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On the origin of receptor microclusters on T cells
Shlukování receptorů na povrchu T buněk

Bachelor´s thesis

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Abstrakt

T buňky hrají důležitou roli jak v specifické, tak vrozené imunitě. Receptory T buněk rozeznávají antigeny presentované MHC glykoproteiny povrchu buněk. Navázání antigenu na receptor vyvolává aktivačních signály. Tyto signály vedou k seskupování T-buněčných receptorů do mikroclusterů a tvorbě imunologické synapse. Imunologická synapse hraje důležitou roli v signalizaci, kostimulaci, aktivaci T buněk a v degradaci receptorů. Tato práce je zaměřena na proces tvorby mikroclusterů T-buněčných receptorů, formování imunologické synapse a na to, jak vývoj ve fluorescenční mikroskopii změnil náš pohled na tvorbu těchto struktur.

Klíčová slova

T buňky, povrchové receptory, plazmatická membrána, mikroclustery, fluorescenční mikroskopie

Abstract

T cells play an important role in both acquired and innate immunity. T cell receptors recognize antigens presented by MHC glycoproteins on cellular surfaces. The binding of the antigen to the T-cell receptor triggers activation signals. This leads to T-cell receptor clustering to microclusters and immunological synapse generation. The IS plays an important role in signalization, co-stimulation, T-cell activation and receptor degradation. This thesis is focused on the process of the T-cell receptor microclusters and immunological synapse formation and how the development in fluorescence microscopy improved our insight into these processes.

Key words

T cells, surface receptors, plasma membrane, microclusters, fluorescence microscopy

Contents

1 Table of abbreviations

TCR	T cell receptor
MC	microcluster
PM	plasma membrane
IS	immunological synapse
APC	antigen presenting cell
MHC	major histocompability complex
pMHC	peptide – MHC complex
SMAC	supramolecular activation cluster
cSMAC	central SMAC
pSMAC	peripheral SMAC
dSMAC	distal SMAC
LFA-1	lymphocyte-function associated antigen 1
ZAP-70	zeta-chain-associated protein kinase 70
LAT	linker for activation of T cells
ICAM-1	intercellular adhesion molecule 1
SLP-76	lymphocyte cytosolic protein 2
WASp	Wiskott-Aldrich syndrome protein
TEM	transmission electron microscopy
IRM	interference reflection microscopy
FSM	fluorescent speckle microscopy
PALM	photoactivated localization microscopy
FCCS	fluorescence cross-correlation spectroscopy
TIRFM	total internal reflection fluorescence microscopy
FRET	Förster resonance energy transfer
FRAP	fluorescence recovery after photobleaching
SMLM	single-molecule localization microscopy
SIM	structured illumination microscopy
STORM	stochastic optical reconstruction microscopy

2 Introduction

T cells play a crucial role in the acquired immune response and cooperate with the innate immunity. TCRs recognize antigens from various environmental sources, e.g., pathogens, damaged cells, and tumors. T cells can influence immune response by activating other immune cells or directly with the apoptotic signal. T cells can control and regulate immune response and provide long-term immunity. These cells were recognized as a major driver of autoimmunity and autoinflammatory diseases.

2.1 T cell development

T cells originate from thymocytes. Thymocytes are produced as stem cells in the bone narrow. They develop in the thymus, where they undergo maturation in the process of thymopoiesis. Thymocytes undertake TCR rearrangement and gain both CD4 and CD8 coreceptors. These double-positive thymocytes go through positive selection in the thymic cortex. During the positive selection, the ability of TCR to bind MHC I or MHC II is probed. Double-positive thymocytes with ability to bind MHC I result in $CD8^+$ T cells, double-positive thymocytes with the ability to bind to MHC II evolve into single-positive $CD4^+$ T cell. In parallel, negative selection eliminates thymocytes, which interact strongly with self-antigens. Successfully matured thymocytes become peripheral naïve T-cells (Takahama, 2006).

2.2 Main T cell subsets

2.2.1 $CD4^+$ T cells

$CD4^+$ T cells, also known as helper T cells, recognize antigens presented by MHC II on the surface of the APC. Activated helper T cells acquire further information in the form of cytokines that determine their differentiation into an effector subset within Th cell line. Th-cell subsets such Th1, Th2, T-17 and Th-22 cells produce various cytokines and influence the immune response (Raphael *et al*., 2015).

2.2.2 $CD8^+$ T cells

$CD8^+$ T cells are cytotoxic cells that can kill damaged, cancer, or infected cells. The TCR of $CD8^+$ T cells binds antigen presented by MHC I on the surface of almost all cells in the body. Cytotoxic T cells are able to trigger apoptosis of the target cell.

2.2.3 Memory T cells

Memory T cells provide long-term protective immune response. Memory T cells originate from the effector T cells. Most of the effector T cells commit apoptosis once pathogen

has been cleared. However, a small fraction of these cells become long-lived memory T cells (Kaech & Cui, 2012).

2.2.4 Regulatory T cells

Regulatory T cells (Tregs) play a crucial role in the regulation of the immune response against innocuous and ubiquitous antigens. Tregs develop in the thymus, their development can be regulated by the Tregs which migrate back to the thymus (Thiault *et al*., 2015).

2.3 T cell activation

The initial activation of the naïve $CD4^+$ and $CD8^+$ T cells is triggered when TCR recognizes, and binds antigen presented by MHC complex on the surface of target cells. Then the CD4 or CD8 molecules bind to the pMHC complex and stabilize the receptor/ligand complex. For full T-cell activation and proliferation a second signal is required. It is provided by the co-stimulatory molecules. The crucial in this process is the co-stimulation provided by CD28, which binds to the CD80 or CD86 on the APC surface. T cells with a strong reaction are given survival signals by several molecules such a OX40 and ICOS. Without the co-stimulatory signals T cells become anergic.

In my thesis. I have focused on one of the essential immunity processes, the process of when antigen-specific T cell and APC encounter that leads to changes of the PM, TCR clustering, IS development and T-cell activation.

3 Immunological synapse discovery

The spatial organization of T cell – APC contact, IS generation and process of T cell activation have been studied through various microscopic methods. Monks *et al.* used 3D fluorescent microscopy and revealed, that after the antigen-specific T cell – APC contact receptors and signaling molecules cluster into spatially segregated and distinct domains, that were named SMACs. Two SMACs were recognized: cSMAC and pSMAC, that formed circle around the cSMAC. In the cSMAC were observed TCR-CD3 complexes, signaling and co-stimulatory molecules such a CD2, CD4 and CD28 (Grakoui *et al*., 1999). In the pSMAC were clustered adhesive molecule LFA-1 and cytoskeletal protein talin (Monks *et al*., 1998). Further was detected dSMAC established at the periphery of the IS and enriched with large regulative molecules CD43 and CD45 (Freiberg *et al*., 2002). Such a bull-eye structure represents the mature IS (Grakoui *et al*., 1999).

4 TCRs on T-cell surface

Yokosuka *et al* proposed that TCRs are not pre-clustered into MCs and clusters are generated upon T cell – APC contact (Yokosuka *et al*., 2005). A deeper insight into the state of TCRs, signaling and adaptor molecules before activation was provided by advanced microscopy methods such a TEM, high-speed PALM and dual-color FCCS. These methods revealed that TCR and adaptor protein LAT are arranged into separate membrane domains that were termed nanoclusters (Lillemeier *et al*., 2010). Using two-color PALM, it was observed, that part of TCR and LAT nanoclusters overlap each other. Thus, overlapping nanoclusters can serve as hot spots for activation (Sherman et al., 2011). On the edge of LAT nanoclusters PALM imaging detected adaptor protein SLP-76 (Sherman *et al*., 2011), that acts in the pathway leading to T-cell activation (Burns *et al*., 2011).

5 Early phase of the immunological synapse development

Grakoui *et al.* used IRM combined with fluorescent microscopy to observe contact area of the T-cell – APC encounter. APC was supplied as a lipid bilayer with fluorescently labeled pMHC and ICAM-1. IRM allowed to observe surface contacts and fluorescent microscopy revealed localization of the labeled molecules (Grakoui *et al*., 1999). As a result of early phase of the IS development, the two domains were segregated. First domain consisted of centrally accumulated LFA-1 clusters bound to ICAM-1 in the center of the contact area. Second domain consisted of TCR MCs which surrounded LFA-1 accumulation (Grakoui *et al*., 1999).

An antigen recognition by TCR initiates TCR clustering (Yokosuka *et al*., 2005), influences actin polymerization (Bunnell et al., 2001, 2002; Wülfing & Davis, 1998) and abated T cell migration (Grakoui *et al*., 1999).

5.1 TCRs clustering

TCRs aggregate into MCs immediately after the antigen recognition. Size of newly generated TCR MCs depend on the density of pMHC on the lipid bilayer (Yokosuka *et al*., 2005). TCRs closely cooperate with actin cytoskeleton. TIRFM studies revealed that actin cytoskeleton was necessary for TCR MCs formation (Campi *et al*., 2005) but not necessary for stability of the formed TCR MCs (Varma *et al*., 2006).

TCR MCs contain adaptor and signaling molecules such a LAT and ZAP-70. ZAP-70 is recruited to TCRζ and phosphorylates LAT (Wang et al., 2010), activated LAT recruits other proteins that participate in T-cell signaling pathway (Bunnell *et al*., 2001; Finco *et al*., 1998). FRAP experiments revealed, that ZAP-70 can rapidly exchange between LAT nanoclusters and spread activation signal over the contact area (Bunnell *et al*., 2002; Douglass & Vale, 2005). This means that newly generated TCR MCs are the place of the origin of signaling pathways including Ca^{2+} signaling(Boniface *et al*., 1998; Bunnell *et al*., 2002; Varma *et al*., 2006).

5.2 Actin cytoskeleton

The activation of co-stimulatory molecule CD28 and adhesive molecule LFA-1 reoriented actin cytoskeleton to the area of encounter (Wülfing & Davis, 1998). TCRζ binds to actin after engagement of TCR and pMHC (Rozdzial *et al*., 1995). Engaged TCRs activate protein Nck that recruits WASp, a linker between TCR and actin polymerization (Gil *et al*., 2002). WASp is initially colocalized with TCRs, afterwards occurs at the periphery (Barda-Saad *et al*., 2005).

Actin polymerization leads to prolongation of filopodia and lamellipodia and thus to the spreading of T cells, that leads to maximalization of the contact area (Bunnell *et al*., 2001, 2002). This T cell expansion facilitates generation of new TCR MCs in the PMs contacts (Bunnell *et al*., 2002).

During the expansion phase actin cytoskeleton delivers signaling molecules into the contact area (Wülfing & Davis, 1998). Inhibition of actin polymerization cease the early T cell activation (Delon *et al*., 1998).

5.3 Microtubule cytoskeleton

After the antigen specific contact of T cell – APC, microtubule cytoskeleton undergoes changes. MTOC is translocated to the area proximal to the contact site (Geiger *et al*., 1982; Kuhn & Poenie, 2002) by a molecular motor dynein (Combs *et al*., 2006; Martín-Cófreces *et al*., 2008). Translocation is regulated by ZAP-70 (Blanchard *et al*., 2002) and dynein remains at the periphery of the IS (Combs *et al*., 2006).

6 Contraction phase of the immunological synapse development

6.1 cSMAC development

After the maximum cell spread the next stage of the IS development occurred as a formation of the distinct SMACs. TCR MCs were translocated and accumulated into cSMAC in the center of the IS. cSMAC is surrounded with pSMAC, a ring of adhesive molecules LFA-1, so, these molecules exchanged their positions after the initial phase (Grakoui *et al*., 1999; K. H. Lee *et al*., 2002). At the distant periphery ring of the IS is located dSMAC formed by CD43 and CD45 molecules (Freiberg *et al*., 2002). This phase was termed as a contraction phase, because TCR MCs are translocated from the periphery to the IS center (Yokosuka *et al.*, 2005).

Majority of TCR MCs is translocated to the IS center. TCR MCs are initially translocated individually, but during the translocation TCR MCs can fuse. This is supported by the number of CD3ζ chains in clusters at the periphery (40 – 110) and at the center (110 – 290) (Yokosuka *et al*., 2005) and with observed decreasing volume of TCR MC – pMHC complexes during translocation (Grakoui *et al*., 1999).

Between 5 - 15 min after the encounter, TCR MCs are immobilized in a newly generated cSMAC (Varma *et al*., 2006). The formation of distinct cSMAC and pSMAC is observable between 5 – 15 min after T cells – encounter activating lipid bilayer (Grakoui *et al*., 1999; Yokosuka *et al*., 2005), however a computational reconstruction of the IS formation after encounter of T cells and B cells shows, that in this system the formation of distinct cSMAC and pSMAC and mature IS formation is detectable in 15 – 30 min (K. H. Lee *et al*., 2002). This suggests that different results can be obtained using different experimental system.

6.2 pSMAC development

The formation of distinct pSMAC can be observed *via* localization of actin protein talin. Talin associates with the adhesive molecule LFA-1 (Monks *et al*., 1998; Smith *et al*., 2005).

Just a little amount of TCR is efficient in talin recruitment into contact area (Kupfer & Singer, 1989).

Talin is observed in the pSMAC in the mature IS (Freiberg *et al*., 2002; Monks *et al*., 1998). 3D analysis using digital deconvoluting microscopy revealed, that 45 s after T cell – APC encounter, talin is uniformly dispersed in the contact site. This indicates that SMACs formation did not start yet. After 4 min, observation of distinguishable talin-based pSMAC is possible (Freiberg *et al*., 2002).

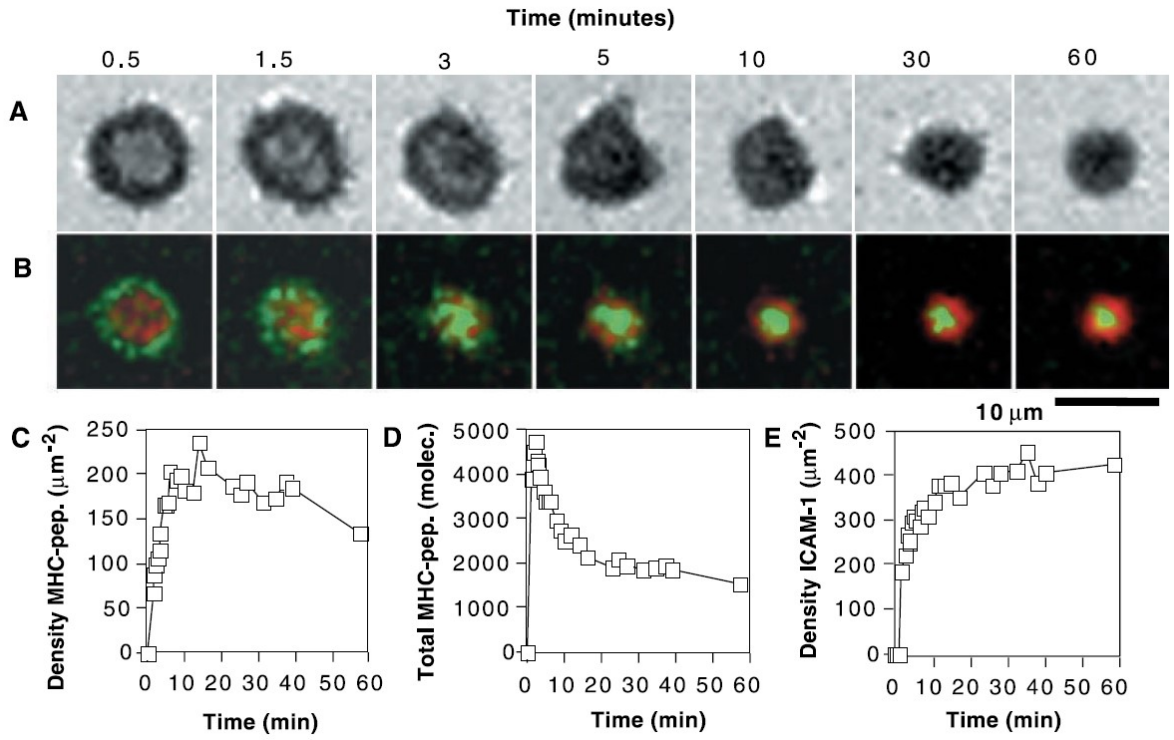

Figure 1: Formation of the immunological synapse. 2B4 T cells in contact with a supported planar bilayer **(A)** Images of contact formation (IRM) at five time points. IRM shows contacts as dark gray on a light background. (**B**) Images of MHC-peptide (green) and ICAM-1 (red) accumulation. (**C**) Density of accumulated Ek(MCC88-103) (**D**). Total accumulated Ek(MCC88-103) (**E**). Density of accumulated ICAM-1 (adopted from Grakoui *et al.* 1999)

6.3 Cluster segregation in SMACs

It was proposed that one of the factors contributing to the SMAC segregation is the size of the clusters. TCR MCs are larger than the LFA-1 clusters, thus, TCR MCs can push LFA-1 clusters out from cSMAC to pSMAC. This was supported by the observation of artificially formed LFA-1 clusters larger than in physiological conditions. These were able to reach cSMAC. Moreover, LFA-1 are separated by size in pSMAC (Hartman *et al*., 2009).

The spatial distribution of actin in the IS was proposed as another factor contributing to the segregation of SMACs. Actin is not distributed homogenously in the IS, pSMAC is based on actin lamella, while in cSMAC the amount of actin is very low (Kaizuka *et al*., 2007). This is

consistent with fluorescent confocal microscopy examination of the F-actin retrograde flow which occurs only in the periphery of the IS (Yu *et al.*, 2010).

Thus, if the cSMAC is the area without F-actin retrograde flow, there must be another mechanism that translocates TCR MCs into cSMAC.

6.4 Dynein-dependent translocation of the TCR MCs

Hashimoto-Tane *et al.* observed that not only actin is involved in the distribution of TCR MCs. After initial activation and formation of the IS, TCR MCs are also associated with dynein, a molecular motor cooperating with the microtubule cytoskeleton. Dynein-dependent translocation mechanism contributes to the centripetal movement of TCR MCs and the one, which can move TCR MCs into low-actin cSMAC (Hashimoto-Tane *et al*., 2011).

6.5 Signaling during contraction phase

Engagement of TCRs by cognate ligands induces phosphorylation of the TCR CD3 intracellular domain by tyrosine kinase Lck from the Src family (Iwashima *et al.*, 1994), this is regulated by CD45 phosphatase (Sieh *et al*., 1993), Lck is inactive in the presence of CD45. FRET spatiotemporal analysis revealed that CD45 is associated with TCR CD3ζ chain. CD45 is later excluded from cSMAC into dSMAC (Freiberg *et al.*, 2002). However, under certain conditions, CD45 might be present in the mature IS cSMAC again (Varma *et al*., 2006).

In the mature IS, Lck is present in the cSMAC (K. H. Lee *et al*., 2002; Monks *et al*., 1998) but only in its inactive form (K. H. Lee *et al*., 2002).

The TCRζ chain associates with ZAP-70, that is phosphorylated by Lck there and that initiates a downstream signaling cascade (Iwashima *et al*., 1994; Thill *et al*., 2016). ZAP-70 phosphorylates SLP-76 (Yablonski *et al*., 1998) and LAT (Finco et al., 1998; Wang et al., 2010). Recruitment of ZAP-70 to TCR MCs is dependent on the MC formation (Varma *et al.*, 2006).

TIRFM and spatiotemporal analysis revealed that ZAP-70 and SLP-76 are present as MCs colocalized with TCR MCs, their amount increases immediately after T-cell – APC encounter until full surface expansion after 3 min at the end of the expansion phase. (Bunnell *et al*., 2002; K. H. Lee *et al*., 2002). However, during next 3 min, in the contractile phase, CD3ζ is translocated to the center, while most of the ZAP-70 remains at the periphery. The ZAP-70 signaling in the initial clusters declines rapidly and is replaced by signaling from newly generated TCR MCs at the periphery (Yokosuka *et al*., 2005). Most of SLP-76

exhibits motility and translocation to cSMAC, but after the contractile phase disappears from signaling MCs (Yokosuka *et al*., 2005).

7 The immunological synapse maintenance and sustained signaling

Yokosuka *et al.* used TIRFM, single-molecule tracing and quantitative signal tracing to observe TCR MCs. The TCR MCs were continuously generated on the lamellipodia-like structure at the periphery of IS and were translocated into cSMAC (Yokosuka *et al*., 2005). This IS maintenance and signaling appear 5 min after the first signaling occurs at the contact site (Campi *et al*., 2005).

In the cSMAC, TCR MCs are immobilized, stabilized (Grakoui *et al*., 1999) and later degraded. Degradation in the cSMAC was observed just after 6 min after T-cell – APC encounter, this means that the degradation occurred during cSMAC formation (Huppa et al., 2003).

For the maintenance of the mature IS structure, a sustained signaling and T-cell activation, are necessary through continuous stimulation by TCR MCs (Huppa *et al*., 2003; K. H. Lee *et al*., 2002). The time required for full activation and proliferation differs in between T-cell subsets (K. H. Lee *et al*., 2002; van Stipdonk *et al*., 2001).

TCR MCs are generated at the periphery and are translocated to the cSMAC (Yokosuka *et al*., 2005). These TCR MCs contain 40 – 150 TCR molecules (Yokosuka *et al*., 2005). For the maintenance of the mature IS, TCR MCs with 11 – 17 TCR molecules are sufficient (Varma *et al*., 2006).

The newly generated peripheral TCR MCs contain signaling and adaptor molecules such as SLP-76 and ZAP-70. While TCR MCs are translocated into cSMAC, most of the ZAP-70 remains in the periphery, only a small part of them moves centripetally and their track is very short. SLP-76 do not move into the cSMAC and remains in the periphery suggesting that cSMAC is not a major signaling site (Yokosuka *et al*., 2005).

Large differences were observed in SMACs dynamics. pSMAC and dSMAC were observed as highly dynamics structures, while cSMAC was observed as a stable structure (Yokosuka *et al*., 2005).

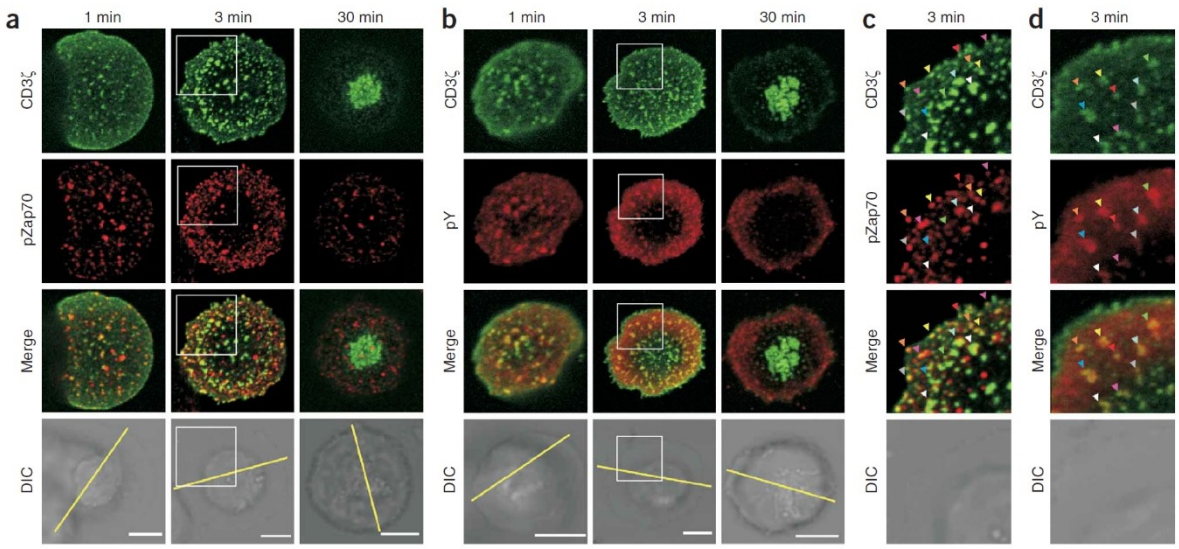

Figure 2: Phosphorylated ZAP-70 and tyrosin-phosphorylated proteins are localized only in CD3ζ clusters at the periphery of immunological synapse (**a,b**). Staining for pZAP-70 (**a**) or pY (**b**) Arrowheads indicated MCs containing both CD3ζ and ZAP-70 (**c**) or pY (**d**) (adopted from Yokosuka *et al*. 2005).

7.1 The role of the actin cytoskeleton in the formation and maintenance of SMACs

Grakoui *et al.* observed cessation of pMHC transport when T cells were treated with an actin inhibitor cytochalasin D suggesting that actin cytoskeleton is necessary for translocation of TCR – pMHC complexes to the center of the contact area (Grakoui *et al*., 1999).

TCR MCs are translocated to the cSMAC during contraction and mature IS maintenance stage (Yokosuka *et al*., 2005). The crucial provider of this centripetal translocation is retrograde F-actin flow (Ilani *et al*., 2009; Kaizuka *et al*., 2007). F-actin continuously polymerizes, which elongates filopodia and lamellipodia. Depolymerization of F-actin provides retrograde flow, that is used to centripetal translocation of TCR MCs and another molecules to the center of IS (Schaefer *et al*., 2008).

Demond *et al.* used TIRFM to observe kinetics of TCR MCs movement by centripetal F-actin retrograde flow. The velocity of F-actin retrograde flow was measured as 20 nm/s (Demond *et al.*, 2008). The velocity is likely dependent on the ability to bind to actin (Demond *et al*., 2008). Combination of TIRFM and FSM studies suggested that the velocity is likely affected by the mechanical binding of other molecules, such as talin to actin cytoskeleton (Hu *et al*., 2007; Jankowska *et al*., 2018).

High resolution imaging and analysis of spatial distribution of actin, TCR and LFA-1 revealed, that these molecules are organized into separate microdomains. During the activation, these molecules are translocated centripetally (Kaizuka *et al*., 2007). Although mechanism of translocation based on F-actin retrograde flow is universal for all these molecules, they are translocated separately (Hartman *et al*., 2009).

7.2 Myosin IIA regulates the IS structure

Non-muscular molecular motor myosin IIA has a regulatory role in the behavior of the IS. Myosin IIA controls the general organization and radial symmetry of the IS and regulates maximal cell spreading during the expansion phase and initiates the contraction phase during the IS development (Babich *et al*., 2012).

8 cSMAC function

8.1 Signaling

Firstly, it was proposed that cSMAC serves as a signaling center using high density accumulation of TCR MCs and pMHC to facilitate TCR – pMHC engagement. This area is supported by the LFA-1 – ICAM-1 ring which holds T-cell and APC in a tight apposition (Grakoui *et al*., 1999). Studies exploring kinetics of TCR – pMHC engagement revealed that high density of TCR and pMHC provides more often engagement with the same half-life of binding and facilitates TCR MCs phosphorylation by Lck (K. H. Lee *et al*., 2003).

However, later it was proposed that a crucial signalization takes place at the periphery of the IS, in the dSMAC. There are newly and continuously generated TCR MCs and sustained signalization events do not occurs in the cSMAC (Yokosuka *et al*., 2005). However, Cemerski *et al.* observed that the phosphorylation is present also in cSMAC but is hidden by downregulation of TCR. Experiments studying the effect of antigen quality revealed that cSMAC can enhance low agonist peptide signalization in later time points (Cemerski *et al*., 2008). Also in this case, as was previously suggested, signalization occurs in the first tens of minutes at the periphery of IS (K. H. Lee *et al*., 2002; Mossman *et al*., 2005).

8.2 Degradation

Another role has been proposed to cSMAC: cSMAC acts as a negative regulator of TCRs and balances signaling and degradation of TCR MCs (K. H. Lee *et al*., 2003). Lee *et al.* observed a faster degradation of those TCR MCs which induced strong phospohorylation. cSMAC uses this mechanism to protect T cell from overactivation (Lee *et al*., 2003). This is supported by the observation of activation signaling before a complete mature IS developed (K. H. Lee *et al*., 2002), suggesting that cSMAC is not necessary for T-cell activation (Yokosuka *et al*., 2005). Also Ca^{2+} signalization is independent on cSMAC, important is continuous TCR MCs generation at the periphery (Varma *et al*., 2006).

Accumulation of TCR MCs into cSMAC facilitates ubiquitination of these TCRs (Cemerski *et al*., 2007). This is supported by colocalization of molecules acting in the degradation pathway such a ubiquitin and LBPA (Vardhana *et al*., 2010; Varma *et al*., 2006). Moreover, in the cSMAC, it was observed invagination enriched with CD2 molecule suggesting that cSMAC acts as a place for resetting signaling molecules (Singleton *et al*., 2006). CD2 is the surface costimulatory and adhesive molecule (Yang *et al*., 2001). In the cSMAC are degraded only fully phosphorylated TCR MCs, others are recycled to the PM (Liu *et al*., 2000).

TCR MCs degradation in cSMAC is dependent on dynein and microtubule transport of TCR MCs to cSMAC (Hashimoto-Tane *et al*., 2011).

8.3 Co-stimulation

CD28 is the co-stimulatory receptor, its ligands are CD80 and CD86 molecules expressed on the APC surface (Rudd & Schneider, 2003; Sharpe & Freeman, 2002). Without CD28 activity T cells become anergic (Harding *et al*., 1992). CD28 contributes to the development of distinct cSMACs (Wülfing *et al*., 2002). CD28 co-stimulation is inhibited by the activity of CTLA-4 receptor, which has a higher affinity for CD80 and CD86 than CD28 (Greenwald *et al*., 2002). CTLA-4 remains in lysosomes under the PM from where it can be secreted (Iida *et al*., 2000) to $CD3^{lo}$ area. It leads to the inhibition of CD28 co-stimulation and dissociation of CD28-associated signaling molecules (Yokosuka *et al*., 2010).

CD28 clusters are formed immediately after T-cell – APC contact, a number of CD28 clusters increases during the expansion phase. The development of these clusters is dependent on CD80 on the APC. In mature IS, CD28 clusters are generated at the periphery and translocated to cSMAC after several minutes (Yokosuka *et al*., 2008).

Yokosuka *et al.* used TIRFM to observe mobility of co-stimulatory molecule CD28 during the IS development and signaling in the IS. This approach revealed that cSMAC consist of two domains with different amount of CD3 and CD28. Outer domain, $CD3^{lo}$ is highly dynamic and enriched with CD28 but has low amount of CD3. Inner domain, $CD3^{hi}$, is enriched with CD3, has low amount of CD28 and a low dynamics occurs here (Yokosuka *et al*., 2008).

8.4 Multifunctional cSMAC

Further roles for cSMAC were proposed. In the IS formed by cytotoxic T-lymphocytes two domains were recognized, secretory and signaling (Stinchcombe *et al*., 2001). Cytotoxic T-lymphocytes localize centrosome to the cSMAC area, contact PM of target cell and deliver lytic granules to target cells (Stinchcombe *et al*., 2006). Choudhuri *et al.* discovered that the center of the IS is extracellular cavity filed with extracellular microvesicles, which are enriched in TCRs. It was proposed that it may be a mechanism of communication between T-cell and APC (Choudhuri *et al*., 2014).

9 Diversity in immunological synapses

Previous chapters described classical bull-eye synapse (Grakoui *et al*., 1999; Monks *et al.*, 1998). However, ISs with different morphologies were observed at the contacts of some T cell subset with target cells.

9.1 Multifocal IS

Richie *et al.* found that thymocytes undertaking process of negative selection cannot form central cumulation of TCR neither early after contact with peptide nor several hours later (Richie *et al*., 2002). Double-positive thymocytes activated by a contact with the lipid bilayer enriched in pMHC and ICAM-1 did not form classical synapse, but formed several foci containing accumulated TCR – pMHC and excluding LFA-1-ICAM-1. ICAM-1 covers rest of the contact area. Such IS structure was termed as a multifocal IS (Hailman *et al*., 2002).

Lee *et al.* proposed that thymocytes forms multifocal IS as a result of low TCR expression on their surface (S. E. Lee *et al.*, 2003).

Further, Th1 and Th2 may not form classical IS under certain conditions. Th1 are able to form classical IS but Th2 cells stimulated with a high antigen density develop a multifocal IS. When stimulated with low antigen density, they develop a compact central TCRs but cannot exclude ICAM-1 from the center (Thauland *et al*., 2008).

Multifocal synapse is predominant synapse between T cells and dendritic cells, specifically between dendritic cells and naïve $CD4^+$ or $CD8^+$ T cells and activated $CD4^+$ T cells (Thauland & Parker, 2010). Dendritic cells can form IS similar to a classical IS but cannot form full LFA-1 ring around the accumulated TCRs MCs (Brossard *et al.*, 2005).

9.2 Immunological kinapses

Sims *et al.* observed periodically disrupted and reassociated ISs during T cell priming. Protein-kinase Cθ in pSMAC can lead to IS disruption and symmetric reassembly is regulated by WASp. After the disruption of the IS, it is relocated and reassembled. LFA-1 – ICAM-1 complexes are organized to the lamella. Lamella lies towards the uropod which is enriched in TCRs (Sims *et al*., 2007).

Immunological kinapses were observed on $CD4^+$ cytotoxic T-lymphocytes, Th cells, anergized Th1 cells and on T cells that are able to recognize antagonist pMHC (Thauland & Parker, 2010).

10 T cell microvilli

A new look at the process of antigen recognition, T cell activation and IS formation between T cell and APC provides PM analysis with respect to its 3D topography. This approach can recognize and demonstrate an arrangement of TCRs and signaling and adhesive molecules on the 3D surface of T cell. This allows us to better understand the spatial aspects of the PM contact with the target cell, recognition of antigens and the processes, which lead to T-cell activation.

Microvilli are actin-dependent membrane protrusions. Experiments using T cells treated with latrunculin A revealed that actin polymerization in microvilli is highly dynamic. Microvillar density obtained by SEM in 2D topography was 2 – 4 microvilli/μm^2 (Majstoravich *et al*., 2004). By 3D SEM is the length of the T cell microvilli from 0.1 μm to few micrometers, diameter is 70 – 100 nm (Jung *et al*., 2016).

Microvilli are mobile structures, which exhibit undulating movement and lateral movement with velocity 5.2 μm/min. This movement serves to scanning the APC surface (Cai *et al*., 2017). The density of the microvilli remains unchanged both in the IS area and in the rest of the cell surface, however, the dwell time is different. In the IS area, the dwell time is circa 8.9 s, in the contact area is dwell time circa 7.69 s and without antigen stimulation 3 – 6 s (Cai et al., 2017).

Jung *et al.* used a combination of variable-angle-TIRFM and SMLM to gain 3D topographical map of microvilli with the localization of TCR, CD44 and CD45. The results suggested that areas with high molecular density of TCR are microvilli tips on the 3D map (Jung *et al.*, 2016). These results are supported by SIM imaging of T cells interacting with lipid bilayer, TCR MCs were closer to the bilayer compared to the mean of all molecules (Cai *et al*., 2017). TCR MCs were found only on microvilli tips. CD44 molecules were localized to the base of microvilli and CD45 molecules were uniformly dispersed across PM (Jung *et al*., 2016). Razvag *et al.* used a combination of PALM and direct-STORM to observe localization of TCR and CD45 clusters and atomic force microscopy to observe the contact sites of microvilli and PM. These experiments revealed segregated localization of CD45 and TCR clusters and spatial role for a close positioning of these MCs in the early T cell response (Razvag *et a*l., 2018).

Ghosh *et al.* studied localization of signaling and adapter molecules on the PM. On the microvilli were found coreceptors CD2 and CD4, kinase Lck, and LAT. Further,

connection between TCR and actin cytoskeleton was probed. It was revealed that TCRs are connected with microvilli *via* proteins erzin, radixin and moesin from the ERM family (Ghosh et al., 2020).

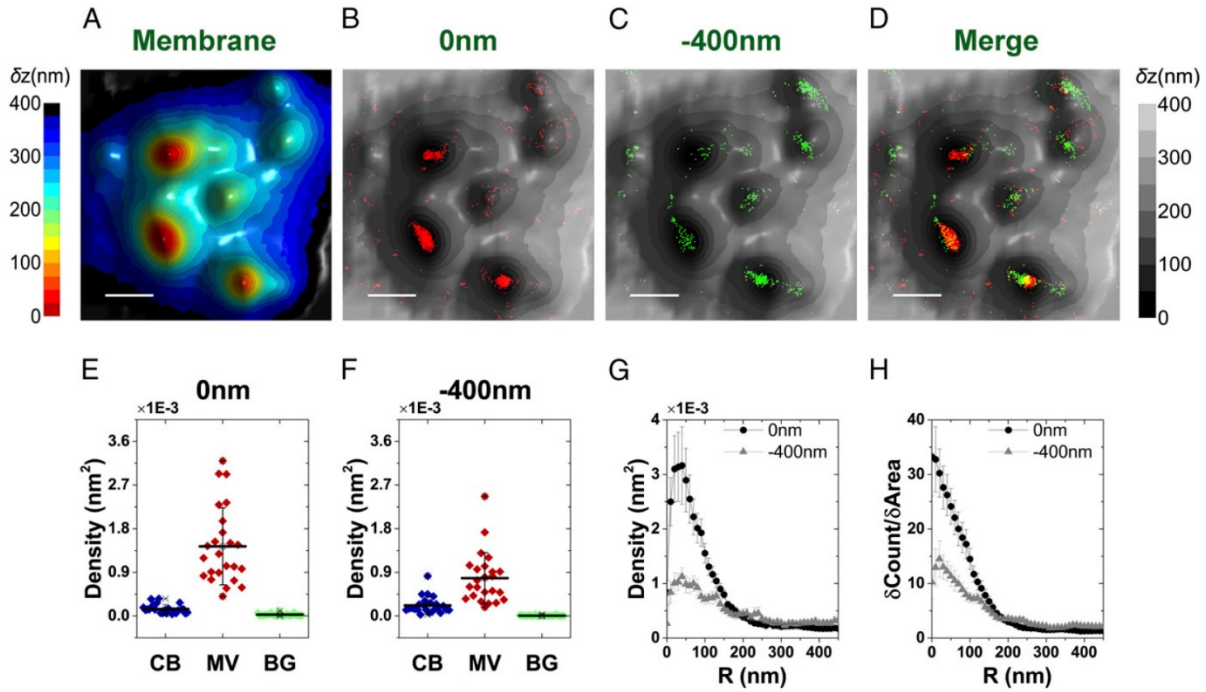

Figure 3: Mapping the distribution of αβTCR molecules in relation to the 3D surface topography of effector T cells. (**A**) Representative 3D surface reconstruction map of a human T-cell membrane obtained from VA-TIRFM measurements. (**B-D**) Localization maps (red dots: 0 nm; green dots: −400 nm) of αβTCR molecules on the cell from A, overlaid with the 3D surface reconstruction map (gray scale). (**E** and **F**) The densities of the αβTCR localizations within the area of cell body (CB) (blue), microvilli (MV) (wine), and background (BG) (green). (**G**) Density plot of αβTCR positions as a function of distance from microvilli tips. (**H**) Cumulative increase of the fraction of total molecules on each cell as a function of the distance from the tips, normalized by the cumulative increase in the fraction of area. (adopted from Jung *et al*. 2016)

11 Conclusion

An antigen specific encounter of T cell and APC triggers clustering of TCRs and adhesive and signaling molecules to MCs. It leads to the formation of the IS. The classical IS was observed as a central accumulation of TCRs termed as cSMAC surrounded by LFA-1 accumulation termed pSMAC and dSMAC at the IS periphery. Some subsets of T cells create morphologically different ISs such a multifocal IS and the immunological kinapse.

It was proposed that cSMAC plays a key role in the T cell signaling. However, molecular tracing experiments revealed that signaling occurs only in the periphery of mature IS and TCRs are then translocated by actin and microtubules to cSMAC for degradation. Thus, cSMAC plays a role in a negative signaling, regulation and in co-stimulation *via* CD28. Some T-cell subsets can use the cSMAC as a secretory site.

Recently developed microscopic methods allow to observe T cells with a high-resolution imaging without damaging the observed T cells. These methods obtained a 3D topographic map of the T-cell surface together with the location of target molecules. It was revealed, that TCRs and some signaling molecules localize preferentially to microvilli, which has a crucial role in triggering T-cell signaling.

12 Literature

Secondary citations are marked with *

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