CELL SCIENCE AT A GLANCE

The cytotoxic T lymphocyte immune synapse at a glance

Nele M. G. Dieckmann, Gordon L. Frazer, Yukako Asano, Jane C. Stinchcombe and Gillian M. Griffiths*

ABSTRACT

The immune synapse provides an important structure for communication with immune cells. Studies on immune synapses formed by cytotoxic T lymphocytes (CTLs) highlight the dynamic changes and specialised mechanisms required to facilitate focal signalling and polarised secretion in immune cells. In this Cell Science at a Glance article and the accompanying poster, we illustrate the different steps that reveal the specialised mechanisms used to focus secretion at the CTL immune synapse and allow CTLs to be such efficient and precise serial killers.

KEY WORDS: Cytotoxic T lymphocyte, Immune synapse, Secretion

Introduction

In its endeavour to fend off infection and cancerous growth, the mammalian immune system relies on both efficient communication

Cambridge Institute for Medical Research, Biomedical Campus, Cambridge CB2 0XY, UK.

*Author for correspondence (gg305@cam.ac.uk)

G.M.G., 0000-0003-0434-5842

between cellular players and the ability to eliminate harmful agents in a precisely focused manner.

Immune cells can communicate directly with each other by forming close cell–cell contacts that have become known as immune synapses. In addition to this internal communication, the immune system makes use of the synapse during direct attack on infected and cancerous cells – the formation of immune synapses allows killer cells to address the challenge of specifically eliminating 'dangerous' cells whilst leaving healthy cells unaffected. Thus, it is only after the establishment of the focused synapse interface that cytotoxic T lymphocytes (CTLs) and natural killer cells deliver a cocktail of cytotoxic substances from specialised secretory lysosomes (cytolytic granules) to destroy the target. In this review, we will provide an 'at a glance' view of the CTL synapse, highlighting features of its structure and roles in signalling, secretion and immunodeficiencies.
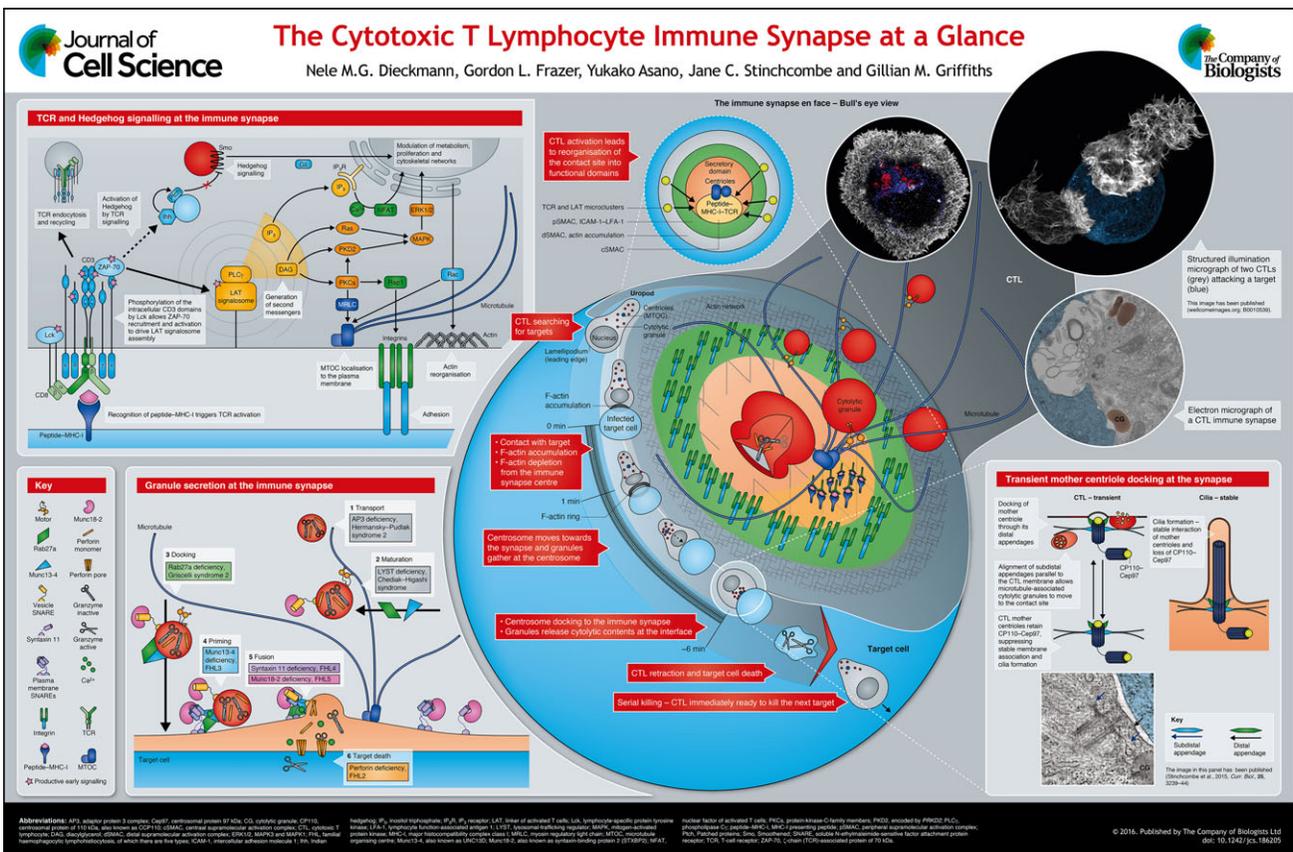
Structural features of the synapse

The formation of immune synapses involves the reorganisation of receptors that are involved in recognition and adhesion to form specialised functional domains at the interface between two cells. The first clear demonstration of distinct structural molecular

patterning in response to target engagement came from work by Kupfer in CD4^{+} T cells (Monks et al., 1998), with the rapid dynamics revealed using artificial bilayers (Grakoui et al., 1999). This showed T-cell receptor (TCR) clustering to the centre of the immune synapse, or central supramolecular activation cluster (cSMAC), with protein kinase C (PKC)-θ and Lck. This c-SMAC is surrounded by a ring of adhesion molecules – lymphocyte function-associated antigen 1 (LFA-1) and its adaptor talin, which are together referred to as the peripheral SMAC (pSMAC). Subsequent immunofluorescence imaging has revealed an accumulation of actin surrounding the pSMAC, sometimes referred to as the distal SMAC (dSMAC), creating a now well-known 'bulls-eye' configuration (see poster).

A similar structure has subsequently been identified in CD8^{+} CTLs with a discrete secretory domain next to the cSMAC and within the pSMAC (Potter et al., 2001; Stinchcombe et al., 2001b). These organised synapses between cells are widely adopted throughout the immune system, with similar layouts being used for both B- and T-cell activation, and even for phagocytosis of particulates by macrophages (Freeman et al., 2016; Goodridge et al., 2011; Niedergang et al., 2016).

Another important feature of the synapse structure is the reorganisation of the cytoskeleton. The microtubule organising centre (MTOC) has long been known to polarise towards the synapse in CD4^{+}, CD8^{+} and natural killer cells (Geiger et al., 1982; Kupfer and Dennert, 1984; Kupfer et al., 1983, 1985), and more recently it has been shown that one of the centrioles at the centre of the MTOC contacts the cell membrane next to the cSMAC, focusing secretion of cytotoxic components next to the point of TCR signalling (Stinchcombe et al., 2001b). This ensures precise secretion of the cytotoxic components perforin and granzymes,

and might also focus the delivery of the alternative cell death mediator FasL (Bossi and Griffiths, 1999; Kägi et al., 1994; Stinchcombe et al., 2006; Tschopp and Nabholz, 1990).

TCR signalling
CTLs identify their target cells through TCRs, whose signalling drives the dramatic reorganisation of the CTL cytoskeleton that goes with the establishment of a synapse.

Over the last 20 years, the biochemical outline of TCR signalling (see Box 1) has been extended with improving microscopy techniques, and the importance of the spatio-temporal dynamics of the process is now widely appreciated. Active TCR signalling is associated with the movement of small groups of TCR and LAT molecules (microclusters) from the synapse periphery toward the cSMAC, where TCRs are endocytosed to a recycling endosome. Once internalised, TCRs might be redelivered to the synapse or selectively trafficked for degradation, and so either enhance or diminish signalling (see poster). Along with direct players in the TCR cascade, inhibitory molecules and cytokines might also be delivered through vesicles to the synapse, both modulating signalling and communicating with the antigen-presenting cell independently of cytolytic granules (Purbhoo, 2013; Soares et al., 2013).

One of the pathways that has most recently been implicated in TCR signalling is the Hedgehog (Hh) pathway (see poster). Hh signalling is the trademark of signalling in the primary cilium, a structure absent only from haematopoietic cells and with surprising structural similarities to the synapse (Wheatley, 1995). In CD8^{+} T cells, Hh signalling is initiated by TCR signalling and causes the intracellular activation of Patched 1 and/or Patched 2 by Indian hedgehog (Ihh) on vesicles within the T cell. This inhibits repression of Smoothened (Smo) by Patched 1 and/or Patched 2, thereby activating Gli1 and driving expression of Hh target genes. In CD8^{+} T cells, a key Hh target gene encodes the protein Rac1, which plays a crucial role both in actin reorganisation and centrosome localisation to the synapse. Inhibition of Smo, either genetically or through use of chemical inhibitors, disrupts CTL-mediated killing (de la Roche et al., 2013).

Recently, the importance of mechanical force in formation of the synapse has become a subject of increasing investigation. To attach to and kill a target cell, the CTL must latch on tightly to its target, and this requires the activation of the integrin LFA-1 (Hogg et al., 2011). Work from the Burkhardt lab in CD4^{+} T cells has shown that full activation of LFA-1 requires F-actin flow, with intercellular adhesion molecule 1 (ICAM-1) on the antigen-presenting-cell side of the synapse providing physical resistance to promote this effect during synapse formation (Comrie et al., 2015a,b). Interestingly, progress in measuring two-dimensional binding kinetics has revealed similar roles for force in promoting adhesion, with catch bonds being formed by P-selectin, an adhesive molecule involved in CTL recruitment to sites of inflammation (Hirata et al., 2002; Marshall et al., 2003). Such catch bonds have recently been implicated in the ability of TCRs to distinguish between agonist and altered peptide ligands, which whilst they remain stimulatory, result in greatly reduced killing efficiency; however, the underlying biology has yet to be fully explored (Liu et al., 2014).

CTL immune synapse formation – sequence of events
The development and refinement of high-speed live-cell imaging techniques has fuelled the investigation of the dynamics of synapse formation and CTL-mediated killing. Over the course of the past

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Box 1. TCR signalling in a nutshell

TCR signalling begins with the activating phosphorylation of the kinases Lck and ζ-chain (TCR)-associated protein of 70 kDa (ZAP-70) when Lck associates with and phosphorylates the TCR, which promotes the recruitment and activation of ZAP-70 (Chan et al., 1992; Iwashima et al., 1994). In brief, active ZAP-70 phosphorylates tyrosine residues on linker of activated T cells (LAT) and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP76; also known as LCP2) to generate the LAT signalosome, a hub for secondary messenger generation (see poster) (Bubeck Wardenburg et al., 1996; Chakraborty and Weiss, 2014; Paz et al., 2001). Associated active phospholipase Cγ1 (also known as PLCG1) generates two of these messengers, inositol triphosphate (IP$_3$) and diacylglycerol (DAG) (Yablonski et al., 1998). IP$_3$ binds to its ER-associated receptor to induce a global Ca^{2+} flux, which is crucial for activation of the nuclear factor of activated T cells (NFAT) complex, whereas DAG recruits other signaling molecules to the membrane. These include protein-kinase-C-family members (PKCs) that activate integrin activity through phosphorylation of Rap guanine nucleotide exchange factor 2 (RapGEF2), thus activating Rap1; in addition, PKCs control myosin regulatory light chain behaviour to ensure efficient MTOC localisation to the immune synapse (Navarro and Cantrell, 2014; Quann et al., 2011). The signalling of PKCs is thought to be further amplified by their ability to phosphorylate the stabilising loop of protein kinase D2 (PRKD2). PRKD2 activity enhances transcription of the key cytokines interferon-γ and interleukin-2, and promotes Ras activity (Navarro et al., 2014a,b). Ras is further activated by the action of Ras guanyl-nucleotide-releasing proteins (RasGRPs), which themselves are recruited by DAG into close proximity of PKCs, and functions by initiating the mitogen-activated protein kinase (MAPK) cascade. This cascade has dramatic effects on CTL metabolism, proliferation, transcription, translation and even the microtubule network through the ERK1/2 complex (Navarro and Cantrell, 2014).

10 years, the order and timing of some key steps in the attack have been unravelled, although some of the details vary somewhat depending on the technique and cell system used.

In vitro, when placed on a glass surface, CTLs migrate with a lamellipodium at the front and a uropod at the rear (see poster). As soon as a target cell is recognised, CTLs stop migrating and accumulate F-actin at the contact site. This is followed by a reduction in F-actin at the centre of the contact site within one minute after initial contact (Ritter et al., 2015). As a consequence, an F-actin ring appears at the edge of the interface, known as the dSMAC. At the same time, TCR microclusters gather at the centre of the interface to form the cSMAC.

During CTL migration, the centrosome (MTOC) is located away from the leading edge, behind the nucleus, in the uropod. When a target encounter triggers TCR signalling, the centrosome starts moving towards the immune synapse (Kuhn and Poenie, 2002). It is thought that 'pioneer' microtubules link the centrosome to the synapse interface, and their shortening and the motor protein dynein act together to reel the centrosome to the synapse (Combs et al., 2006; Yi et al., 2013). The centrosome finally docks at the plasma membrane next to the cSMAC, in a region where F-actin is depleted. It takes about six minutes from the cell–cell contact to centrosome docking at the synapse (Ritter et al., 2015) (see poster).

As cytolytic granules cluster around the centrosome, they move together with the centrosome towards the synapse where they release perforin and granzymes into the space between the CTL and the target (Ritter et al., 2015). Following the release of granule contents, perforin facilitates transport of granzymes into the target, which trigger rapid target cell death. Finally, the CTL detaches from the dying target cell and moves on to find the next target. A new lamellipodium is formed distant from the immune synapse. The centrosome detaches from the synapse membrane and a new uropod is formed as the CTL moves away (Ritter et al., 2015). Intriguingly, the signal to detach appears to be dependent upon the demise of the target cell through caspase activity (Jenkins et al., 2015).

The role of CTL centrosome localisation to the plasma membrane in killing, and similarities with cilia

Precise targeting of cytolytic granules towards exocytic sites opposite the target is mediated by an unusual mechanism that involves centrosome positioning to the immune synapse membrane. On CTL activation, the centrosome moves from the back of the cell around the nucleus and docks with the plasma membrane within the immune synapse, at the boundary between the cSMAC and secretory domain (Ritter et al., 2015; Stinchcombe et al., 2006; Yi et al., 2013).

Centrosome localisation at the plasma membrane is unusual, but also occurs in cells with cilia and flagella; here, the centrosome docks with the plasma membrane through the distal appendages of the mother centriole before extending to form a cilium or flagellum (Azimzadeh and Bornens, 2007). Intriguingly, centrosome docking at the CTL synapse appears to be remarkably similar to that during ciliogenesis, although a cilium does not form (Stinchcombe et al., 2015).

Centrosomes comprise an older, more mature, mother centriole that is characterised by two rings of appendages at the distal end of the centriole, and a younger daughter centriole, which is derived from the mother during centriole replication and lacks appendages. The distal-most appendages are involved in membrane association, whereas the subdistal appendages are involved in microtubule organisation. CTL centrosomes dock at the immune synapse during target killing, with the mother attaching to the membrane through the distal appendages of the mother centriole (Stinchcombe et al., 2015) (see poster). This organisation aligns the subdistal appendages and associated microtubules under the plasma membrane at the secretory domain where granule contents are released. The mechanisms of centrosome docking at the CTL synapse and during cilia formation are also similar, with both processes requiring Cep83 (Stinchcombe et al., 2015; Tanos et al., 2013). However, once the centrosome has docked, the pathways diverge. In ciliated cells, the centrosome-end-regulating proteins CP110 (also known as CCP110) and Cep97 are lost, and cilia formation proceeds, whereas CTL mother centrioles retain the CP110–Cep97 complex on docking during killing and show no signs of cilia formation (Stinchcombe et al., 2015). Because it has been recently shown that lymphocytes have the capacity to form cilia if CP110 is depleted (Prosser and Morrison, 2015), it is likely that the mechanisms involved in CP110 retention act to prevent cilia formation at the CTL synapse during killing (see poster). Preventing cilia formation prevents stabilisation of CTL centrosomes and ensures centrosome docking is only transient, thereby allowing the multiple polarisation events that are required for sequential killing of several targets.

Other similarities between cilia and the immune synapse have also been found in CD4$^+$ T cells, including a role for intraflagellar transport (IFT) proteins, which are required for cilia formation, in TCR recycling (Finetti et al., 2009, 2014; Vivar et al., 2016). Electron microscopy tomography reveals a very similar organisation of the centrosome and secretory compartments in CD4$^+$ T cells (Ueda et al., 2011), although centrosome docking is yet to be studied in CD4$^+$ T cells.

Targeted granule secretion at the immune synapse

The release of granule contents at the synapse is tightly controlled by a sophisticated protein machinery that coordinates the delivery, docking and fusion of granules at the plasma membrane (see poster). Malfunctioning of this machinery as a result of genetic defects in its components leads to the devastating immune deficiency condition familial haemophagocytic lymphohistiocytosis (FHL) with five subtypes (FHL1 to FHL5), and the related conditions Griscelli syndrome type 2 (GS2), Hermansky–Pudlak syndrome type 2 (HPS2) and Chediak–Higashi syndrome (CHS) (Chediak, 1952; Farquhar and Claireaux, 1952; Griscelli et al., 1978; Hermansky and Pudlak, 1959; Higashi, 1954). Mouse models of these conditions show that upon pathogen challenge, the genetic mutation impairs the secretion of pro-apoptotic factors from CTL (and natural killer) granules, whereas the production of cytokines and their release through a different secretory pathway appears to be enhanced (Brisse et al., 2015; de Saint Basile et al., 2015 preprint; Jenkins et al., 2015; Reefman et al., 2010). The inability of CTLs and natural killer cells to clear the infection whilst continuously secreting cytokines promotes the activity of effector immune cells, leading to a life-threatening hyper-inflammatory state (haemophagocytic lymphohistiocytosis, HLH) that requires immunosuppressive therapy and ultimately bone-marrow transplantation (Sieni et al., 2014). To date, four FHL-associated proteins have been identified and a fifth disease-linked genetic locus awaits further investigation (Côte et al., 2009; Feldmann et al., 2003; Ohadi et al., 1999; Stepp et al., 1999; zur Stadt et al., 2009, 2005). The known secretion factors at the CTL immune synapse are the putative vesicle-tethering protein Munc13-4 (also known as UNC13D; implicated in FHL3), the soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) protein syntaxin 11 (implicated in FHL4), the

syntaxin-binding protein Munc18-2 (also known as STXBP2; implicated in FHL5), the Munc13-4 binding partner Rab27a (implicated in GS2), the adaptor protein subunit AP3-β3A (also known as AP3B1; implicated in HPS2) and the lysosomal-trafficking regulator protein (LYST; implicated in CHS) (Barbosa et al., 1996; Barrat et al., 1996; Côte et al., 2009; Dell'Angelica et al., 1999; Feldmann et al., 2003; Fukai et al., 1996; Ménasché et al., 2000; Nagle et al., 1996; Perou et al., 1996; zur Stadt et al., 2009, 2005). These proteins are thought to act in successive steps during the maturation, transport and secretion of cytolytic granules (see poster).

HPS2 CTLs that lack the AP3 complex cannot transport their granules to the immune synapse, suggesting that AP3 might coordinate the delivery of a motor protein or a motor adaptor to the granule membrane (Clark et al., 2003). In CTLs from individuals with GS2, the loss of functional Rab27a means that granules polarise but fail to detach from microtubules and, therefore, cannot reach the plasma membrane (Haddad et al., 2001; Stinchcombe et al., 2001a). In CTLs, Rab27a functions in granule docking through its interaction with the vesicle tether Munc13-4, whereas in melanocytes, it has been shown to link to the actin-bound motor myosin Va to 'capture' melanosomes at their target membrane in the cell periphery (Elstak et al., 2011; Hume et al., 2001; Neeft et al., 2005; Shirakawa et al., 2004; Wu et al., 2001).

Munc13-4 associates with the cytolytic granules in attacking CTLs. In Munc13-4-deficient CTLs, granules reach the plasma membrane but cannot be 'primed' for secretion (Elstak et al., 2011; Feldmann et al., 2003). It has been suggested that in addition to the vesicle tethering interaction with Rab27a, Munc13 proteins also interact with SNARE complexes, the helical protein bundles that drive membrane fusions, through a MUN-domain (Basu et al., 2005; Guan et al., 2008).

Syntaxin 11 and Munc18-2 are binding partners that localise to the plasma membrane of CTLs and neutrophils; this strongly suggests that they cooperate to drive the final steps of granule fusion (Brochetta et al., 2008; Côte et al., 2009; Dieckmann et al., 2015; Hackmann et al., 2013; Halimani et al., 2014; Hellewell et al., 2014; zur Stadt et al., 2009). The loss of syntaxin 11 from the plasma membrane of Munc18-2-deficient CTLs supports the notion that Munc18-2 acts as a syntaxin 11 chaperone, similar to Munc18-1 chaperoning of syntaxin 1A; however, Munc18-2 has also been found to associate with granules in CTLs, mast cells and neutrophils where it may perform a yet unknown function, potentially in association with granule SNAREs (Brochetta et al., 2014, 2008; Dieckmann et al., 2015; Han et al., 2011; Martin-Verdeaux et al., 2003; Rowe et al., 2001).

Mutations in the LYST protein that give rise to CHS have been suggested to cause a fission defect that entails the formation of enlarged lysosomes, whose excessive size appears to prevent fusion at the immune synapse (Baetz et al., 1995; Durchfort et al., 2012). A recent report shows that overexpression of either Rab27a alone or Rab27a together with Slp3 (also known as SYTL3) partially restores granule secretion [measured by appearance of the lysosomal membrane protein CD107a (also known as LAMP1) at the cell surface] and that coexpression of Rab27a, Munc13-4 and Slp3 rescues the secretion defect of CHS CTLs. This gives rise to the suggestion that LYST might be involved in the trafficking of effectors that drive the maturation of perforin-containing vesicles into granules that are fully secretion competent (Sepulveda et al., 2015).

Two additional genetic defects have been linked to the immune synapse and can trigger the development of HLH, but they do not directly affect secretory factors. For instance, FHL2 arises owing to loss of the pro-apoptotic factor perforin from the cytolytic granules (Stepp et al., 1999). Interestingly, immune synapses formed by perforin-deficient CTLs and natural killer cells persist much longer than normal synapses; here, the FHL2 CTLs appear to become stuck on the targets that they fail to kill (Jenkins et al., 2015). Finally, in X-linked lymphoproliferative disease type 1 (XLP-1), the mutation of the signalling lymphocyte activation molecule (SLAM)-associated protein (SAP; also known as SH2D1A) disturbs key intracellular signalling processes that are exerted by SLAM (also known as SLAMF1) in natural killer cells and CTLs, thereby resulting in a defective killer response against Epstein-Barr-virus-infected cells (Coffey et al., 1998; Dupré et al., 2005), reviewed by (Tangye, 2014).

Concluding remarks

In this review, we have focused on the formation of the CTL immune synapse, which is a highly dynamic process that relies on the close interplay of signalling factors, cytoskeletal elements and membrane fusion machinery to deliver a rapid cytotoxic hit, which allow CTLs to be effective serial killers. There are many more aspects that are currently being explored, including the roles of motor proteins, positive and negative receptor signalling, mechano-sensing and CD4$^+$ cells that acquire cytolytic potential. In addition, the understudied role of the target cell in forming the synapse and the signals that tell the CTL when to depart remain to be uncovered, along with a full understanding of how the CTL manages not to kill itself as it releases its deadly cytolytic load.

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