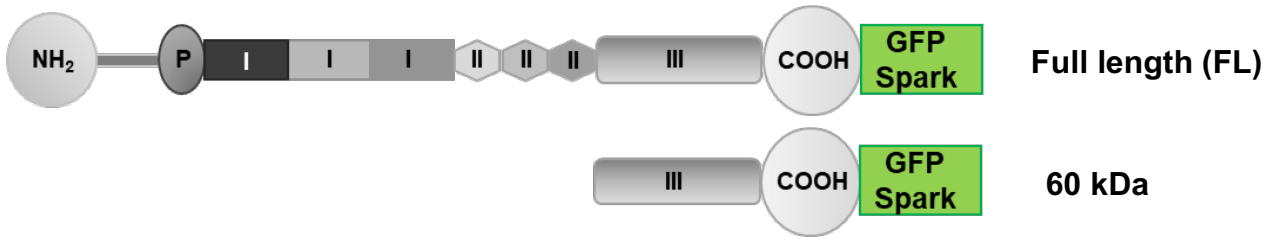


**Figure 6 | Schematic representation of the fusion proteins TSP-1 FL-GFP Spark and TSP-1 60kDa-GFP Spark**

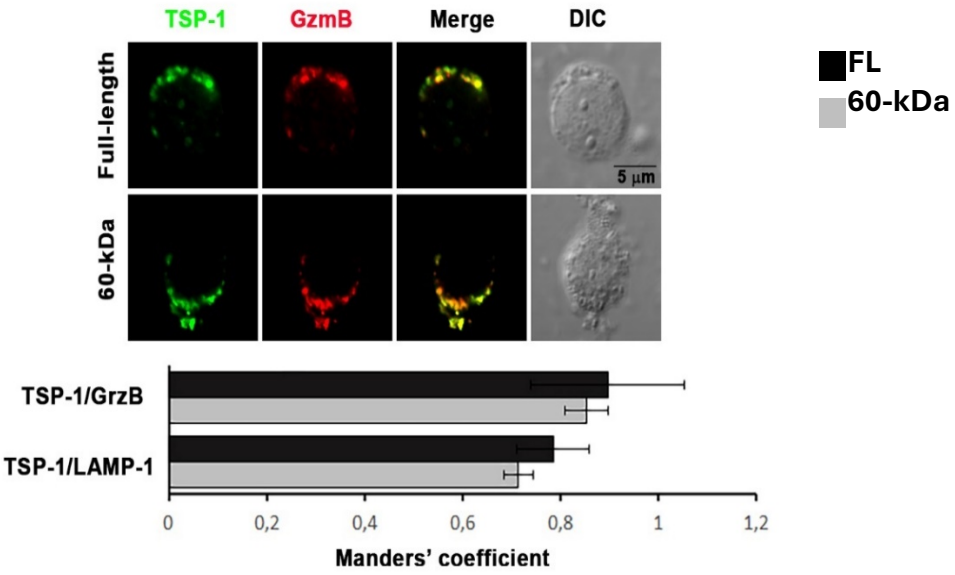


**Figure 7 | Intracellular localisation of TSP-1 in T CD8$^+$ lymphocytes**

Confocal images of CTLs transiently transfected with constructs encoding the 60 kDa and FL TSP-1 (green), stained with anti- GzmB (red). Scale bar: 5 µm. Quantification of the colocalization using the Manders' coefficient between TSP-1 FL (black bar) and TSP-1 60 kDa (grey bar) with GzmB and LAMP-1.

## TSP-1 engineering

The results reported above show that the 60 kDa C-terminal portion of TSP-1 correctly localizes to the lytic granule compartment. Importantly, TSP-1 represents the outermost component of the glycoprotein shell of SMAPs (Bálint et al., 2020). Based on this evidence, we started an engineering process working on the 60 kDa C-terminal part of the protein to remove the TSP-1 intrinsic adhesive sites.

## Adhesive site removal

One particular feature of TSP-1 is the ability to interact with several cellular types and matrix components and regulate different cellular process. For this reason, we decided to mutagenize two intrinsic adhesive sites of the protein within the C-terminal 60 kDa portion: the RGD motif in the type III repeat region, representing the integrin binding site, and a region of 8 amino acids in the C-terminal globular domain corresponding to the CD47 binding site.

To this aim we generated three constructs encoding the following 60 kDa TSP-1 mutants: a mutant lacking the 8 amino acids corresponding to the CD47 receptor binding sites (60 kDa ΔCD47 BS); a mutant with the RGD motif mutated to RGE (60 kDa D928E); a double mutant combining the two (60 kDa D928E + ΔCD47 BS) (Figure 8).

$CD8^+$ T lymphocytes were transiently transfected with the mutants described above. To analyse the intracellular localisation of TSP-1 we co-stained the cells with GzmB and LAMP-1. The results showed that all the mutants correctly localise with both the GzmB and the lysosomal compartment (Figure 9), indicating that mutagenesis does not interfere with the intracellular localization of the protein.

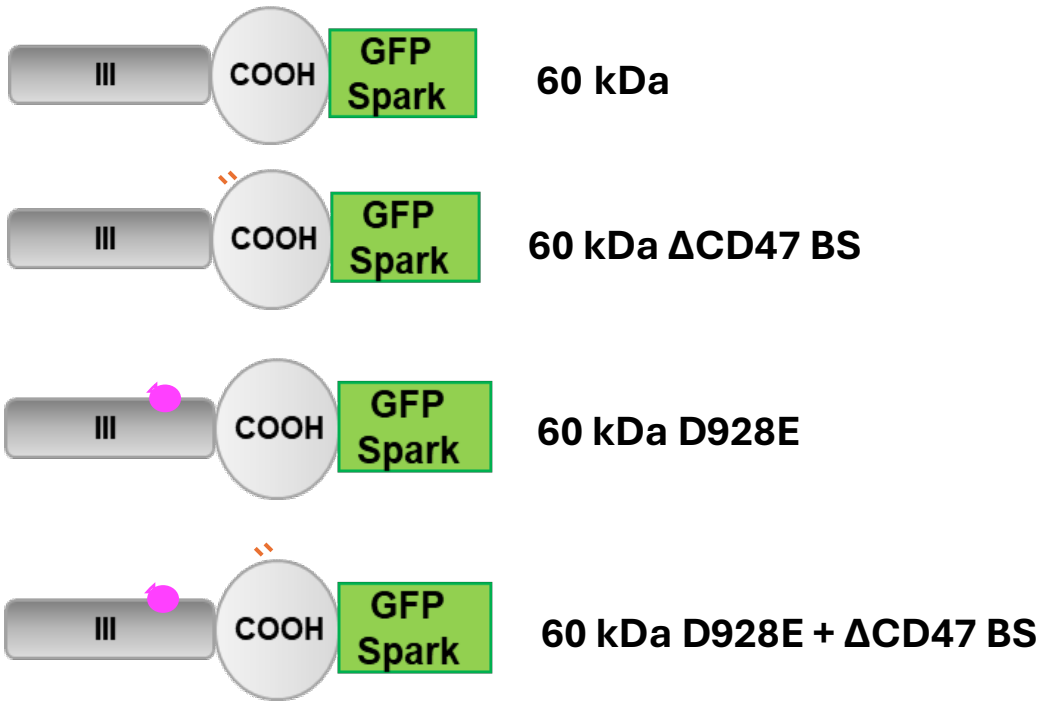


**Figure 8 | Graphic representation of TSP-1 mutants.**

Starting from the pMax 60 kDa-GFP Spark vector, the following constructs were generated: pMax-60kDa Δ CD47 BS GFP Spark; pMax-60kDa D928E-GFP Spark; pMax-60kDa D928E+ΔCD47 BS-GFP Spark

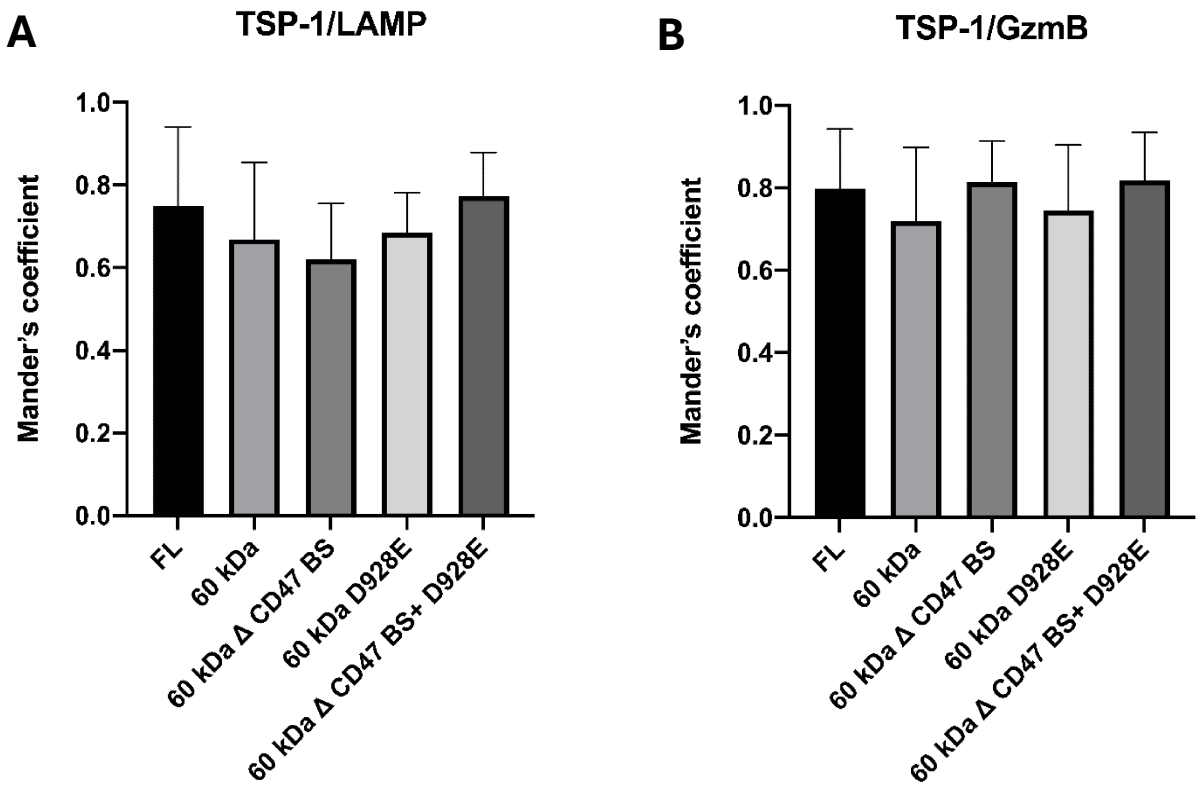


**Figure 9 | Intracellular localization of the TSP-1 adhesion mutants in CD8$^+$ T lymphocytes**
Colocalization analysis between TSP-1 mutants with either LAMP-1 (A) or GzmB (B).

In conclusion, the data obtained show that the mutagenesis of the C-terminal portion of TSP-1 through the removal of the adhesive sites does not affect the correct intracellular localization of the protein. This suggests that it could be included, even when mutated, in SMAPs particles.

# DISCUSSION AND CONCLUSIONS

SMAPS have been recently characterised as a new mechanism of CTLs mediating cytotoxicity, functioning alongside the canonical killing mechanisms based on the release of LGs and through the Fas/FasL pathway, and challenging the current scenario of CTLs-mediated killing (Bálint et al., 2020). The SMAPs are stored in MCGs, differing form SCGs for size, morphology and protein composition (Chang et al., 2022). How they are targeted to MGCs and how they are assembled and released at the IS, are all processes that need to be further investigated. At the structural level, they consist of a core of Prf and Gzm enclosed in a glycoprotein shell, of which TSP-1 is a major component and appears to contribute significantly to CTLs-mediated killing (Cassioli et al., 2025) . SMAPs are able to kill target cells within hours and independently of the CTLs that released them. This peculiarity opens the possibility of exploiting SMAPs as a weapon that functions independently of T cells.
In this work, we engineered TSP-1 as the outer SMAP component, in order to set the foundation for the generation of SMAPs able to specifically target leukemic B cells. The first step, which has been the objective of this thesis, was to assess the ability of a recombinant C-terminal 60 kDa portion of TSP-1 to localize to LGs and to remove its natural adhesive determinants to limit non-specific killing. In particular, having demonstrated that the 60 kDa TSP-1 construct correctly localizes to LGs, we have generated three constructs encoding, respectively, a 60 kDa TSP-1 mutant lacking the 8 amino acids corresponding to the CD47 receptor binding site, a 60 kDa TSP-1 mutant lacking the RGD motif that mediates integrin binding, and a double mutant with both the mutations previously described.

These results have set the basis for the next phases of this project. First, the functionality of the engineered SMAPs will be determined. Specifically, we aim to determine whether CTLs can release SMAPs containing the engineered forms of TSP-1 at the same manner to those containing the wild-type protein, as well as the killing ability of engineered SMAPs. Second, the ability of SMAPs lacking non-specific binding and displaying an anti-CD19 scFv to specifically kill CLL cells will be assessed. Demonstrating both efficient release and target specificity would represent a significant step toward the development of engineered SMAPs as a novel, cell-free immunotherapeutic strategy against B-cell malignancies. Moreover, by tailoring SMAPs to this specific cell type, we sought to improve the precision of therapeutic strategies, ensuring that the SMAPs would attack exclusively B lymphocytes

while minimizing off-target interactions with healthy cells. This targeted approach is crucial for increasing the effectiveness of treatments while reducing potential side effects.

The development of such engineered SMAPs could open new paths for the treatment of B-CLL, and also other type of tumors, enabling more personalized and precise medical interventions for cancer patients.

Part II

# Role of the ciliogenesis protein CCDC28B in the regulation of lysosome biogenesis in T cells

# INTRODUCTION

## Role of ciliary proteins in T lymphocytes

Primary cilia are immotile organelles known for their role in development and cell signalling. They are present in almost all cell types, with the exception of lymphoid cells. The primary cilium is a slim microtubule-based organelle assembled by most eukaryotic cells in response to various developmental signals (Goetz & Anderson, 2010). Although historically regarded as a vestigial structure, increasing evidence demonstrates how primary cilia are essential signalling hubs, acting as "sensing antennae" to detect and process multiple extracellular cues (Pedersen et al., 2016). Dysfunctions of primary cilia are associated with numerous human inherited developmental degenerative diseases termed ciliopathies (Reiter & Leroux, 2017).

Investigations of ciliopathy-associated phenotypes have enhanced our current understanding of the cilium and the mechanisms regulating this particular structure. Although lymphocytes are among the few cell types that do not form primary cilia (Hildebrandt & Otto, 2005), subsequent studies have revealed that immune cells appear to form "frustrated cilia" with centrosome docking without cilium formation (Griffiths et al., 2010).

In recent years many ciliary proteins have been found to localise at extraciliary sites and be involved in function outside the primary cilium. These functions extent from cytoskeleton regulation to vesicular trafficking (Hua & Ferland, 2018). Perhaps, the most striking example is represented by the non-ciliated T lymphocytes, in which components of the ciliary machinery are repurposed for the assembly and function of the IS even in the absence of a primary cilium (Cassioli & Baldari, 2019).

At first sight, the IS and the primary cilium seem to be very different structures. Nevertheless, a large body of evidence has pointed out that these two structures have several similarities. A striking parallel was first noticed at the ultrastructural level, when images of centrosome polarization at the IS in CTLs were compared with images of basal body docking during ciliogenesis (Stinchcombe et al., 2006). Indeed, a characteristic event during the assembly of both the IS and the primary cilium is the translocation of the centrosome close to the plasma membrane. Additionally, both the Golgi apparatus and the recycling compartments, which are closely associated with the centrosome, were shown to polarize towards the plasma membrane, both at the IS and at the base of the cilium. These structural similarities extend also to the actin cytoskeleton on which the formation of the IS and the primary cilium rely on (Sütterlin & Colanzi, 2010). The initial encounter of CTLs with their target cell results

in a rapid accumulation of actin across the contact site (Jenkins et al., 2014) and during killing, the membrane reorganizes to form a tight contact. As a result, the initial actin accumulation at the IS is rapidly followed by its depletion. Before CTLs dissociate and move on to new other targets, actin returns to the contact site (Ritter et al., 2015) (Stinchcombe et al., 2001). The loss of cortical actin is essential for granule delivery, whereas its restoration facilitates the termination of secretion (Ritter et al., 2017). Actin rearrangement is a process that takes place also in primary cilia formation (Copeland, 2020), and several studies have reported the identification of actin-binding proteins in the cilium (Ishikawa et al., 2012) (Kohli et al., 2017) (Liu et al., 2007).

Over the last decade, a large body of evidence has revealed that the cilium and the IS also share common mechanisms to orchestrate polarized trafficking. One of the main regulators of this trafficking is represented by the intraflagellar transport (IFT) protein family. In ciliated cells, the most relevant role of IFT proteins is to carry cargos, through anterograde transport, along the axonemal microtubules from the cell body to the tips of cilia, followed by retrograde transport back to the cell body. Cargo trafficking is mediated by the molecular motors kinesin and dynein for anterograde and retrograde transport, respectively (Finetti et al., 2022).

Notably, the activity of the IFT system extends beyond the primary cilium, contributing to key cellular processes such as cell cycle progression (Vitre et al., 2020), microtubule and actin cytoskeleton dynamics (Ishikawa & Marshall, 2017), intracellular vesicular trafficking and autophagy (Finetti et al., 2021). In support of these evidence, it has been demonstrated that IFT proteins are localized not only at the base of the cilium, but also in the cell cytoplasm.
Interestingly, a number of IFT components have been found to be expressed in hematopoietic cells and shown to participate in T lymphocyte activation (Finetti et al., 2009) (Finetti et al., 2014).
For instance, IFT20 acts together with other IFT proteins to promote IS assembly in T cells (Finetti et al., 2009). This process is based on the ability of IFT20 to interact with trafficking regulators and molecular motors for the polarized delivery of TCRs and membrane-bound signalling mediators associated with recycling endosomes (Finetti et al., 2014) (Onnis et al., 2016) (Vivar et al., 2016). In this regard, IFTs recruit small GTPases to orchestrate receptor recycling at both the IS and primary cilia. For instance, Rab8 is an important player in cilium assembly and trafficking, and colocalizes with IFT20 in $Rab11^{+}$ endosomes in activated lymphocytes (Patrussi & Baldari, 2016). Moreover, Rab29 was also identified as an interactor of IFT20, Rab8, and Rab11, and they all participate in the vesicular recycling to the IS (Onnis et al., 2015). In a report by Cassioli and colleagues, the potential role of Bardet-Biedl syndrome 1 protein (BBS1), an essential core component of the BBS complex

that cooperates with the IFT system in ciliary protein trafficking, in IS assembly was investigated. Indeed, BBS1 was found to promote centrosome polarization towards the IS through the clearance of centrosomal F-actin and its regulator WASH (Cassioli et al., 2021).

Beside the role of ciliary proteins in IS assembly and function, other fundamental processes have been documented in the context of extraciliary functions of the IFT, among which the cellular degradation pathway.
In this regard, recent studies have highlighted a pivotal role for IFT20 in the regulation of autophagy, a process exploited by all eucaryotic cells to regulate cytoplasmic turnover of proteins and organelles (Finetti et al., 2022). It is known that autophagy is orchestrated by the autophagy-related (ATG) proteins (Glick et al., 2010). IFT20 contributes to this process by regulating the localization of ATG16L1 at early endosomes to promote autophagosome biogenesis in T cells (Finetti et al., 2021).
In addition, IFT20 was found to be implicated in another central degradation process that also controls the latest steps in autophagy, requiring lysosome function, by regulating the CI-MPR dependent lysosomal targeting of acid hydrolases, on which lysosome biogenesis and function depend. In this pathway IFT20 acts as an adaptor coupling the CI-MPR to dynein for retrograde transport to the trans-Golgi network (TGN) (Finetti et al., 2020)

Whitin the pool of ciliary proteins that play fundamental roles in non-ciliated cells, CCDC28B (coiled-coil domain containing 28B) needs to be added to the list. CCDC28B is a coiled-coil (CC) domain-containing protein that interacts and colocalizes with the Bardet-Biedl syndrome (BBS) proteins at the basal body. CCDC28B has been shown to be essential for cilium formation, as depletion of CCDC28B results in shortened or absent cilia, however the underlying mechanism remains elusive (Cardenas-Rodriguez et al., 2013). CCDC28B was found to be expressed in T-cells where, similar to ciliated cells, it showed a centrosomal localization. A recent study has revealed a role for CCDC28B in the IS assembly, as the absence of CCDC28B resulted in impaired TCR and tyrosine-phosphoprotein accumulation at the IS. Moreover, CCDC28B was demonstrated to have a role in regulating the actin cytoskeleton by coupling TCR signalling to actin polymerization. In particular, CCDC28B regulates TCR recycling by promoting the FAM21-dependent recruitment of the actin regulator WASH to early endosomes (Capitani et al., 2022).

The growing number of cellular processes that are shared between the IS and the cilium, from actin cytoskeleton rearrangements to membrane trafficking to signalling pathways, supports the idea that lymphocytes have repurposed the ciliary machinery to perform their cytotoxic functions, indicating

a possible evolutionary connection between these structures (Douanne et al., 2021). Thus, the homology between the primary cilium and the IS offers a valuable resource for uncovering mechanisms with significant implications in both physiological and pathological contexts.

# AIM OF THE THESIS

Recently cellular degradation pathways have emerged as new extraciliary functions of the IFT system, with a key role again for IFT20 in a central degradation step controlling lysosome function, by regulating the mannose-6-phosphate receptor (M6PR)-dependent lysosomal targeting of acid hydrolases.

The aim of this work is to investigate whether, similar to IFT20, the ciliary protein CCDC28B could be implicated in the regulation of M6PR retrograde trafficking in T cells and its possible contribution to lysosome biogenesis and function.

# Material and methods

## Gene editing of Jurkat cells using CRISPR-Cas9 technology

A guide RNA (gRNA) targeting Cas9 nuclease activity to CCDC28B gene (GAACTTGGCCCGCTGCTTGG) was designed using the web-based tool CRISPOR (Haeussler et al., 2016) and cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid (kindly gifted by F. Zhang; Addgene, 48138) as described elsewhere (Ran et al., 2013). Jurkat cells were transfected with either empty vector or the gRNA-encoding construct using the Nucleofector Solution 2M [5 mM KCl, 15 mM MgCl2, 15 mM HEPES, 150 mM Na2HPO4/NaH2PO4 pH 7.2, 50 mM Manitol] (Chicaybam et al., 2013) and the Amaxa Nucleofector II system (Lonza), using the Program X-005. Single Green Fluorescent Protein (GFP)-expressing cells were sorted and screened for gene knockdown/knockout by immunoblotting.

## Cell cultures and immunoblot

Jurkart T cell lines were grown at 37 °C in the presence of 5% $CO_2$, in RPMI 1640 medium supplemented with 10% iron-enriched bovine calf serum (BCS; HyClone, GE Healthcare). All primary commercial antibodies employed in the study are listed in the Table 3. Secondary horseradish peroxidase (HRP)-labelled antibodies were purchased from Jackson ImmunoResearch Laboratories and Alexa Fluor 488-, 555-labeled secondary antibodies from ThermoFisher Scientific.

Cells ($2*10^6$/sample) were lysed in 1%(v/v) Triton-X100 in the presence of protease and phosphatase inhibitors for 5 min on ice. The homogenates were centrifuged at 16.000 x g for 20 min at 4 °C and the soluble fractions were recovered. Protein concentration from post-nuclear supernatants was quantified using the Quantum Protein Bicinchoninic Acid (BCA) Assay (Euroclone) and the iMark™ microplate reader (Bio-Rad) to measure the absorbance at 570 nm. Protein extract was denaturated in 4x Bolt LDS sample buffer (Invitrogen) supplemented with 10x Bolt sample reducing buffer (Invitrogen) for 5 min at 100°C. Proteins (10 μg) were subjected to SDS-Page on Bolt Bis- Tris mini protein gels (Invitrogen) and transferred to nitrocellulose (GE HealthCare) under wet conditions. Blocking was performed in PBS containing 5% non-fat dry milk, 0.2% Tween 20 (Sigma-Adrich). Membranes were incubated in primary antibodies for 3 h at room temperature (20-25°C) or overnight

at 4°C, followed by incubation in with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 45 min at room temperature. Secondary antibodies were detected using the chemiluminescent kit SuperSignal™ West Pico PLUS Chemilumiscent Substrate (Thermo Fisher Scientific). Chemiluminescence signals were acquired using Alliance Q9-ATOM imaging system and NineAlliance x64 software (both from UVITEC, Cambridge, UK). The densitometric analysis of protein bands was carried out using ImageJ (National Institutes of Health, USA).

## Immunofluorescence and image analysis

Jurkat cells were seeded on to poly-L-lysine (Merck)-coated slides (ThermoFisher Scientific) and fixed and permeabilized in methanol at -20°C for 10 min. After washing with PBS, samples were stained with primary antibodies (table 3) at 4°C overnight and then incubated at room temperature for 45 min with Alexa fluor 488- and 555-labeled secondary antibodies and mounted with 90% glycerol/PBS. Confocal microscopy was carried out on a Zeiss LSM700 microscope (Carl Zeiss, Jena, Germany) using a 63x/1.40 oil immersion objective. Co-localization analyses were performed on a medial optical section of single cells using ImageJ and the JACoP plugin to calculate Mander's coefficient M1, which indicates the proportion of the green signal coincident with a signal in the red channel over its total intensity, and M2, which is defined conversely for red (Manders et al., 1992). Mander's coefficients range from 0 to 1, corresponding to non-overlapping images and 100% co-localization between both images, respectively. The CI-MPR dispersion was measured using ImageJ by calculating the distance of CI-MPR + dots from the centrosome (identified by γ-tubulin staining). The number and area of LAMP-1$^+$ dots were determined in individual medial confocal sections using ImageJ ("Analyze particles" tool).

## Statistics and reproducibility

Each experiment is the result of at least 3 independent repetitions. Statistical analyses were performed using Prism software (GraphPad Software). Pairwise or multiple comparisons of values with normal distribution were carried out using Student's t-test (unpaired), one-sample t-test (theoretical mean=1), whereas values without Gaussian distribution were analysed with Mann-Whitney test. A *P-value* < 0.05 was considered as statistically significant.

| Antibody | Host Species | Catalogue number | Source | Dilution WB | Dilution IF |
|---|---|---|---|---|---|
| Anti-actin | Mouse | MAB1501 | EMD Millipore | 1:10000 | - |
| Anti-cat D | Rabbit | Ab75852 | Abcam | 1:1000 | 1:100 |
| Anti-CI-MPR | Rabbit | Ab124767 | Abcam | 1:100000 | 1:400 |
| Anti- LAMP-1 | Mouse | 328602 | BioLegend | 1:1000 | 1:400 |
| Anti-TGN38 | Mouse | Sc-166224 | Santa Cruz | - | 1:400 |

**Table 3 | List of the primary antibodies used in this work.**

# RESULTS

## CCDC28B controls the lysosomal targeting of acid hydrolases by regulating MPR traffic

The secretory pathway is responsible for the synthesis, folding and delivery of a different range of proteins (Barlowe & Miller, 2013). Through this pathway, newly synthesized proteins travel from the ER to their final destination. This highly organised system consists of a series of membranous compartments that include the ER, Golgi cisternae, the TGN, different types of secretory vesicles and the plasma membrane (Bonifacino & Rojas, 2006). In particular, the TGN has a crucial role as a site of protein sorting representing a hub of the membrane trafficking network of the cell (Lu & Hong, 2014). In fact, proteins from TGN can reach different possible destinations such as the extracellular space, the plasma membrane, secretory vesicles and endosomes (Spang, 2015). Usually, secretory cargos are delivered to the plasma membrane for exocytosis, while cargos with sorting signals are able to reach endosomes via the TGN-to-endosome pathway. Retrograde transport is essential in order to recycle the secretory machinery components, such as cargo adaptors or receptors, thus preventing their routing to lysosomes for degradation (Tu et al., 2020).

The trafficking of lysosomal hydrolases relies on specific sorting signals. Lysosomal hydrolases are synthesized in the ER and subjected to glycosylation on asparagine residues. Once in the secretory pathway, these proteins are recognized by a phosphotransferase, the GlcNA-1-phosphotransferase, which starts the reaction resulting in the addition of a M6P moiety to specific N-linked oligosaccharides. This enzyme catalyses the transfer of a GlcNac-1-phosphate residue from UDP-GlcNAc to C6 positions of selected mannose in high mannose type oligosaccharides of the hydrolases. Then, the so-called uncovering enzyme, the N-acetylglucosamine-1-phosphodiester α N-acetyl-glucosaminidase, removes the terminal GlcNAc, resulting in the exposure of the M6P recognition signal. Subsequently, the protein carrying the modification can be recognised by specific receptors that bind the M6P residue of the newly synthesized lysosomal hydrolases in the TGN (Du et al., 2022). The receptors responsible for the recognition of the newly M6P tagged proteins are the 46 kDa cation-dependent (CD) MPR, and the 300 kDa CI-MPR. These receptors are type I integral membrane proteins, where CD-MPR is described as an intracellular receptor that functions only

within the cell, while the CI-MPR is also present at the cell membrane where it promotes the uptake of extracellular ligands bearing M6P residues. Both receptors are deputed to sort acid hydrolases to lysosomes (Meraş et al., 2022)

As a result, lysosomal hydrolases tagged with M6P are packed into coated vesicles destined for lysosomes following the TGN-to-endosome pathway (Coutinho et al., 2012). The binding of M6P-tagged proteins to receptors occurs in a pH-dependent manner, in fact the neutral pH of the TGN enables the interaction with M6P-tagged cargos, while in the late endosomes, the acidic pH induces the release of the lysosomal hydrolases into the endosomal lumen, followed by fluid phase transport to lysosomes (Olson et al., 2020). In contrast, the MPR goes back to the TGN to engage in further rounds of hydrolase sorting (Braulke & Bonifacino, 2009). Failure in the recycling of the MPR results in the secretion of lysosomal hydrolases into the extracellular space, leading to aberrant lysosomal functions (Lu & Hong, 2014).

Finetti and colleagues demonstrated that the ciliary protein IFT20 participates in the biogenesis and function of lysosomes by regulating the MPR mediated lysosomal targeting of acid hydrolases, as the retrograde transport of MPRs from endosomes back to the TGN in Jurkat T cells requires IFT20 (Finetti et al., 2020).

Considering the role of the ciliary protein CCDC28B in various fundamental processes of T lymphocytes, similarly to what observed for IFT20, the aim of this work is to provide insights into the involvement of the ciliary protein CCDC28B in intracellular CI-MPR traffic.

To address the role of CCDC28B protein in these processes, we used as cellular model a Jurkat T cell line knocked out for CCDC28B expression by CRISPR/Cas9 gene editing, and the respective control Jurkat T cells edited with non-specific gRNAs. CCDC28B protein levels were evaluated in edited cells by immunoblot analysis (Figure 10).

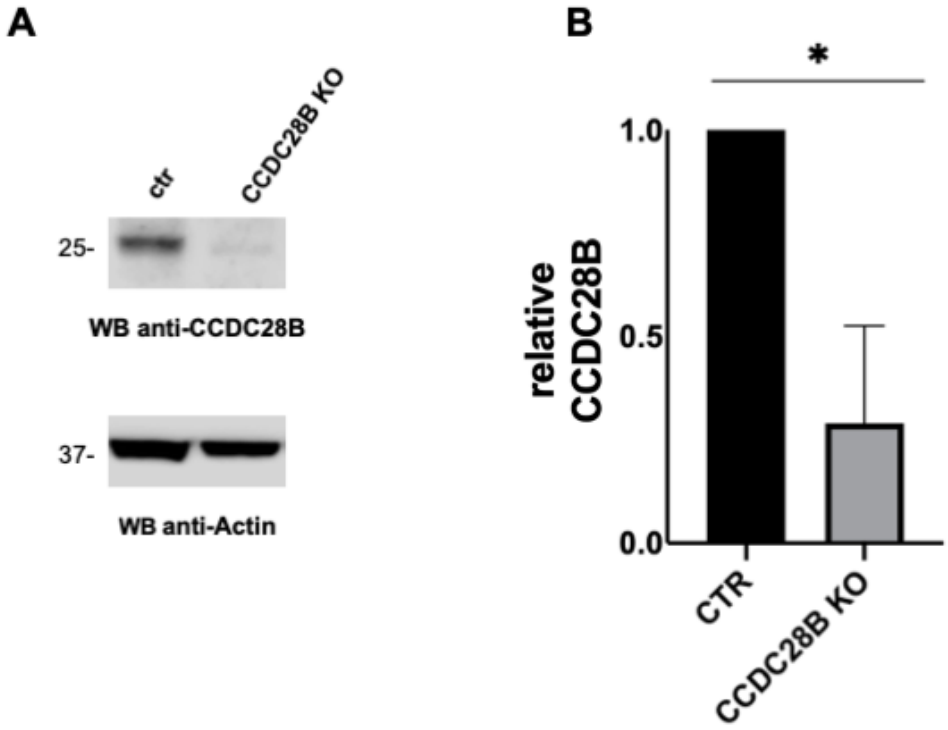


**Figure 10**

Representatives immunoblot analysis of CCDC28B in lysates of control (ctr) and CCDC28B KO Jurkat T cells with the respective loading control (actin). The migration of molecular mass markers is shown. **B.** The histograms show the quantification of CCDC28B protein levels (n = 4); mean fold ± SD. *P ≤ 0.05.

To investigate the role of CCDC28B in the retrograde traffic of the CI-MPR, we carried out a colocalization analysis of CI-MPR with the TGN marker TGN38. A decrease in the TGN38 colocalization of CI-MPR was observed in CCDC28B KO cells, while no defects in the total expression levels of CI-MPR were observed by immunoblot analysis in CCDC28 KO cells compared to control cells (Figure 11). These data indicate a defect in CI-MPR intracellular localization in CCDC28B KO cells and suggest that CCDC28B might be implicated in the retrograde transport of CI-MPRs to the TGN.

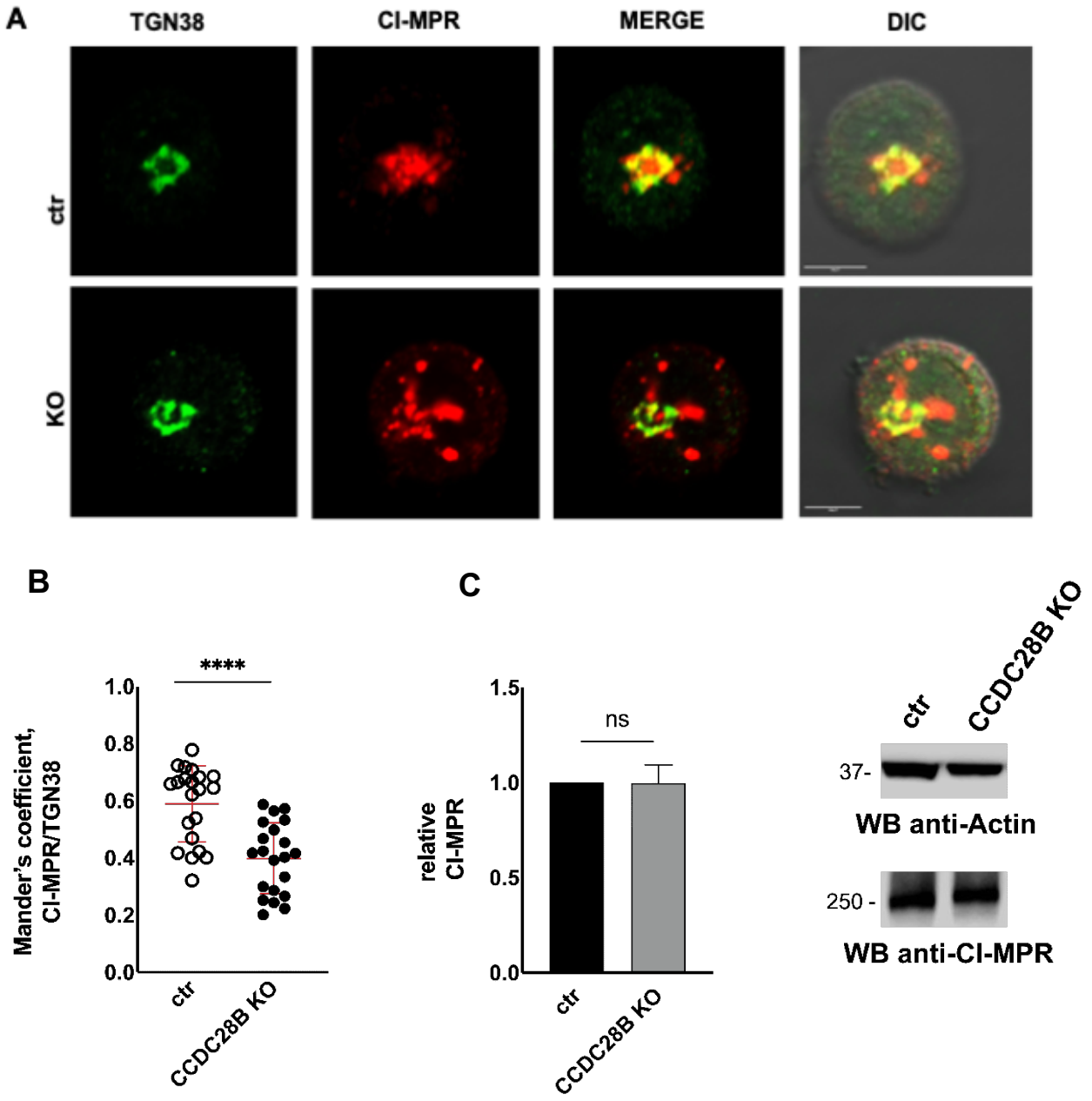


**Figure 11**

**A**. Immunofluorescence analysis of the trans-Golgi marker TGN38 and CI-MPR in ctr and CCDC28BK Jurkat cells and quantification of the weighted colocalization using Mander's coefficient (≥10 cells/sample, n ≥ 3). The data are expressed as mean ± SD (Mann–Whitney test, **B**). **C** Immunoblot analysis of CI-MPR in in lysates of control (ctr) and CCDC28B KO Jurkat T cells. Actin was used as loading control. The migration of molecular mass markers is shown. The histogram shows the quantification of CI-MPR protein levels (n = 4); mean fold ± SD. Representative images are shown. Size bar: 5 μm ****P ≤0.0001

To test this hypothesis, we analysed the retrograde transport of antibody-tagged CI-MRP from the cell surface to the TGN. To do this, we set up a recycling assay that exploits the pool of MPRs that follows the secretion pathway and is thus exposed at the plasma membrane. Cells were incubated with anti-CI-MPR antibodies at 37 °C for 60 min in order to allow the internalization and recycling of the antibody-tagged MPRs, and then CI-MPR-antibody complexes were imaged in permeabilized cells using fluorescently labelled secondary antibodies CI-MRP-positive vesicles remained dispersed in CCDC28BKO Jurkat cells, compared with the grouped distribution observed in ctr cells, as quantified by measuring the distance of CI-MPR-positive vesicles from the centrosome (Figure 12), indicating a block in retrograde transport in the absence of CCDC28B. In support of this notion, imaging internalized CI-MPR in cells co-stained for TGN38 showed that CI-MPRs co-localized with the TGN in ctr cells, but not in CCDC28B KO cells (Figure 12). These data indicate a key role of CCDC28B in controlling the retrograde transport of the CI-MPR to the TGN.

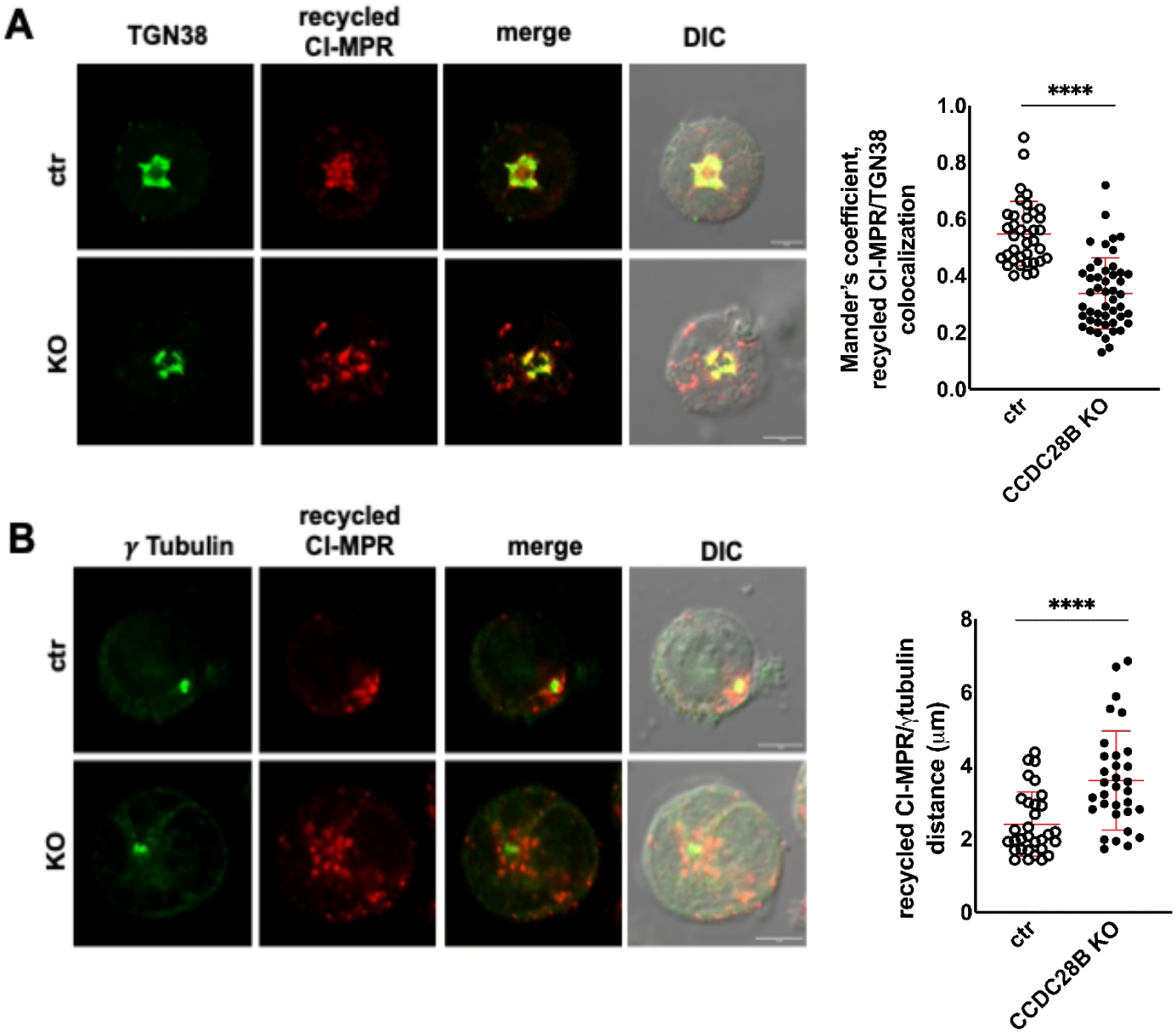


**Figure 12**

Immunofluorescence analysis of recycled CI-MPR and TGN38 (**A**) or γTubulin (**B**) in ctr and CCDC28B KO Jurkat. The graphs show the Mander's colocalization coefficient between CI-MPR$^+$ vesicles and TGN38 after antibody-induced CI-MPR internalization (at least 16 cells from three independent experiments were analysed, Mann–Whitney test) (**A**) or the distance of CI-MRP$^+$ vescicles from centrosome (≥10 cells/sample, n ≥ 3, Mann–Whitney test) **(B)**. Representative images are shown. Size bar: 5 μm. ****P ≤0.0001

As previously described, MRPs bound to their M6P-tagged cargoes bud from the TGN, transit through the early endosomes and finally reach to the late endosomes. The progressive drop in pH promotes dissociation of the cargoes which enter the lysosomes, while the MPRs are recycled back to the TGN by retrograde trafficking. Defects in this process lead to the formation of dysfunctional lysosomes, as observed for example in IFT20-deficient T cells, showing lysosomal compartment abnormalities, consisting in an increased size and reduced number of lysosomes (Finetti et al., 2020). At variance from what observed for IFT20 deficient T cells, lysosome analysis in CCDC28B KO cells reveals a slightly reduction in lysosome size in CCDC28B KO cells compared to control cells, while no differences in lysosome number per cell were detected (Figure 13). This slight reduction in lysosome size in CCDC28B depleted T cells might be a result of a disruption in lysosome biogenesis. However, the lack of changes in lysosome number might indicate that the lysosomal production and degradation is balanced, with the defect affecting in the first instance the size and maturation of lysosomes.

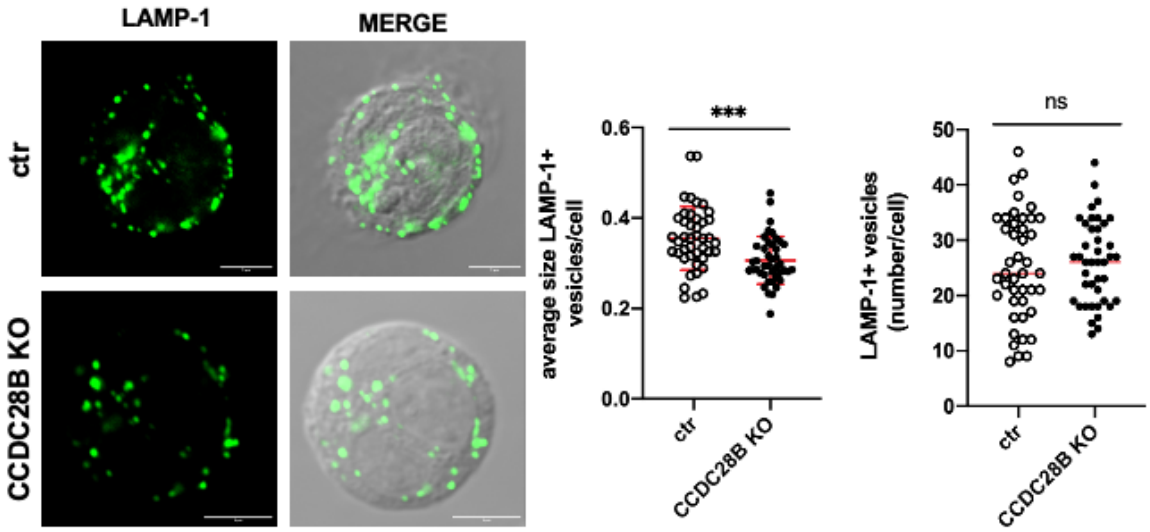


**Figure 13**

Immunofluorescence analysis of LAMP-1 in ctr and CCDC28B KO Jurkat cells. Representative images (medial optical sections) are shown (left). Size bar: 5µm. The graphs show their average size (µm2) (Mann–Whitney test) and the quantification of the number of LAMP-1$^+$ vesicles/cell (Mann–Whitney test). At least 15 cells from three independent experiments were analysed. ***P ≤ 0.001, ns.

# DISCUSSION AND CONCLUSIONS

Lysosome biogenesis is an essential process that guarantees the presence of the right number of lysosomes to support cellular degradation processes and metabolism. The generation of new lysosomes is regulated by the coordination of lysosomal protein biosynthesis and endosome-lysosome trafficking (Luzio et al., 2003). Lysosomal hydrolases are soluble enzymes that are synthesized and modified through a linkage with oligosaccharides in the ER. Subsequentially, in the Golgi apparatus they acquire M6P modification. This modification acts as a specific sorting signal that is recognized by MPRs at the TGN. The resulting hydrolase-MPR complex is enclosed in clathrin coated vesicles and travels through early endosomes to late endosomes. The low pH characteristic of late endosomes allows for the release of the hydrolase into the lysosome through fluid-phase transport, while the MPR is then recycled back to the TGN through retrograde transport for new cycles of transport (Braulke & Bonifacino, 2009). The MPR retrograde transport to TGN requires the retromer complex, several Rab GTPase and their effectors, the SNARE complex, as well as the interaction with the cytoskeleton components (Lu & Hong, 2014).

Finetti and colleagues demonstrated that the ciliary protein IFT20 is required for lysosome biogenesis and function by controlling the lysosomal targeting of acid hydrolases. This function involves its ability to act as an adaptor coupling the CI-MPR to dynein for retrograde transport to the TGN (Finetti et al., 2020). Moreover, regulation of lysosome biogenesis is operated at both transcriptional and translational levels. The existence of transcriptional regulation of lysosome biogenesis was demonstrated by the identification of gene transcription factor EB (TFEB) as a master regulator of several genes encoding lysosomal membrane proteins and hydrolases, known as the CLEAR (Coordinated Lysosomal Expression and Regulation) gene network (Sardiello et al., 2009) (Palmieri et al., 2011). In fact, IFT20 deficiency in T cells resulted in a reduction of lysosome number and an increase in their size, concomitant to an upregulation of genes belonging to the CLEAR gene network as a compensation attempt to restore lysosomal function (Finetti et al., 2019)

These findings strengthened the key role of ciliary proteins in non-ciliated T cells, extending beyond their documented role in the assembly of the IS.

In the present work, we aimed to investigate the contribution of the ciliogenesis protein CCDC28B in lysosome biogenesis and function.

CCDC28B is coiled-coil (CC) domain-containing protein that that modulates cilia length in vitro and in vivo. A recent study from our lab showed that CCDC28B protein is expressed in non-ciliated T

cells where it promotes actin polymerization at endosomal TCR through the recruitment of the actin regulator WASH and retromer complex component, thereby promoting their recycling to the IS (Capitani et al., 2022). Considering the role of the ciliary protein CCDC28B in regulating the actin cytoskeleton and its role in coordinating the endosomal recycling machinery, here we investigated the possible involvement of CCDC28B in the regulation of CI-MPR retrograde trafficking in T cells. The retrograde transport pathway of CI-MPR, following the release of acid hydrolases in late endosomes, has been partially elucidated. Recycling CI-MPR are sorted into tubular endosomes by the retromer complex and eventually reach the TGN through the coordinated action of different Rab GTPases and their effectors, and of STX16, Vit1A, and VAMP3 containing SNARE complex (Pfeffer, 2011).

The results of this thesis reveal that CCDC28B could be considered as an additional component of the CI-MPR retrograde pathway, as shown by the decrease in CI-MPR colocalization with the TGN under basal conditions and the inability of antibody-tagged recycling CI-MPRs to reach the TGN. Similar defects are consistent with the defects observed in cells with disrupted retromer function or impaired retrograde transport machinery (Progida et al., 2010) (Simonetti et al., 2017)

Defects in this process may also lead to the formation of dysfunctional lysosomes, as suggested by the size reduction in CCDC28B KO cells. These preliminary results suggest that CCDC28B, similar to the ciliary protein IFT20, plays a role in the retrograde transport of the CI-MPR, however how this affects lysosome biogenesis and function remains to be investigated.

The finding of this thesis may provide a foundation for further investigations regarding the implication of CCDC28B in the pathway leading to the biogenesis of LGs in CTLs, since the CI-MPR pathway is also exploited by these cells for the LGs transport of the Gzms (Griffiths & Isaaz, 1993).

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