Biochimica et Biophysica Acta 1853 (2015) 802–809

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Nanoclusters of the resting T cell antigen receptor (TCR) localize to non-raft domains ☆

Katharina Beck-García [a,b,c,d], Esmeralda Beck-García [a,c,d,e], Sheila Bohler [a,d], Carina Zorzin [a,c], Erdinc Sezgin [f], Ilya Levental [g], Balbino Alarcón [h], Wolfgang W.A. Schamel [a,b,c,d,*]

[a] Department of Molecular Immunology, Faculty of Biology, BIOSS Center for Biological Signalling Studies, Freiburg, Germany
[b] Spemann Graduate School of Biology and Medicine (SGBM), Freiburg, Germany
[c] Centre for Chronic Immunodeficiency CCI, Albert-Ludwigs-University Freiburg, Germany
[d] Max Planck-Institute of Immunobiology and Epigenetics, Freiburg, Germany
[e] International Max Planck Research School for Molecular and Cellular Biology (IMPRS-MCB), Freiburg, Germany
[f] Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
[g] Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, USA
[h] Centro de Biología Molecular Severo Ochoa, CSIC-Universidad Autónoma de Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 1 August 2014
Received in revised form 4 December 2014
Accepted 15 December 2014
Available online 20 December 2014

Keywords:
TCR
Nanoclustering
Membrane
Lipid
Cholesterol

ABSTRACT

In the last decade an increasing number of plasma membrane (PM) proteins have been shown to be non-randomly distributed but instead forming submicron-sized oligomers called nanoclusters. Nanoclusters exist independently of the ligand-bound state of the receptors and their existence implies a high degree of lateral organisation of the PM and its proteins. The mechanisms that drive receptor nanoclustering are largely unknown. One well-defined example of a transmembrane receptor that forms nanoclusters is the T cell antigen receptor (TCR), a multisubunit protein complex whose nanoclustering influences its activity. Membrane lipids, namely cholesterol and sphingomyelin, have been shown to contribute to TCR nanoclustering. However, the identity of the membrane microdomain in which the TCR resides remains controversial. Using a GFP-labeled TCR we show here that the resting TCR localized in the disordered domain of giant PM vesicles (GPMVs) and PM spheres (PMSs) and that single and nanoclustered TCRs are found in the high-density fractions in sucrose gradients. Both findings are indicative of non-raft localization. We discuss possible mechanisms of TCR nanoclustering in T cells. This article is part of a Special Issue entitled: Nanoscale membrane organisation and signalling.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

T cells are a crucial component of the adaptive immune system. They are activated when the T cell antigen receptor (TCR) recognizes foreign antigens, i.e. foreign peptides presented on MHC molecules (peptide-MHC). The TCR consists of six transmembrane (TM) proteins that assemble in dimers: the antigen-binding TCRαβ dimer and the signal-transducing CD3γε, CD3δε and ζζ dimers to form a single complex [1,2]. However, the stoichiometry of the minimal TCR complex is still not resolved [3]. Multivalent binding of peptide-MHC or anti-TCR antibodies to TCRαβ results in a change in the conformation of the CD3 and ζ subunits, called CD3 conformational change (CD3CC) [4,5]. The CD3CC leads to an opening of the proline-rich region in the cytoplasmic tail of CD3ε, enabling the adaptor protein Nck to bind to this region using its first SH3 domain [4]. Induction of the CD3CC is required for the phosphorylation of the CD3 and ζ subunits, and thus for the activation of the T cell [6–8]. Other models of antigen-induced TCR phosphorylation have also been proposed [9]. In this article, we will focus on the pre-assembly of TCRs on the cell surface that occurs independently and prior to antigen-binding. In order to distinguish these pre-clusters from antigen- and signaling-induced microclusters [10,11], they were called TCR nanoclusters [3,12].

1.1. The TCR forms nanoclusters

On the PM of T cells, single TCR complexes [13] and nanoclustered TCRs (multimers of the single TCRs) coexist independently of TCR activation (Fig. 1A). A wide variety of techniques have been used to demonstrate the organization of the TCR in nanoclusters: Blue Native PAGE (BN-PAGE), immuno-gold electron microscopy (EM) staining of the cell surface TCR on fixed cells [14], immuno-gold EM staining of the TCR cytoplasmic tails, high speed photoactivated localization microscopy (PALM) [15], in single and double-colour [16], staining with

☆ This article is part of a Special Issue entitled: Nanoscale membrane organisation and signalling.

* Corresponding author at: Albert-Ludwigs-University Freiburg, BIOSS Center for Biological Signalling Studies, Schänzlestraße 18, 79104 Freiburg, Germany. Tel.: +49 761 203 67511.
E-mail address: wolfgang.schamel@biologie.uni-freiburg.de (W.W.A. Schamel).

http://dx.doi.org/10.1016/j.bbamcr.2014.12.017
0167-4889/© 2014 Elsevier B.V. All rights reserved.

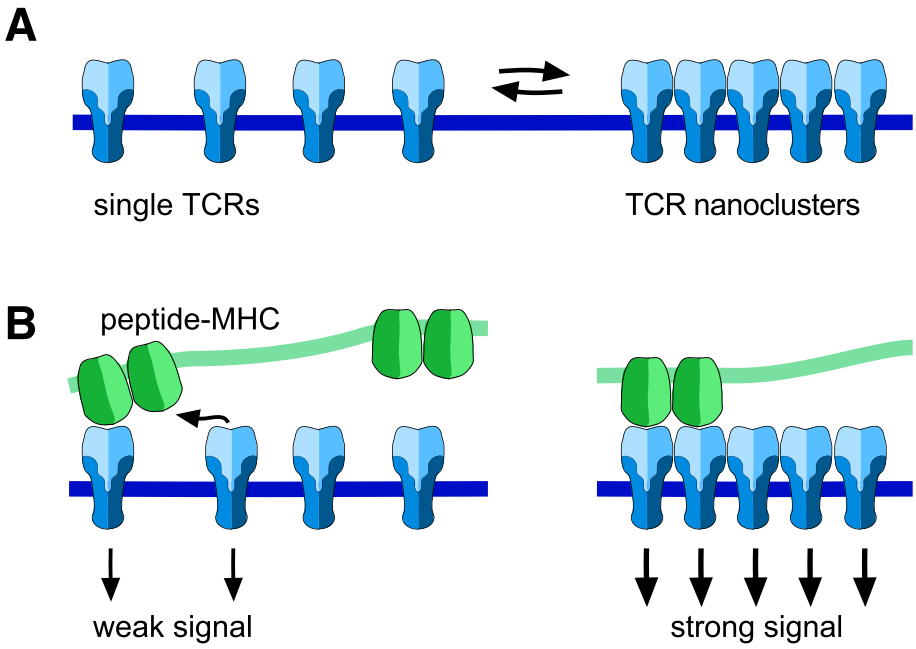

Fig. 1. The TCR co-exists as single and nanoclustered complexes. (A) Single TCRs of a possible αβγεδεζζ or γεαβζζαβδε stoichiometry can form nanoclusters which contain up to 20 TCRs. The arrows indicate the dynamics of these associations. (B) The single TCRs possess low avidity towards multimeric peptide-MHC and only those TCRs are activated that bind to the antigen (left). The TCR nanoclusters possess high avidity towards multimeric antigens and all TCRs within a cluster are activated even when only two TCRs are antigen-bound. This renders the nanoclusters highly responsive even to low concentrations of antigen.

quantum dots [17] and by dual-color fluorescence cross-correlation spectroscopy (FCS) [15]. An in-depth discussion of this data can be found in recent reviews [3,18] and in the accompanying reviews in this issue. Interestingly, different techniques show that the TCR oligomerizes to reach a maximum size of ~20 TCRs per nanocluster [14–16]. To date the dynamics of the TCRs in nanoclusters is not known. Thus, it could be that individual TCRs rapidly exchange between the nanoclustered pool and the "single TCR" pool (Fig. 1A).

The degree of TCR nanoclustering differs among different T cell lines and T cell populations. This was first noted when the sizes of a murine Vα17Vβ16 TCR were studied using BN-PAGE [19]. The original T cell clone contained mostly single TCRs; a hybridoma of this clone using the fusion line BW5147$\alpha^{-}\beta^{-}$ contained a substantial proportion of nanoclustered TCRs; and a transfectant in which the Vα17 and Vβ16 chains were expressed in the TCR$\alpha\beta^{-}$ cells 54ζ17 contained mostly TCR nanoclusters. Later we found that naïve T cells express mostly small-sized TCR oligomers and that the nanoclustering is strongly enhanced in antigen-experienced T cells [12]. Thus, T cell blasts and memory T cells express highly nanoclustered TCRs.

Interestingly, the disparity in TCR nanoclustering among different T cells can help to explain its functional consequences. It was shown that the large TCR nanoclusters are preferentially tyrosine phosphorylated in response to low concentrations of antigen [14] (Fig. 1B). This preferential phosphorylation could be due to a higher avidity of large TCR nanoclusters towards a multimeric peptide-MHC [20] and/or to the existence of cooperativity phenomena within nanoclusters [6]. In this regard, we proposed that dimeric pMHC-binding to as few as two TCRs in a nanocluster caused all TCRs within the nanoclusters to undergo the CD3CC and adopt the active conformation, independent of their binding to a cognate pMHC [6,12] (Fig. 1B). Implications of these findings were extensively discussed before [3,18] and could be behind the higher sensitivity of effector and memory T cells (high degree of nanoclustering) compared to naïve T cells (low degree of nanoclustering) [21–23].

1.2. Membrane lipids are involved in forming TCR nanoclusters

Since T cells can regulate the degree of TCR nanoclustering and thereby the sensitivity of the T cells towards antigenic stimulation [3,12], it is crucial to understand the molecular mechanism(s) that regulate TCR nanoclustering.

Initially, we noticed that detergents that sequester cholesterol, such as digitonin, disassembled the TCR nanoclusters to single TCRs as detected by BN-PAGE [14,24,25]. In contrast, detergents that do not extract cholesterol, such as Brij96, kept the TCR nanoclusters intact. This motivated us to modulate cholesterol levels in intact cells by using methyl-β-cyclodextrin (MβCD), which extracts cholesterol from membranes. In fact, most TCR nanoclusters were disrupted by MβCD treatment as seen by BN-PAGE and immuno-gold EM [14,20]. The effect of MβCD could be reverted by adding cholesterol, clearly indicating that cholesterol is required for TCR nanoclustering [20]. The cholesterol-based TCR nanoclustering is of functional relevance, since MβCD- and cholesterol-treatments modulate the avidity of the TCR for pMHC tetramers [20,26,27].

In 1997 it was proposed that the membrane can be divided into lipid rafts and non-rafts [28]. Rafts are enriched in cholesterol and glycosphingolipids whereas non-rafts mostly contain unsaturated phospholipids. The data to support the "raft hypothesis" came from detergent extractions of cells with detergent and then testing whether a protein or lipid is present in the detergent-resistant membrane (DRM) fraction, initially synonymous with rafts, or not. However, DRMs should not automatically be equated with rafts in intact membranes. For a deeper discussion on this issue we would like to refer to a recent review [29].

In order to understand how cholesterol contributes to TCR nanoclustering, it is crucial to determine in which PM microdomains the single and the nanoclustered TCRs are located. The localization of the resting TCR in PM microdomains is controversially discussed in the literature. On the one hand, it was suggested that the resting TCR is localized in non-raft membranes, since it was not present in detergent-resistant membrane (DRM) fractions [30,31]. Likewise it

was concluded that the resting TCR is present in non-rafts in plasma membrane fragments of T cells [32], on the PM surface [33], or in giant unilamellar vesicles (GUVs) [20]. On the other hand, using a different DRM extraction protocol as before, it was found that the resting TCR is localized in a subset of the DRM fractions [34]. Furthermore, the TCR colocalized with the ganglioside raft-marker GM1 on the resting T cell after GM1 clustering [35,36] and the authors suggest that this is indicative of raft localization in the resting state. Thus, the aim of this study was to clarify whether the TCR is in the non-raft or the raft subdomain of the native PM. To this end we used here two physiological model systems, the giant plasma membrane vesicle (GPMV) and the plasma membrane sphere (PMS), to study the localization of the resting TCR.

1.3. Giant PM vesicles (GPMVs) and PM spheres (PMSs)

Lipid model systems like GUVs, GPMVs and PMSs segregate into a liquid-disordered (l_d, corresponding to the non-raft domain) and a liquid-ordered (l_o, corresponding to the raft domain) phase enriched in unsaturated and saturated fatty acids, respectively [37,38]. We previously reported that the TCR localized in the l_d domain of GUVs [20]. However, the biological significance of GUV experiments is limited due to the compositional simplicity of the lipid mixture and the exclusion of TM proteins from the l_o phase [39–41]. By contrast, GPMVs and PMSs contain a physiological sampling of lipids [42,43] and some TM proteins in the ordered phase [43–46], thus comprising a powerful and up-to-date best model system to study TM protein partitioning between coexisting liquid phases in a biological membrane.

2. Materials and methods

2.1. Reagents

The following antibodies were used: anti-CD3ε (M20ε, Santa Cruz Biotechnology), anti-ζ antiserum 448 [47], anti-mouse TCRβ (H57-597, Abcam) and secondary antibodies for Western blot (WB).

2.2. Vectors and cells

PMiDsRed2_mζ was generated by amplifying mouse ζ (mζ) by PCR from pcDNA3_mζ-SBP [20] and cloned at the XhoI/MfeI site in pMiDsRed2 (provided by S. Herzog). In parallel, GFP was amplified from pMiG and fused to SBP by PCR. The GFP-SBP PCR product was cloned into the MfeI/BamHI site of pMiDsRed2_mζ yielding the final plasmid pMiDsRed2_mζ-GFP-SBP. This plasmid was transfected into the mouse 2B4-derived ζ-deficient line MA5.8 to yield the mouse T cell line M.mζ-GFP-SBP which was then used for the experiments in Fig. 2. The M.mζ-SBP/mζ-GFP cell line had been described previously [20] and was used for the experiments in Fig. 3. The GFP-GPI expressing RBL-2H3 cells were also described [45]. Furthermore, the human T cell line Jurkat was used. All cells were cultured in complete RPMI-1640 media supplemented with 5% fetal calf serum.

2.3. Cell lysis and WB

Five to thirty million cells were lysed in 1 ml lysis buffer containing 20 mM TrisHCl (pH 8), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1× protease inhibitor cocktail (Sigma #P2714), 1 mM PMSF, 5 mM iodoacetamide, 0.5 mM sodium orthovanadate, 1 mM NaF and Brij96V or digitonin as indicated. SBP-tagged TCR was eluted from streptavidin beads with 4 mM biotin for 30 min at 4 °C. BN-PAGE was done as before [48].

2.4. Preparation of GPMVs and determination of the partition coefficient ($K_{p,\ raft}$)

To induce GPMV formation, cell blebbing was induced as described [42,44] with either 25 mM paraformaldehyde (PFA) and 2 mM dithiothreitol (DTT) or 2 mM N-ethylmalemide (NEM) for 4–6 hours at 37 °C in a Ca^{2+}-containing buffer (150 mM NaCl, 10 mM Hepes and 2 mM $CaCl_2$, pH 7.4). GPMVs were recorded with the confocal microscope LSM780 from Zeiss and quantified with the ZEN2010 software as reported [45]. $K_{p,\ raft}$ ($K_{p,\ raft} = int_{raft}/int_{non\text{-}raft}$) was determined by line scans through the l_o and the l_d domain, and the resulting maximal intensities were divided by each other. Imaging was done at 10 °C for the PFA/DTT method and at 4 °C for the NEM method, since there is no observable microscopic phase separation above these temperatures.

2.5. Preparation of plasma membrane spheres (PMSs)

PMSs were prepared as described previously [43]. Briefly, the cells were incubated in PMS Buffer (1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5 mM Hepes, 1 mg/ml glucose in 1x PBS (pH 7.4) for 4 hours at 37 °C. Then

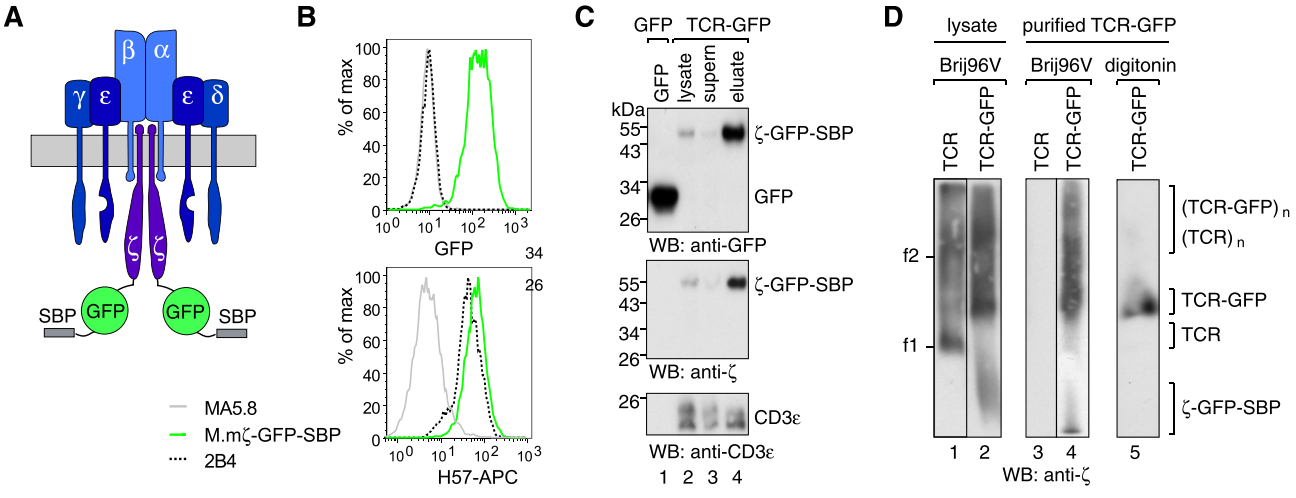

Fig. 2. The TCR-GFP-SBP forms nanoclusters. (A) Schematic representation of the TCR-GFP-SBP. (B) The parental MA5.8, M.mζ-GFP-SBP and 2B4 T cells were stained with an APC-coupled anti-TCRβ (H57-597) and analyzed by flow cytometry. Histograms of the GFP and the APC fluorescence intensity are shown. (C) After lysis of the M.mζ-GFP-SBP cells in 0.5% Brij96V, the TCR-GFP-SBP was purified by a streptavidin PD and after washing eluted with 4 mM biotin. Proteins of the lysate, the depleted lysate after the PD (supern.) and the eluate were separated by reducing SDS-PAGE. Purifed GFP was also analyzed (lane 1). WB was performed using anti-GFP, anti-ζ and anti-CD3ε antibodies. (D) 2B4 (TCR) and M.mζ-GFP-SBP cells (TCR-GFP-SBP) were lysed with either 0.5% Brij96V or 1% digitonin. Cell lysates were dialyzed against BN lysis buffer and separated by BN-PAGE (lanes 1, 2). Alternatively, the TCR-GFP-SBP was purified as in (C) and separated by BN-PAGE (lanes 3–5). WB was performed using anti-ζ antibodies. The marker protein was ferritin in its 24-mer and 48-mer forms (f1, 440 kDa; f2, 880 kDa).

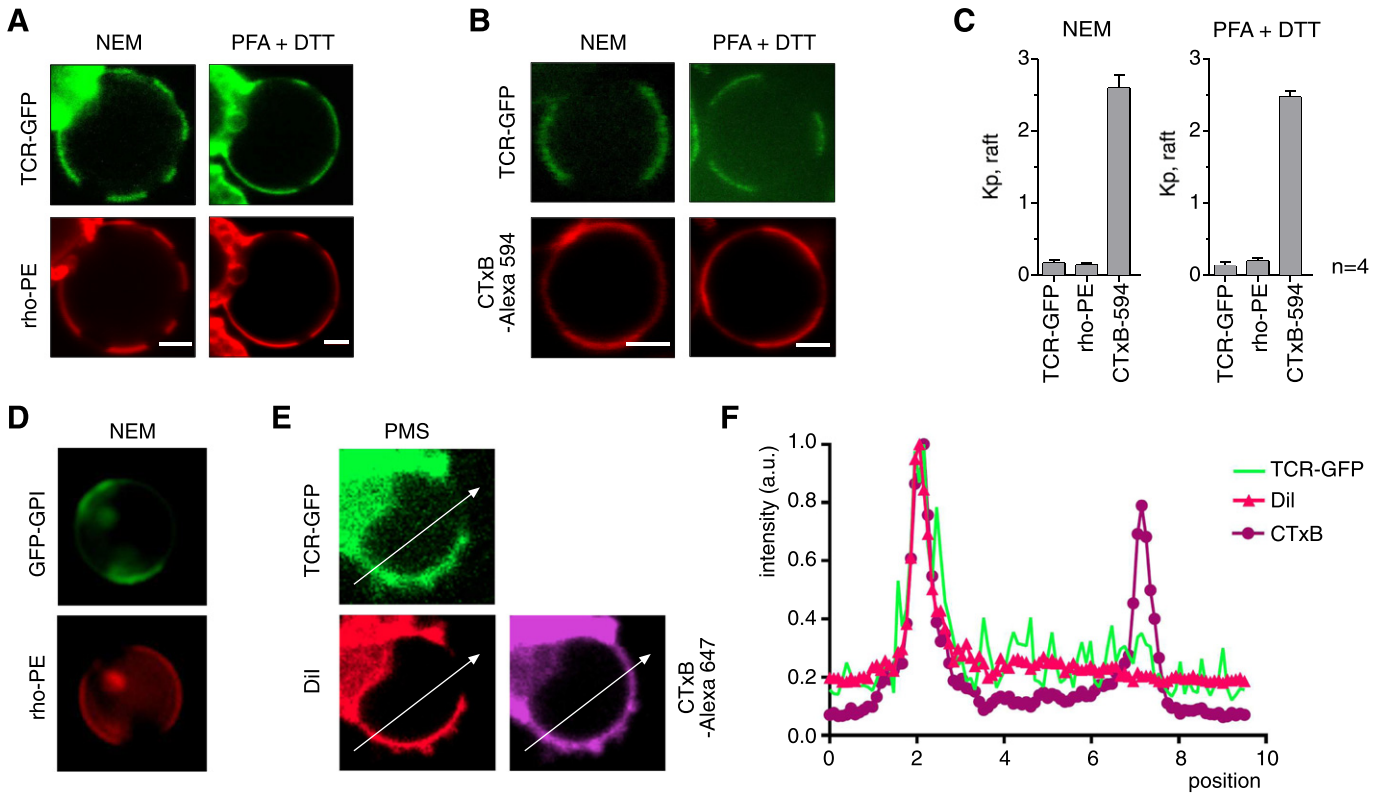

Fig. 3. The TCR-GFP localizes in the l_d domain of GPMVs and PMS. GPMVs from M.mζ-SBP/mζ-GFP cells were induced with NEM or PFA plus DTT and recorded with a confocal microscope. The white scale bars represent 3 μm. The fluorescence of the TCR-GFP is represented in green (A, B), the one of rho-PE (A) and CTxB-Alexa 594 (B) in red. (C) The partition coefficients $K_{p,\,raft}$ of the TCR-GFP-SBP, rho-PE and CTxB-Alexa 594 were determined. (D) GPMVs from GFP-GPI expressing cells were induced with NEM and recorded with a confocal microscope. The fluorescence of GFP-GPI is displayed in green and the one of rho-PE in red. (E) PMSs were prepared from M.mζ-SBP/mζ-GFP cells, labeled with the non-raft probe, Fast DiI, and CTxB-Alexa 647 and recorded by confocal microscopy. The TCR-GFP is shown in green, Fast DiI in red and CTxB in deep purple. (F) Line scans as indicated in (E) were taken and the intensity profiles are shown for the TCR-GFP, Fast DiI and CTxB.

the cells were labeled with the non-raft probe, Fast DiI, and cholera toxin subunit B for 5 minutes at room temperature. Afterwards, PMSs were recorded by confocal microscopy at room temperature.

3. Results

3.1. The TCR-GFP forms nanoclusters

To study the localization of the TCR in GPMVs, a mouse T cell line expressing a chimeric ζ chain comprising full length mouse ζ fused to GFP and a streptavidin-binding peptide (SBP) was established (Fig. 2A). GFP was used for the localization studies, and SBP for TCR purification. To this end, the mouse 2B4-derived ζ-deficient cell line MA5.8 was transduced with a plasmid encoding for murine ζ-GFP-SBP, resulting in the M.mζ-GFP-SBP cell line. The expression of ζ-GFP-SBP was verified by flow cytometry (Fig. 2B, top panel). The expression of ζ-GFP-SBP allowed the TCR to assemble completely (TCR-GFP), as demonstrated by reconstitution of TCR surface expression in the TCR-negative MA5.8 cell line (Fig. 2B, lower panel). Next, we directly examined the integrity of the TCR complex. M.mζ-GFP-SBP cells were lysed and ζ-GFP-SBP was purified by pull-down with streptavidin-coupled beads. After elution with biotin, an aliquot of the lysate, the depleted supernantant obtained after pull-down, and the biotin eluate were analyzed by SDS-PAGE and WB. The ζ-GFP-SBP chimera was purified efficiently and was intact as indicated by the appearance of a signal at the expected molecular weight of 44 kDa in the anti-GFP and the anti-ζ WB (Fig. 2C, lanes 2–4), which was 18 kDa higher than purified GFP (lane 1). Purification of ζ-GFP-SBP led to the copurification of CD3ε (lane 4). Since ζ and CD3 co-purify only in the complete TCR complex [1], these results showed that ζ-GFP-SBP is integrated in a fully assembled TCR.

To test whether the ζ-GFP-SBP-containing TCR (TCR-GFP) can form nanoclusters, a BN-PAGE analysis was conducted (Fig. 2D). Wild-type 2B4 T cells or M.mζ-GFP-SBP cells (containing TCR-GFP) were lysed in the detergent Brij96V (lanes 1–4), which keeps the TCR nanoclusters intact, or digitonin (lane 5), which disrupts TCR nanoclusters [14,24]. Then, the cell lysates (lanes 1 and 2) and streptavidin-purified material (lanes 3-5) were separated by BN-PAGE and analyzed by anti-ζ WB. In the presence of Brij96V, single TCRs and nanoclustered complexes were formed by the TCR-GFP (lanes 2, 4), which were larger in size than the complexes formed by the wild-type TCR (lane 1). As expected, only the tagged TCR was purified with streptavidin beads (lane 4) whereas the wild-type TCR was not (lane 3). In the presence of digitonin (lane 5), only single TCR-GFP (and no nanoclusters) were detected. Small amounts of non-assembled ζ-GFP-SBP were also detected.

In conclusion, single TCRs and nanoclustered complexes were formed by the TCR-GFP, resembling the wild type TCR.

3.2. The TCR is localized in the disordered phase of GPMVs and PMSs

GPMVs are cytoskeleton-free plasma membrane vesicles, which maintain the protein and lipid diversity of native membranes, and therefore present a coherent model system to determine protein localization in domains of biological membranes by confocal microscopy [49]. To study the partitioning of the TCR in GPMVs, two distinct methods were applied to induce formation of the GPMVs: one was based on N-ethylmaleimide (NEM) and the other on paraformaldehyde (PFA) plus dithiothreitol (DTT). The TCR-GFP colocalized with rhodamine-PE (rho-PE), a lipid dye that strongly partitions to the non-raft phase (Fig. 3A). In contrast, no overlap was observed between the GFP-TCR and the raft marker, fluorescently-labeled cholera toxin B

subunit (CTxB-Alexa 594) (Fig. 3B). The partitioning coefficients (K_p) for TCR-GFP-SBP, rho-PE, and CTxB were determined (Fig. 3C). The K_p of TCR-GFP-SBP is similar to that of rho-PE, and very different to that of CTxB. Hence, the TCR-GFP-SBP partitioned to the non-raft domain of GPMVs. As a control, GFP linked to glycosylphosphatidylinositol (GFP-GPI) was enriched in the raft domain (Fig. 3D), as expected [45].

These data were corroborated by the use of plasma membrane spheres (PMS) [43–46]. We observed that the TCR-GFP partitioned into the Fast DiI-rich phase, which corresponds to the disordered phase, but is not enriched in the CTxB-positive phase (Fig. 3E and F). Together these findings support the idea that the TCR is preferably found in the non-raft phase in T cells.

3.3. Both single and nanoclustered TCRs are in detergent-soluble membrane fractions

Previous studies observed that the resting TCR is present in the soluble protein fractions after DRM preparations [30,31]. However, these studies did not differentiate between the single and the nanoclustered TCR. Here, we made use of the characteristic of lipid rafts to float on a sucrose gradient when lysed on ice with a polyoxyethylene detergent [28,50–52]. To investigate whether both TCR forms localize to the same membrane domain, we lysed Jurkat T cells in 0.75% Brij96V (a polyoxyethylene detergent) on ice and performed a sucrose gradient centrifugation to separate the DRM fraction from the soluble protein fraction. After centrifugation, individual fractions were separated by BN-PAGE (Fig. 4). Single TCRs and nanoclusters were both exclusively found in the fractions corresponding to soluble protein (lanes 10–12, upper panel), whereas the control molecule GM1 was, as expected, specifically found in the DRM fractions (lanes 2–6, lower panel).

4. Discussion

4.1. All TCR forms are localized to the non-raft domain

In this work we show that the resting TCR is localized in the disordered/non-raft phase of physiological plasma membrane mixtures.

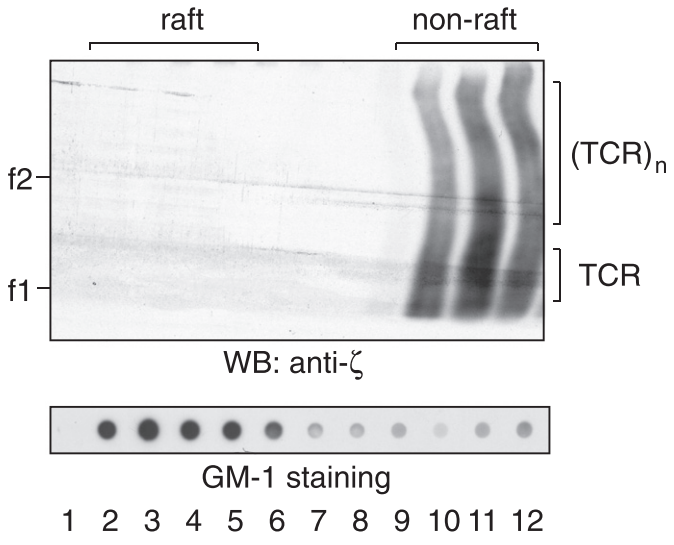

Fig. 4. The single and nanoclustered TCR is present in non-raft membranes. To separate detergent resistant from soluble membranes, Jurkat T cells, expressing a wild type TCR, were lysed in ice cold 0.75% Brij96V and a sucrose gradient centrifugation was conducted at 0 °C. The individual fractions (1 to 12, from top to bottom of the gradient) were analyzed by BN-PAGE followed by WB using anti-ζ antibodies (top panel). With the same fractions a dot blot was prepared and the cholera toxin B subunit (CTxB) was used to detect the lipid raft marker ganglioside GM1.

To do so we used state-of-the-art cell biology techniques, such as the generation of GPMVs and PMSs, demonstrating that the GFP-tagged TCR co-localizes with the rhodamine-PE and Fast DiI-rich phases, and not with the CTxB-stainable phase. This observation is consistent with recent studies demonstrating that the resting TCR is located in the l_d phase of GUVs [20]. Since the GUVs were composed of few defined lipids the physiological relevance of the data was not clear.

Non-raft localization of the TCR is in line with the finding that GPI-anchored proteins, which are constitutively present in raft domains, were not concentrated in TCR domains [33] and that TCR immunoisolates are not enriched in the raft-marker GM1 [32]. In contrast to these findings, it was reported that the TCR colocalized with GM1 in resting T cells [35,36]. In these experiments, GM1 was stained with CTxB, which predominantly binds to GM1, but also to other sugar structures with terminal galactose [53]. Importantly, binding of CTxB to T cells influenced T cell signaling [54–56]. Thus, Janes et al. might have studied a partially activated rather than a resting T cell. Further, the apparent colocalization of GM1 and the TCR might be due to convolutions of the PM at these sites [33]. Together, with these data, our results suggest that the resting TCR is localized in the non-raft microdomains phase in natural lipid mixtures and in defined lipid compositions. Consistent with our findings, the lipid environment around the TCR is not condensed in the resting state [57], and unsaturated fatty acid levels are elevated in the vicinity of the TCR, as shown by lipid mass spectrometry of TCR immunoisolates [58].

Finally, we found that both the single and nanoclustered TCRs were present in the detergent soluble membranes fractions when Jurkat cells were lysed in Brij96V at 0 °C. This detergent was chosen because it keeps the nanoclustered TCRs intact [14,24] and at the same time does not solubilize detergent-resistant membranes, as shown with Brij98V [34]. Since the TCR (and especially the nanoclustered TCR) binds to cholesterol and most likely also to sphingomyelin [20], and since cholesterol and sphingomyelin are a crucial component of lipid rafts, it is remarkable that all TCRs were found in the detergent soluble membranes (Fig. 4) and likewise localized to the non-raft domains (Fig. 4). In the following paragraphs we aim to formulate molecular mechanisms that might contribute to TCR nanoclustering.

4.2. Protein-based TCR nanoclustering

For a number of TM (and soluble) proteins it has been shown that homotypic protein–protein interactions are involved in forming oligomeric assemblies. For example, self-association of syntaxin 1 relies on weak protein–protein interactions, forming nanoclusters of approximately 60 nm containing around 75 syntaxin molecules [59]. Certain protein domains in the extracellular region of TNF receptor family members have been identified to self-associate to form stable trimers [60–62]. Similarly, protein–protein interactions between ectodomains of the erythropoietin receptor (EPOR) cause stable receptor dimerization [63,64]. In case of the TCR, we found by using immuno-gold EM that TCRs often have a distance of 10 nm in the nanoclusters [12,14,65]. Since TCRs have a diameter of approximately 10 nm [66], TCRs might be in direct contact to each other, allowing for direct protein–protein interactions. Possible subunits to be involved have been identified: TCRα and ζ. When the ectodomains of TCRα and TCRβ linked to the TM and cytoplasmic regions of the EPOR were expressed, at least two TCRαβ interacted [67]. The responsible regions were the C and F strands as well as the AB loop of the constant immunoglobulin domain of TCRα (the Cα domain, Fig. 5A). Concerning ζ, we showed that a mutation in the TM region (L19A) in the context of a complete TCR, significantly reduced the formation of TCR nanoclusters [12] (Fig. 5B). However, whether leucine 19 of ζ is involved in protein–protein interactions within the membrane, or in binding to lipids that mediate the clustering (see below) is not known.

Biochemically we did not detect any other protein that would have been part of the TCR nanoclusters in stoichiometric amounts [14]. However, we cannot exclude that an additional protein (such as a scaffold

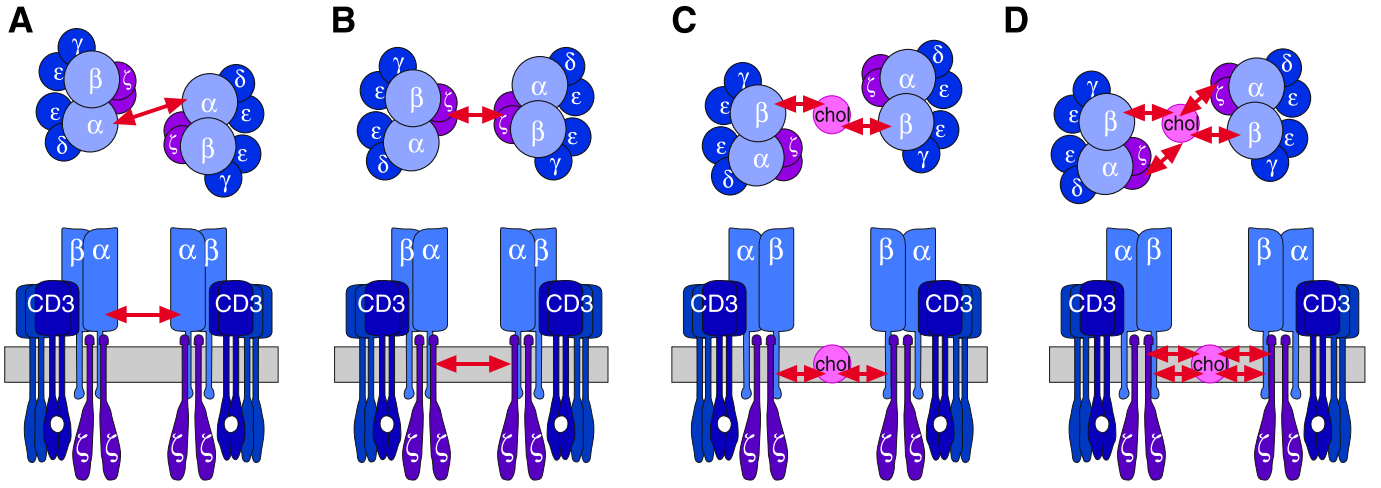

Fig. 5. Mechanisms to control the TCR-TCR interaction. The TCR subunits and the cholesterol/sphingomyelin islet (chol) are shown from the top view (upper panels) and the lateral view (lower panels). (A) The C and F strands as well as the AB loop of Cα mediate the homotypic TCR-TCR interaction. (B) In the membrane L19 of ζ mediates the interaction. (C) Cholesterol (and sphingomyelin) bind specifically to TCRβ, forming a raft islet. Since the TCR is located to the non-raft domain, the raft islet needs to be shielded from the non-raft surroundings. This can be achieved by TCR nanoclustering. (D) It might also be that TCRβ and ζ together bind to cholesterol.

protein) mediates TCR-TCR interactions that might be present in substoichiometric amounts.

4.3. Lipid-based TCR nanoclustering

The most familiar concept of membrane organization is based on the finding that lipids can segregate into distinct micro- (or nano-)domains [28,68]. In lipid rafts cholesterol, sphingomyelin and lipids with saturated fatty acids form a liquid-ordered phase, whereas unsaturated fatty acids predominate in the liquid-disordered phase. Although the existence of lipid rafts in living cells was questioned [53], it now becomes clear that they exist [58,69,70], although smaller and more transient than originally thought. Hence, proteins that segregate into lipid rafts, such as glycosylphosphatidylinositol (GPI)-linked proteins, might form lipid-based clusters. However, sometimes homophilic protein interactions are also involved [71].

In several studies we could show that cholesterol and sphingomyelin are required to form TCR nanoclusters (see introduction). Thus, the most straightforward model could have been that TCRs nanocluster when localized to lipid rafts, and stay single TCRs when localized to the non-raft domain. However, this is not the case, since we show here that nanoclustered TCRs are not present in lipid rafts.

Another important hint for the formulation of a model of how TCRs nanocluster, comes from our earlier finding, that the TCRβ chain specifically binds to cholesterol. In the complete TCR complex, only TCRβ and not any other subunit was cross-linked to radioactive cholesterol [20]. We suggest the following model of TCR nanoclustering addressing the possible role of lipids in this process (Fig. 5C).

The single TCRs localize in the non-raft phase. In addition, the TCRβ chain specifically binds to cholesterol within the membrane [20], which might recruit lipids with saturated fatty acids, such as sphingomyelin. Thus, as small raft islet forms at one defined site of the TCR. Even when a small raft islet has formed at the TCRβ chain, the rest of the TCR is still present in the non-raft domain. However, to shield the raft islets from the non-raft domain, TCRs form nanoclusters (such as dimers, Fig. 5C). Indeed, TCR dimer formation is dependent on cholesterol and sphingomyelin [20].

Whether TCRβ alone binds to cholesterol or whether in addition ζ also binds to cholesterol (e.g. using amino acid L19, Fig. 5D) is not known. Please note that the cross-linker group in cholesterol might only have faced TCRβ, so that a possible interaction with ζ might not have been resolved in this assay.

4.4. Factors that control the size of TCR nanoclusters

So far three potential TCR-TCR interaction sites have been identified and thus a number of possible TCR-TCR arrangements could be envisaged (Fig. 6). According to the suggestion by Kuhns and Davis of how the individual subunits are arranged [67,72], which is reflected in the upper panels of Fig. 5, all three sites are located on the same side of the TCR. This could result in the formation of either dimers (Fig. 6A), implying that additional mechanisms need to cause larger nanoclusters, or strings of TCRs (Fig. 6B). In favor of the second possibility, our immunogold EM pictures often show linear strings of TCRs [14]. These linear arrangements could also be in line with the model shown in Fig. 6C, in which each TCR contacts its neighbour TCR by only one interaction site. In addition we also saw irregular TCR nanoclusters as depicted in Fig. 6D. However, other segregation mechanisms such as involvement of the TCR's sugar groups, the actin cytoskeleton, or additional TCR-TCR contact sites cannot be excluded as additional driving forces for TCR nanoclustering. In analogy to the syntaxin 1 nanoclusters [59], we suggest that the TCR nanoclusters are dynamically regulated, so that individual TCRs can leave and join the cluster.

In contrast to ζ–ζ or TCRα–TCRα interactions, the cholesterol-based TCR nanoclustering seems to be a mechanism that can be manipulated by the cells. Thus, by altering the concentration of cholesterol the size and number of TCR nanoclusters can be controlled. In fact naïve T cells have low cholesterol levels in their PM and few TCR nanoclusters, whereas the PMs of memory T cells have higher cholesterol content and more TCR nanoclusters [12,73]. A deeper discussion is found in a recent review [3].

So far we have only discussed mechanisms that contribute to TCR nanoclustering. But how about mechanisms that limit the size of the clusters? Firstly, reduced cholesterol concentrations cause less and smaller TCR clusters (see above). Secondly, it was observed that disruption of the actin cytoskeleton led to larger TCR nanoclusters [15]. This is in line with the idea the cortical actin cytoskeleton beneath the plasma membrane forms fences, that TM proteins cannot easily pass [74]. According to this model, smaller TCR nanoclusters could not reach each other, since they would be separated by the actin network. Alternatively, depolymerization of actin could enhance TCR nanoclustering due to the lack of appropriate anchoring in the membrane. Therefore, increasing cholesterol content and the cortical actin cytoskeleton could be exerting opposing effects on the size of TCR nanoclusters, resulting in a regulation of the minimum and maximum limits of TCR oligomerization. This regulation of TCR nanocluster size could be essential to control

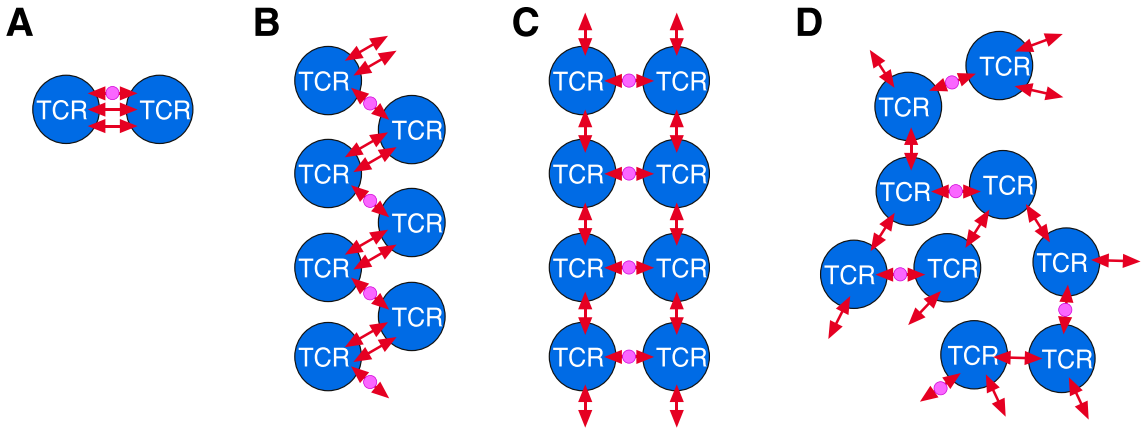

Fig. 6. Mechanisms of TCR nanoclustering. The three so far identified inter-TCR–TCR interactions (TCRβ-raft islet-TCRβ, ζL19-ζL19 and Cα-Cα) are indicated by red arrows. (A) All three interactions are used within one TCR dimer. (B) One TCR can simultaneously interact with two TCRs, leading to linear or zig-zagged TCR strings. (C, D) One TCR can simultaneously interact with three TCRs, leading either to ordered arrangements (C) or to irregular TCR assemblies (D).

the extent of cooperativity between the TCRs and the avidity towards multivalent ligands and thereby the sensitivity of T cells for antigen.

Declaration of interest statement

The authors report no declarations of interest.

Acknowledgements

This study was supported by the Excellence Initiative of the German Research Foundation (GSC-4, the Spemann Graduate School and EXC294, the BIOSS Center for Biological Signalling Studies, by the German Research Foundation grant SCH 976/2-1, and by the European Union through grant FP7/2007-2013 SYBILLA (Systems Biology of T-Cell Activation).

References

[1] B. Alarcon, D. Gil, P. Delgado, W.W. Schamel, Initiation of TCR signaling: regulation within CD3 dimers, Immunol. Rev. 191 (2003) 38–46.
[2] M.M. Davis, J.J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, Y. Chien, Ligand recognition by alpha/beta T cell receptors, Annu. Rev. Immunol. 16 (1998) 523–544.
[3] W.W. Schamel, B. Alarcon, Organization of the resting TCR in nanoscale oligomers, Immunol. Rev. 251 (2013) 13–20.
[4] D. Gil, W.W. Schamel, M. Montoya, F. Sanchez-Madrid, B. Alarcon, Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation, Cell 109 (2002) 901–912.
[5] R.M. Risueno, W.W. Schamel, B. Alarcon, T cell receptor engagement triggers its CD3epsilon and CD3zeta subunits to adopt a compact, locked conformation, PLoS ONE 3 (2008) e1747.
[6] N. Martinez-Martin, R.M. Risueno, A. Morreale, I. Zaldivar, E. Fernandez-Arenas, F. Herranz, A.R. Ortiz, B. Alarcon, Cooperativity between T cell receptor complexes revealed by conformational mutants of CD3epsilon, Sci. Signal. 2 (2009) ra43.
[7] S. Minguet, M. Swamy, B. Alarcon, I.F. Luescher, W.W. Schamel, Full activation of the T cell receptor requires both clustering and conformational changes at CD3, Immunity 26 (2007) 43–54.
[8] S. Minguet, W.W.A. Schamel, A permissive geometry model for TCR-CD3 activation, Trends Biochem. Sci. 33 (2008) 51–57.
[9] P.A. van der Merwe, O. Dushek, Mechanisms for T cell receptor triggering, Nat. Rev. Immunol. 11 (2011) 47–55.
[10] T. Yokosuka, K. Sakata-Sogawa, W. Kobayashi, M. Hiroshima, A. Hashimoto-Tane, M. Tokunaga, M.L. Dustin, T. Saito, Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76, Nat. Immunol. 6 (2005) 1253–1262.
[11] R. Varma, G. Campi, T. Yokosuka, T. Saito, M.L. Dustin, T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster, Immunity 25 (2006) 117–127.
[12] R. Kumar, M. Ferez, M. Swamy, I. Arechaga, M.T. Rejas, J.M. Valpuesta, W.W. Schamel, B. Alarcon, H.M. van Santen, Increased sensitivity of antigen-experienced t cells through the enrichment of oligomeric T cell receptor complexes, Immunity 35 (2011) 375–387.
[13] M. Swamy, S. Minguet, G.M. Siegers, B. Alarcon, W.W. Schamel, A native antibody-based mobility-shift technique (NAMOS-assay) to determine the stoichiometry of multiprotein complexes, J. Immunol. Methods 324 (2007) 74–83.
[14] W.W. Schamel, I. Arechaga, R.M. Risueno, H.M. van Santen, P. Cabezas, C. Risco, J.M. Valpuesta, B. Alarcon, Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response, J. Exp. Med. 202 (2005) 493–503.
[15] B.F. Lillemeier, M.A. Mortelmaier, M.B. Forstner, J.B. Huppa, J.T. Groves, M.M. Davis, TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation, Nat. Immunol. 11 (2010) 90–96.
[16] E. Sherman, V. Barr, S. Manley, G. Patterson, L. Balagopalan, I. Akpan, C.K. Regan, R.K. Merrill, C.L. Sommers, J. Lippincott-Schwartz, L.E. Samelson, Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor, Immunity 35 (2011) 705–720.
[17] L. Zhong, G. Zeng, X. Lu, R.C. Wang, G. Gong, L. Yan, D. Huang, Z.W. Chen, NSOM/QD-based direct visualization of CD3-induced and CD28-enhanced nanospatial coclustering of TCR and coreceptor in nanodomains in T cell activation, PLoS One 4 (2009) e5945.
[18] E. Molnar, S. Deswal, W.W. Schamel, Pre-clustered TCR complexes, FEBS Lett. 584 (2010) 4832–4837.
[19] S. Hellwig, W.W. Schamel, U. Pflugfelder, B. Gerlich, H.U. Weltzien, Differences in pairing and cluster formation of T cell receptor alpha- and beta-chains in T cell clones and fusion hybridomas, Immunobiology 210 (2005) 685–694.
[20] E. Molnar, M. Swamy, M. Holzer, K. Beck-Garcia, R. Worch, C. Thiele, G. Guigas, K. Boye, I.F. Luescher, P. Schwille, R. Schubert, W.W. Schamel, Cholesterol and sphingomyelin drive ligand-independent T-cell antigen receptor nanoclustering, J. Biol. Chem. 287 (2012) 42664–42674.
[21] M. Pihlgren, P.M. Dubois, M. Tomkowiak, T. Sjogren, J. Marvel, Resting memory CD8+ T cells are hyperreactive to antigenic challenge in vitro, J. Exp. Med. 184 (1996) 2141–2151.
[22] K. Kimachi, M. Croft, H.M. Grey, The minimal number of antigen-major histocompatibility complex class II complexes required for activation of naive and primed T cells, Eur. J. Immunol. 27 (1997) 3310–3317.
[23] P.O. Ericsson, P.L. Orchansky, D.A. Carlow, H.S. Teh, Differential activation of phospholipase C-gamma 1 and mitogen-activated protein kinase in naive and antigen-primed CD4 T cells by the peptide/MHC ligand, J. Immunol. 156 (1996) 2045–2053.
[24] B. Alarcon, M. Swamy, H.M. van Santen, W.W.A. Schamel, T-cell antigen-receptor stoichiometry: pre-clustering for sensitivity, EMBO Rep. 7 (2006) 490–495.
[25] G.M. Siegers, M. Swamy, E. Fernandez-Malave, S. Minguet, S. Rathmann, A.C. Guardo, V. Perez-Flores, J.R. Regueiro, B. Alarcon, P. Fisch, W.W. Schamel, Different composition of the human and the mouse gammadelta T cell receptor explains different phenotypes of CD3gamma- and CD3delta-immunodeficiencies, J. Exp. Med. 204 (2007) 2537–2544.
[26] M. Uhlin, M.G. Masucci, V. Levitsky, Pharmacological disintegration of lipid rafts decreases specific tetramer binding and disrupts the CD3 complex and CD8 heterodimer in human cytotoxic T lymphocytes, Scand. J. Immunol. 57 (2003) 99–106.
[27] T.M. Fahmy, J.G. Bieler, M. Edidin, J.P. Schneck, Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen, Immunity 14 (2001) 135–143.
[28] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[29] B. Diaz-Rohrer, K.R. Levental, I. Levental, Rafting through traffic: membrane domains in cellular logistics, Biochim. Biophys. Acta 1838 (2014) 3003–3013.
[30] R. Xavier, T. Brennan, Q. Li, C. McCormack, B. Seed, Membrane compartmentation is required for efficient T cell activation, Immunity 8 (1998) 723–732.
[31] C. Montixi, C. Langlet, A.M. Bernard, J. Thimonier, C. Dubois, M.A. Wurbel, J.P. Chauvin, M. Pierres, H.T. He, Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains, EMBO J. 17 (1998) 5334–5348.
[32] T. Harder, M. Kuhn, Selective accumulation of raft-associated membrane protein LAT in T cell receptor signaling assemblies, J. Cell Biol. 151 (2000) 199–208.
[33] O.O. Glebov, B.J. Nichols, Lipid raft proteins have arandom distribution during localized activation of the T-cell receptor, Nat. Cell Biol. 6 (2004) 238–243.

[34] P. Drevot, C. Langlet, X.J. Guo, A.M. Bernard, O. Colard, J.P. Chauvin, R. Lasserre, H.T. He, TCR signal initiation machinery is pre-assembled and activated in a subset of membrane rafts, EMBO J. 21 (2002) 1899–1908.
[35] P.W. Janes, S.C. Ley, A.I. Magee, Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor, J. Cell Biol. 147 (1999) 447–461.
[36] P.W. Janes, S.C. Ley, A.I. Magee, P.S. Kabouridis, The role of lipid rafts in T cell antigen receptor (TCR) signalling, Semin. Immunol. 12 (2000) 23–34.
[37] C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid rafts reconstituted in model membranes, Biophys. J. 80 (2001) 1417–1428.
[38] S.L. Veatch, S.L. Keller, Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol, Biophys. J. 85 (2003) 3074–3083.
[39] K. Bacia, C.G. Schuette, N. Kahya, R. Jahn, P. Schwille, SNAREs prefer liquid-disordered over "raft" (liquid-ordered) domains when reconstituted into giant unilamellar vesicles, J. Biol. Chem. 279 (2004) 37951–37955.
[40] N. Kahya, D.A. Brown, P. Schwille, Raft partitioning and dynamic behavior of human placental alkaline phosphatase in giant unilamellar vesicles, Biochemistry 44 (2005) 7479–7489.
[41] H. Shogomori, A.T. Hammond, A.G. Ostermeyer-Fay, D.J. Barr, G.W. Feigenson, E. London, D.A. Brown, Palmitoylation and intracellular domain interactions both contribute to raft targeting of linker for activation of T cells, J. Biol. Chem. 280 (2005) 18931–18942.
[42] R.E. Scott, Plasma membrane vesiculation: a new technique for isolation of plasma membranes, Science 194 (1976) 743–745.
[43] D. Lingwood, J. Ries, P. Schwille, K. Simons, Plasma membranes are poised for activation of raft phase coalescence at physiological temperature, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 10005–10010.
[44] T. Baumgart, A.T. Hammond, P. Sengupta, S.T. Hess, D.A. Holowka, B.A. Baird, W.W. Webb, Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 3165–3170.
[45] I. Levental, D. Lingwood, M. Grzybek, U. Coskun, K. Simons, Palmitoylation regulates raft affinity for the majority of integral raft proteins, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 22050–22054.
[46] P. Sengupta, A. Hammond, D. Holowka, B. Baird, Structural determinants for partitioning of lipids and proteins between coexisting fluid phases in giant plasma membrane vesicles, Biochim. Biophys. Acta 1778 (2008) 20–32.
[47] S. Deswal, K. Beck-Garcia, B. Blumenthal, E.P. Dopfer, W.W. Schamel, Detection of phosphorylated T and B cell antigen receptor species by Phos-tag SDS- and Blue Native-PAGE, Immunol. Lett. 130 (2010) 51–56.
[48] M. Swamy, G.M. Siegers, S. Minguet, B. Wollscheid, W.W. Schamel, Blue native polyacrylamide gel electrophoresis (BN-PAGE) for the identification and analysis of multiprotein complexes, Sci. STKE 2006 (2006) l4.
[49] E. Sezgin, I. Levental, M. Grzybek, G. Schwarzmann, V. Mueller, A. Honigmann, V.N. Belov, C. Eggeling, U. Coskun, K. Simons, P. Schwille, Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes, Biochim. Biophys. Acta 1818 (2012) 1777–1784.
[50] D.A. Brown, E. London, Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem. Biophys. Res. Commun. 240 (1997) 1–7.
[51] J. Bohuslav, T. Cinek, V. Horejsi, Large, detergent-resistant complexes containing murine antigens Thy-1 and Ly-6 and protein tyrosine kinase p56lck, Eur. J. Immunol. 23 (1993) 825–831.
[52] A.I. Magee, I. Parmryd, Detergent-resistant membranes and the protein composition of lipid rafts, Genome Biol. 4 (2003) 234.
[53] S. Munro, Lipid rafts: elusive or illusive? Cell 115 (2003) 377–388.
[54] H. Sommermeyer, R. Schwinzer, V. Kaever, K. Resch, Cholera toxin-mediated inhibition of signalling in Jurkat cells is followed by, but not due to a loss of T cell receptor complex, Immunobiology 182 (1991) 266–276.
[55] H. Gouy, P. Deterre, P. Debre, G. Bismuth, Cell calcium signaling via GM1 cell surface gangliosides in the human Jurkat T cell line, J. Immunol. 152 (1994) 3271–3281.
[56] B.M. Haack, F. Emmrich, K. Resch, Cholera toxin inhibits T cell receptor signaling by covalent modification of the CD3-zeta subunit, J. Immunol. 150 (1993) 2599–2606.
[57] K. Gaus, E. Chklovskaia, B. Fazekas de St Groth, W. Jessup, T. Harder, Condensation of the plasma membrane at the site of T lymphocyte activation, J. Cell Biol. 171 (2005) 121–131.
[58] T. Zech, C.S. Ejsing, K. Gaus, B. de Wet, A. Shevchenko, K. Simons, T. Harder, Accumulation of raft lipids in T-cell plasma membrane domains engaged in TCR signalling, EMBO J. 28 (2009) 466–476.
[59] J.J. Sieber, K.I. Willig, C. Kutzner, C. Gerding-Reimers, B. Harke, G. Donnert, B. Rammner, C. Eggeling, S.W. Hell, H. Grubmuller, T. Lang, Anatomy and dynamics of a supramolecular membrane protein cluster, Science 317 (2007) 1072–1076.
[60] F.K. Chan, H.J. Chun, L. Zheng, R.M. Siegel, K.L. Bui, M.J. Lenardo, A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling, Science 288 (2000) 2351–2354.
[61] R.M. Siegel, J.K. Frederiksen, D.A. Zacharias, F.K. Chan, M. Johnson, D. Lynch, R.Y. Tsien, M.J. Lenardo, Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations, Science 288 (2000) 2354–2357.
[62] K. Pieper, M. Rizzi, M. Speletas, C.R. Smulski, H. Sic, H. Kraus, U. Salzer, G.J. Fiala, W.W. Schamel, V. Lougaris, A. Plebani, L. Hammarstrom, M. Recher, A.E. Germenis, B. Grimbacher, K. Warnatz, A.G. Rolink, P. Schneider, L.D. Notarangelo, H. Eibel, A common single nucleotide polymorphism impairs B-cell activating factor receptor's multimerization, contributing to common variable immunodeficiency, J. Allergy Clin. Immunol. 133 (2014) 1222–1225.
[63] O. Livnah, E.A. Stura, S.A. Middleton, D.L. Johnson, L.K. Jolliffe, I.A. Wilson, Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation, Science 283 (1999) 987–990.
[64] I. Remy, I.A. Wilson, S.W. Michnick, Erythropoietin receptor activation by a ligand-induced conformation change, Science 283 (1999) 990–993.
[65] G.J. Fiala, M.T. Rejas, W.W. Schamel, H.M. van Santen, Visualization of TCR nanoclusters via immunogold labeling, freeze-etching, and surface replication, Methods Cell Biol. 117 (2013) 391–410.
[66] I. Arechaga, M. Swamy, D. Abia, W.A. Schamel, B. Alarcon, J.M. Valpuesta, Structural characterization of the TCR complex by electron microscopy, Int. Immunol. 22 (2010) 897–903.
[67] M.S. Kuhns, A.T. Girvin, L.O. Klein, R. Chen, K.D. Jensen, E.W. Newell, J.B. Huppa, B.F. Lillemeier, M. Huse, Y.H. Chien, K.C. Garcia, M.M. Davis, Evidence for a functional sidedness to the alphabetaTCR, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 5094–5099.
[68] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science 327 (2010) 46–50.
[69] C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V.N. Belov, B. Hein, C. von Middendorff, A. Schonle, S.W. Hell, Direct observation of the nanoscale dynamics of membrane lipids in a living cell, Nature 457 (2009) 1159–1162.
[70] D.M. Owen, D.J. Williamson, A. Magenau, K. Gaus, Sub-resolution lipid domains exist in the plasma membrane and regulate protein diffusion and distribution, Nat. Commun. 3 (2012) 1256.
[71] K.G. Suzuki, R.S. Kasai, K.M. Hirosawa, Y.L. Nemoto, M. Ishibashi, Y. Miwa, T.K. Fujiwara, A. Kusumi, Transient GPI-anchored protein homodimers are units for raft organization and function, Nat. Chem. Biol. 8 (2012) 774–783.
[72] M.S. Kuhns, M.M. Davis, TCR signaling emerges from the sum of many parts, Front. Immunol. 3 (2012) 159.
[73] S.M. Kaech, S. Hemby, E. Kersh, R. Ahmed, Molecular and functional profiling of memory CD8 T cell differentiation, Cell 111 (2002) 837–851.
[74] A. Kusumi, C. Nakada, K. Ritchie, K. Murase, K. Suzuki, H. Murakoshi, R.S. Kasai, J. Kondo, T. Fujiwara, Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules, Annu. Rev. Biophys. Biomol. Struct. 34 (2005) 351–378.