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Spatiotemporal regulation of Lck activity in the initiation of TCR signalling

Časoprostorová regulace aktivity kinázy Lck při zahájení TCR signalizace

Ph.D. Thesis

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Prague, 2017

Declaration:

I hereby declare that my thesis is a presentation of my original research work. Wherever contribution of others is involved, every effort is made to indicate this clearly, with reference the literature. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

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V Praze, 20. 3. 2017

Podpis (Signature)

ACKNOWLEDGEMENTS

I would like to thank to all the people who made this thesis possible. At first place, my utmost gratitude belongs to my supervisor Dr. Dominik Filipp, who led my work for all the years of my study. I am sincerely grateful for his support, encouragement, critical discussion and many advises.

My big thanks also belongs to all my colleagues from Laboratory of Immunobiology, to Honza, Matouš, Iva, Aleš, Adéla, Jasper and Tomáš, for their suggestions, help and unforgettable friendly environment. Especially I would like then acknowledge Martina Dobešová and Jana Balounová as well as my former lab colleagues Adéla Broučková and Jan Valečka for theirs experimental and other support.

My appreciation is also going to members of Laboratories of Leucocyte Signalling and Molecular Immunology, to prof. Václav Hořejší, Tomáš Brdička and Aleš Drobek. For help with FACS sorting and microscopy I give my thanks to Zdeněk Cimburek and Ivan Novotný.

Last, but not least, I would like thank to my family for their support. My sincere gratitude belongs to my dear Alenka, particularly for hers strong support, love, help and patience with me.

ABSTRACT

Arguably, the most studied cell types of immune system are T-cells. They are key players of adaptive immunity responsible for targeted action against pathogens or other danger signals. Due to their central importance, any alteration in the regulation of their activity leads often to immunopathology. Thus, the knowledge how to harness their bio-destructive effector functions is of critical importance. Up today, there is only limited consensus on the nature of molecular mechanisms controlling the initiation of T-cell activation. When T-cell receptor (TCR) recognizes its cognate antigen presented on antigen presenting cell (APC), the activation signal is transmitted through the plasma membrane and subsequent phosphorylation of cytoplasmic chains of TCR complex ensues. This is commonly considered as the first biochemical sign of T-cell activation, the process called TCR triggering. How the activation signal gets into the cell and which molecular mechanisms control TCR triggering are two fundamental, yet still unanswered questions. In this study we focused mainly on the latter one. Working within this experimental framework, we investigated three particular problems. The first one concerns the spatiotemporal organization of critical signalling molecules before and after TCR engagement in the context of lipid microdomains that, as we posited, act as an important membrane organizational principal in the regulation of TCR triggering. We mainly focused on Lck kinase which is considered as the main signal-generating element initiating TCR signalling. Using a biochemical approach, we determined membrane distribution of the active form of Lck ($pY394^{Lck}$), the key factor in TCR triggering. In this context we not only showed that in naïve T-cells a limited pool of $pY394^{Lck}$ almost exclusively partition into high molecular weight complexes, but also that after TCR engagement, this pool is significantly increased together with its redistribution to lipid microdomains. Unfortunately, quantitative discrepancies between these and previously reported studies, where $pY394^{Lck}$ levels were found significantly higher and invariant after TCR activation, lead to different conclusions about the role and steady-state levels of active Lck in T-cells. Because $pY394^{Lck}$ drives TCR triggering, the question of the basal level of $pY394^{Lck}$ in naïve T-cell is of central interest. To reconcile these results, in the second line of our research, we provided evidence that most of these discrepancies stemmed from inconsistencies within technical procedures used for sample preparations and that highly saturated levels of $pY394^{Lck}$ were results of uncontrollable spontaneous activation of Lck during cell lysis. Lastly, we previously demonstrated that activation-induced Lck redistribution within plasma membrane was critical for delivery of its function, yet, the mechanism has been unknown. Thus, in the third line of research, we identified, for the very first time, the transient formation of Lck-RACK1-cytoskeleton complexes able to affect Lck redistribution process. The involvement of cytoskeleton in the spatiotemporal organization of signalling molecules provides yet another level of complexity in the regulation of TCR signalling. Taken together, this study provides strong evidence for the contribution of membrane organization to spatiotemporal regulation of Lck activity as well as other signalling components involved in the initiation of TCR signalling.

ABSTRAKT (CZ)

T-lymfocyty jsou bezpochyby nejvíce studovanými buňkami imunitního systému. Jsou klíčovými hráči specifické imunity zodpovědnými za cílenou odpověď proti patogenům a jiným signálům nebezpečí. Díky jejich centrálnímu významu vede často jakákoliv porucha regulace jejich aktivity k imunopatologiím. Znalost jejich bio destruktivní efektorové funkce je tedy velice zásadní. Dodnes panují rozpory, jakým molekulárním mechanismem probíhá zahájení T-buněčné aktivace. Po rozeznání specifického antigenu prostřednictvím antigen-prezentující buňky a T-buněčného receptoru je aktivační signál přenesen přes plasmatickou membránu, kde následuje fosforylace cytoplasmatických částí T-receptorového komplexu. Tato událost je obecně považována za první biochemický znak aktivace T-lymfocytů v procesu zvaném „TCR triggering“. Jak se však aktivační signál dostane do buňky a jaké mechanismy regulují samotné zahájení signalizace, jsou dvě základní, avšak dosud nezodpovězené, otázky. V této práci jsme se zaměřili především na mechanismy regulace T-buněčné aktivace a tři související problémy. V prvním jsme se zabývali časoprostorovou organizací důležitých signálních molekul před a po aktivaci T-lymfocytů v kontextu tzv. lipidových mikrodomén, u kterých předpokládáme, že hrají důležitou úlohu v organizaci membrány během jejich aktivace. Zkoumali jsme především kinázu Lck, která je považována za hlavní generátor signálu zahajujícího T-buněčnou signalizaci. Pomocí biochemických metod jsme studovali distribuci aktivní formy kinázy Lck ($pY394^{Lck}$) v membráně. Nejenže jsme ukázali, že v naivních T-lymfocytech je $pY394^{Lck}$ v omezeném množství přítomna téměř výlučně ve vysokomolekulárních komplexech, ale také jsme zjistili, že po aktivaci T-lymfocytů dojde ke značnému nárůstu množství této aktivované formy Lck a zároveň jejímu přesunu do lipidových mikrodomén. Podstatné rozdíly v kvantifikaci $pY394^{Lck}$ v naší a v předchozích studiích, které naopak poukazují na velké množství již aktivní Lck v nestimulovaných T-buňkách jež se po aktivaci nemění, vedou k odlišným závěrům ohledně úlohy $pY394^{Lck}$. Vzhledem k tomu, že právě $pY394^{Lck}$ iniciuje samotné zahájení signalizace, je otázka bazální Lck aktivity velice důležitá. Dále jsme se proto zabývali tím, co způsobuje takovéto rozpory. Prokázali jsme, že většina těchto nesrovnalostí pramení z nekonsistence postupů při přípravě vzorků a vysoká úroveň detekované $pY394^{Lck}$ je tedy důsledek nekontrolované spontánní aktivace kinázy Lck během buněčné lýze. Již dříve jsme ukázali, že aktivací T-lymfocytů způsobený přesun kinázy Lck v rámci plasmatické membrány je důležitý pro její samotnou funkci, avšak mechanismus tohoto procesu není znám. V další části našeho výzkumu jsme se proto zaměřili na objasnění tohoto redistribuční procesu kinázy Lck a poprvé jsme identifikovali formování dočasného Lck-RACK1-cytoskeleton komplexu. Účast cytoskeletu v organizaci signálních molekul v rámci T-buněčné aktivace tak přináší další úroveň komplexity regulace T-receptorové signalizace. Závěrem, tato práce přináší řadu nových poznatků týkajících se příspěvku membránové organizace k časoprostorové regulaci aktivity kinázy Lck, stejně jako k dalším signálním komponentám účastnících se zahájení T-buněčné signalizace.

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LIST OF ABBREVIATIONS

ACTN1 – α-actinin-1

APC – antigen presenting cell

Csk – C-terminal Src kinase

DPC – distal pole complex

DRM – detergent resistant membrane

F-actin – filamentous actin

FLMNa – filamin A

GADS – Grb2-related adaptor downstream of Shc

HMW – high molecular weight

IS – immunology synapse

ITAM – immunoreceptor tyrosine-based activation motif

K-S model – kinetic-segregation model

LAT – linker for activated T-cells

Lck – lymphocyte-specific protein tyrosine kinase

ld – liquid disordered

lo – liquid ordered

LR – lipid raft

MHC – major histocompatibility complex

pMHC – peptide-bounded to major histocompatibility complex

MTOC – microtubule-organizing centre

PAG – phosphoprotein associated with glycosphingolipid-enriched microdomains

PKC – protein kinase C

RACK1 – receptor for activated C kinase 1

SFK – Src family kinase

SH3 – Src homology domain 3

SMA – styren-maleic acid

SMAC – supramolecular activation cluster

TCR – T-cell receptor

TRAP – transmembrane adaptor protein

INTRODUCTION

The immune system protects organisms against various pathogens or danger signals which they encounter every moment of their life. In vertebrates it could be divided into two major arms, the innate and the adaptive. The innate immunity is evolutionary older, has limited memory and is usually involved in the first immediate reaction to danger. This includes non-specific immune responses like the activation of complement cascade, induction of inflammation, production of reactive oxygen species or release of various proinflammatory cytokines. The adaptive arm of immunity then closely and irreplaceably cooperates with the innate arm in a process called antigen-presentation. Some innate immune cells, mostly dendritic cells, serve as professional antigen presenting cells (APCs). They are able to engulf pathogens, derive short peptides from them and then present them as antigens in the context of major-histocompatibility protein complex (MHC). MHC-antigen complex could be then recognized by a specific T-cell receptor (TCR) protein complex expressed on the surface of T-cells. There are distinct subtypes of T-cells. Two major subgroups, helper T-cells and cytotoxic T-cells, are characterized by the expression of surface CD4 or CD8 co-receptor, respectively. Whereas cytotoxic T-cells mainly act directly on infected cells by induction of apoptosis via production and secretion of cytotoxins (e.g. perforins, granzymes), the role of helper T-cells is of supportive nature. When activated, they provide signal to other immune cells to induce their effector function (for example, the activation of B-cells which then commit to produce specific antibodies). In conclusion, contact between T-cells and APCs and engagement of specific TCR with a cognate antigen are basic prerequisites for T-cell activation.

Molecular mechanisms of T-cell activation have been widely studied for nearly three decades, nevertheless the question how precisely is this process regulated and what are the very first events accompanying TCR triggering are not fully understood. The initiation of T-cell signalling *per se* is critically dependent on the function of the member of Src family tyrosine kinases (SFKs), the Lck kinase. This key enzyme sits on the top of the signalling cascade leading to the activation of T-cells. The very first detectable biochemical event in this process is the phosphorylation of immunoreceptor based activation motifs (ITAMs) within cytoplasmic chains of TCR/CD3 complex for which Lck kinase is almost exclusively responsible. Although, the regulation of Lck activity has been intensively studied and discussed in the past 30 years, there are still many unknowns. This also include elementary questions such as how Lck kinase get activated and how is this process integrated into TCR triggering event and downstream signalling? What is the role of spatial organization and changes in distribution of Lck and various signalling molecules in plasma membrane prior to and upon TCR triggering? All these and many other questions are still open and often debated without clear consensus and resolution.

In this study I will give a short overview of current knowledge of molecular mechanisms involved in T-cell activation. I especially focused on the role of Lck kinase in this process, regulation of its kinase activity and importance of spatial segregation of signalling molecules, including Lck, within various plasma membrane domains. I strongly believe that experimental data generated during the course of this work will have a long lasting contribution to the research focused on the initiation of TCR signalling.

LITERATURE OVERVIEW
THE ROAD TO T-CELL ACTIVATION

The process of T-cell activation is very complex whereby T-cells have to receive three extracellular signals to become activation competent.

All begins with T-cell receptor (TCR) priming – specific recognition of antigens presented by APCs. Antigens, short peptides with length of 10-20 amino acids are derived from endo- (self) or exogenous (non-self) material. They are presented in the context of MHC class I or II.

The first activation signal for T-cells is provided when TCR recognizes its cognate antigen presented on MHC. This leads to the initiation of signal transduction pathway downstream of TCR. The second signal is delivered by interaction of co-stimulatory or co-inhibitory ligands of B7 family members expressed on the same APC (e.g. CD80/86,) with their receptors on T-cells (e.g. CD28, CTLA-4). When this second signal is lacking, T-cell could not be fully activated and become unresponsive, a physiological stage called T-cell anergy. However, while the concomitant delivery of the two signals is sufficient for the initiation of T-cell activation and clonal expansion, nevertheless a third signal which is provided later in the form of various cytokines, is also important for T-cell differentiation and effector function (Curtsinger and Mescher, 2010).

FROM CELLS TO MOLECULES – FORMATION OF IMMUNOLOGY SYNAPSE

IMMUNOLOGY SYNAPSE

From cellular point of view, T-cell activation is initiated by a contact between APC and T-cell and subsequent formation of immunological synapse (IS) at their interface (Paul and Seder, 1994). With development of new microscopy techniques it was shown that this interface is highly dynamic and well-organized structure. (Grakoui et al., 1999; Monks et al., 1997). During the formation and maturation of IS, various signalling components are spatially segregated into distinct clusters or domains within IS which authors called supra molecular activation clusters (SMACs) *(Monks et al., 1997)*. They were able to distinguish that these clusters form three concentric rings – a central (cSMAC), a peripheral (pSMAC) and a distal (dSMAC), containing functionally specific sets of proteins (Fig. 1). Because proteins segregation does not occur without agonist recognition through TCR/MHC, it was hypothesised that IS serves as a platform for spatial control of protein-protein interactions needed for T-cell activation.

DYNAMICS OF IS FORMATION

The use of artificial planar lipid bilayer membrane system allowed scientists to follow spatiotemporal distribution of signalling components within IS in real-time manner *in vitro* (Grakoui et al., 1999). Further analysis revealed that formation of IS is a very dynamic process and can be dissected into several stages (Friedl et al., 2005).

During the first phase TCR specifically recognizes agonist peptide on MHC (pMHC), T-cell gets stop signal and ceases its migration (Dustin et al., 1997). This occurs within few seconds after T-cell/APC contact. TCRs are localized at periphery whereas adhesion molecules LFA-1 accumulate in the centre of newly forming IS, thus providing the physical anchor, stopping migration and helping T-cells to scan for other suitable TCR/MHC complex pairs.

The second phase, characterized as immature IS, is the most important one, especially for the initiation of signal transduction. Within few seconds upon TCR engagement several signalling pathways are triggered, resulting in the phosphorylation events, calcium responses and cytoskeleton reorganisation. Molecular details are described below in the chapter *"Molecular mechanisms of TCR triggering"*. Interestingly, this key step in the initiation of signalling was detected at the periphery of IS and lasts only for few minutes (Lee et al., 2002). Later findings support this conclusion by the observation of small protein islets called microclusters which are continuously generated at synapse periphery and contain besides TCRs also other signalling components (Yokosuka et al., 2005). Due to their highly dynamic nature, it was suggested that these microclusters are important for sustaining of TCR signalling and for regulation of co-stimulation signals in later stages (Varma et al., 2006; Yokosuka et al., 2008).

Mature IS with typical bull-eye structure is formed in next few minutes (15-30 min) when synapse development proceeds to its third stage (Fig. 1), reviewed in (Huppa and Davis, 2003). TCR complexes (microclusters) along with co-stimulation molecules like CD28 and its downstream target PKCθ kinase moves towards the centre of IS (cSMAC) whereas LFA-1 which provides stabilization of IS structure is translocated to the periphery (pSMAC). Molecules with large extracellular domains like CD45 and CD44 are excluded to even more distal periphery (dSMAC). This third stage is essential for T-cell effector function. Nevertheless, this canonical bull-eye mature synapse structure is not the only type of IS arrangement and many other forms exist depending on the cell type, developmental stage or agonist strength; reviewed and discussed in (Friedl et al., 2005; Jacobelli et al., 2004; Mitxitorena et al., 2015).

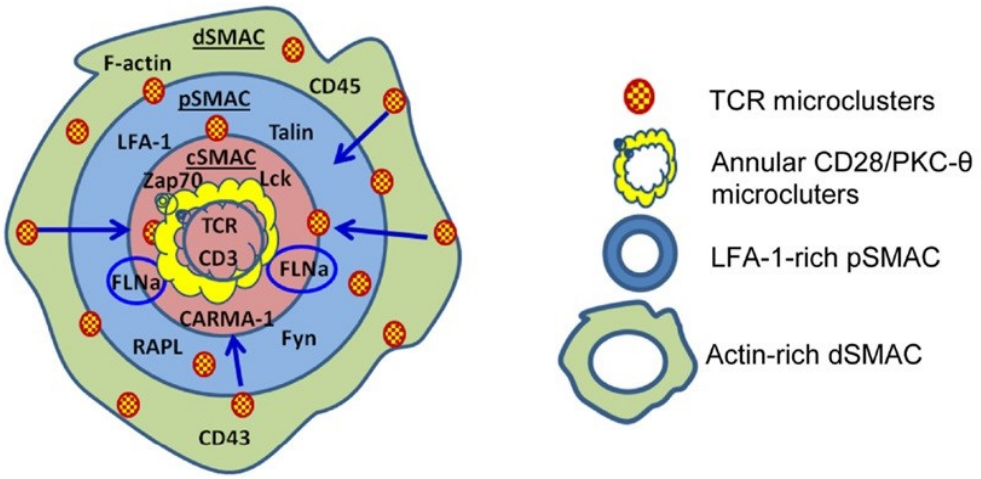

Figure 1: Structure of immunological synapse. *During maturation of IS three distinct SMACs are formed – distal (dSMAC), periphery (pSMAC) and central (cSMAC). TCR microclusters containing Lck, ZAP-70, PKCθ etc. are generated in dSMAC, and migrate to the cSMAC, where they are later internalized. Adhesion ring containing LFA-1 provides IS stabilization and is generated in pSMAC, whereas proteins with large extracellular domains (CD45, CD43) are excluded to dSMAC. Adopted from (Nassef Kadry Naguib Roufaiel et al., 2015)*

The last two stages of IS fate involve termination of signalling processes by internalization of TCRs from cSMAC and upregulation of negative regulators like CTLA-4 which ultimately leads to T-cell/APC contact resolution and T-cell detachment from APC. T-cells motility is then renewed and they begin to proliferate.

IN VIVO STUDIES OF IS

In contrast to *in vitro* studies where the mature immunological synapse with typical SMACs is formed within 30 minutes (Lee et al., 2002), *in vivo* studies showed that T-cell priming and formation of IS takes much longer time – up to 24-48 hours (Mempel et al., 2004; Miller et al., 2004). Three-phase sequence of T-cell activation process through priming by APCs was revealed by an intravital two-photon microscopy in *in vivo* studies conducted in mouse lymph nodes. (Henrickson and von Andrian, 2007; Mempel et al., 2004).

The first phase which lasts for several hours after T-cells homing into lymph nodes is characterized by short-lived interactions with APCs (approx. 5-30min). During these encounters, T-cells are primed and upregulate early activation markers – glycoproteins CD69 and CD44. The second phase lasts for next 16 hours and during this stage, T-cell motility is slowed down and contact between APC and T-cell is stabilized for at least 1 hour. Mature immunological synapse with typical contact zone structures (corresponding to SMACs) is formed, all activation markers including CD25 are upregulated and T-cells start to produce

cytokines IL-2 and IFN-γ. In the third phase, a day after naïve T-cells entry into lymph node, T-cells are fully active, dissociate from APCs, start to proliferate and migrate outside the lymph node.

Cytoskeleton also plays an irreplaceable role in the dynamics of IS formation. Whereas actin filaments are important in the initial polarization and movement of T-cells, microtubules are mainly responsible for cargo delivery to IS, reviewed in (Burkhardt et al., 2008; Comrie and Burkhardt, 2016). The actin-rich leading edge of T-cells provides the initial contact with target cell. Dynamics shown by high-resolution live cell imaging revealed that within one minute after T-cell/APC contact, actin depletion from the centre of IS was initiated concurrently with its polymerization on periphery and reposition of centrosome (MTOC – microtubule-organizing centre) towards IS. In next 2-5 minutes, MTOC localized beneath IS orchestrated the transport of vesicles containing various cytokines or granules to the synapse (Ritter et al., 2015). This rapid process is controlled by several sub-membrane cytoskeletal components which are important in the regulation of spatiotemporal organization dynamics of various proteins in IS.

In conclusion, the kinetics of T-cell priming and formation of immunological synapse seem to be different *in vivo* and *in vitro*. Nevertheless, the main features remain similar. For the activation of T-cells, critically important are the very first seconds of T-cell/APC interaction resulting in the initiation of signal transduction pathways. Then it depends on the "quality and quantity" of this signal, i.e., if it leads to a sustained T-cell/APC contact, T-cell maturation and full activation of its effector functions or if it leads to a signal abortion.

The current knowledge of IS formation is more or less limited to available imaging tools and techniques. However, with advanced microscopy we are able to better understand a spatiotemporal organization and dynamics of particular signalling processes on a single molecule level (Dustin and Depoil, 2011; Rossy et al., 2013b).

MOLECULAR MECHANISMS OF TCR TRIGGERING

From molecular point of view, the recognition of pMHC by TCR complex is the key event in the activation of T-cell. It was shown that as few as one to ten agonist peptides are sufficient to trigger T-cell activation and subsequent IL-2 cytokine production (Huang et al., 2013). Moreover, there are many self-peptide loaded

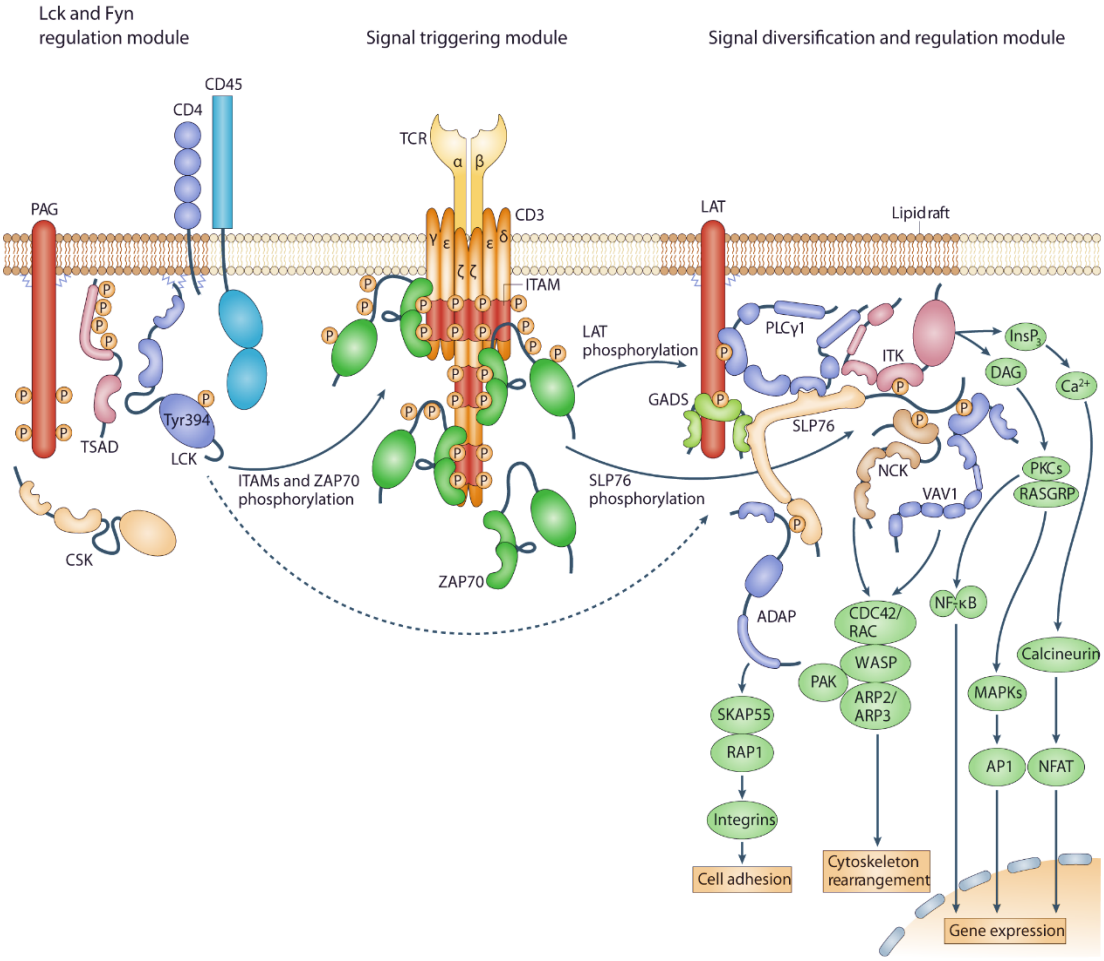

Figure 2: Molecular mechanisms of TCR triggering. *Three signalling modules can be distinguish:* ***(a)*** *Lck and Fyn regulation module (left); It contains master regulators of Lck kinase – CD45 phosphatase which dephosphorylates its inhibitory pY505 residues and Csk kinase which phosphorylates the same residue and thus counteracts the action of CD45.* ***(b)*** *Signal triggering module (middle); This module contains TCR/CD3 complex, which is responsible for transition of activation signal downstream to the effectors. ITAM motif in intracellular parts of CD3 molecules are phosphorylated by active Lck resulting in recruitment and activation of ZAP-70 kinase which in turn phosphorylates adaptor protein LAT.* ***(c)*** *Signal diversification and regulation module (right); Phosphorylated LAT serves as and scaffold protein and docking site for several other proteins. The signal is then conveyed into several signalling pathways leading to cell adhesion (integrin pathway), cytoskeleton rearrangement (NCK/VAV1 pathway) or specific gene expression (PLCγ pathway). Distinct lipid microenvironment (e.g. lipid rafts) could also play a specific regulatory role in this process. Figure was adopted from Acuto et al., 2008.*

MHC complexes on the surface of APCs that have to be discriminated from foreign-peptides loaded MHCs to avoid T-cell improper response. What does cause such a high sensitivity and selectivity? Several models of TCR triggering were postulated but still no general agreement was accomplished. What is known is that TCR engagement launches a whole range of signalling events within seconds (Huse et al., 2007), leading to the activation of various transcription programs. Activated signalling molecules initiate a network of signalling cascades and pathways which can cross-talk with each other and must be tightly regulated. However, for overview purposes of this study, the schematics of this network can be simplified. According to Acuto and colleagues, molecular aspects of early T-cell signalling can be functionally divided into three TCR signalosome modules which are sequentially connected and physically segregated (Acuto et al., 2008): Lck and Fyn regulation module, TCR triggering module and Signal diversification and regulation module (Fig. 2).

LCK AND FYN REGULATION MODULE

Lck and Fyn are protein tyrosine kinases belonging to a conserved Src kinase family (SFK) that provide the crucial enzymatic activity required for early stages of TCR triggering. Deletion or loss of their function leads to defects in thymocytes development during $CD4^{-}CD8^{-}$ stage (Molina et al., 1992; van Oers et al., 1996). Thus the timing of their activation and delivery of function must be tightly controlled.

Structure, function and mechanisms of activation as well as molecules regulating their activity will be discussed on a prototypical member of SFK family, Lck kinase. This lymphocyte-specific non-receptor kinase is around 56 kDa protein with a structure highly conserved among other SFK members (Boggon and Eck, 2004; Ventimiglia and Alonso, 2013). We can distinguish six structural parts: an N-terminal end, an unique, SH3, SH2 and kinase domains and a regulatory C-terminal tail (Fig. 3).

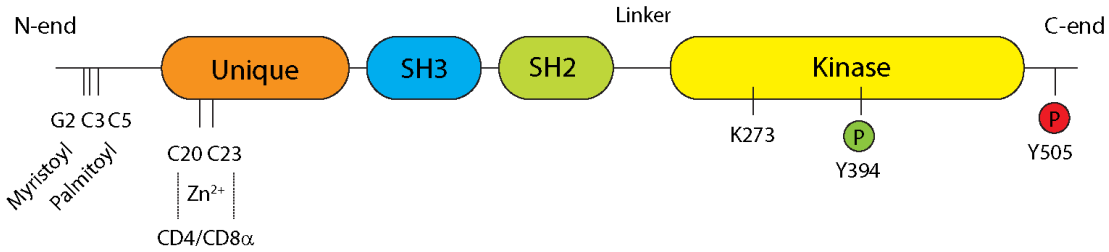

Figure 3: Structure of Lck kinase. *Lck kinase consists of N-terminal end with amino acid responsible for membrane targeting through myristoylation (G2) and palmitoylation (C3 and C5), an unique domain with cysteins contributing to CD4/CD8α binding, SH3, SH2 domains connected by a linker to the kinase domain and a C-terminal tail. Positive (Y394, green) and negative (Y505, red) regulatory tyrosines are depicted.*

N-terminal end is important for the localization of Lck to an inner leaflet of plasma membrane through palmitoylation (Cys3 and 5) and myristoylation (Gly2). This double acylation provides a docking signal to specific membrane structures called lipid microdomains. The unique domain is the least homologous segment within SFK family. In case of Lck, there are cysteines at position 20 and 23 which facilitate non-covalent binding of Lck to its co-receptor CD4 or CD8α through the coordination of zinc atom (Huse et al., 1998). Src homology 3 (SH3) and SH2 domains mediate the binding to other proteins via proline-rich sequences or phosphotyrosine motifs, respectively. They are also involved in intramolecular interactions, where they participate in regulation of kinase catalytic activity. SH2 domain is connected by a short amino acid linker region to the kinase domain. The kinase domain of Lck is highly conserved among other protein kinases with typical smaller N-terminal and bigger C-terminal lobes, ATP binding site in between them, catalytic loop, and phosphotransfer motif around lysine 273 (K273). Essential importance for regulation of Lck activity has the activation loop in kinase domain with "activatory" tyrosine 394 residue (Y394) and "inhibitory" tyrosine 505 residue (Y505) within kinase C-terminal tail segment. The phosphorylation and dephosphorylation of these tyrosine residues influence the conformation of Lck kinase and its catalytic activity (Hermiston et al., 2002) (Fig. 4).

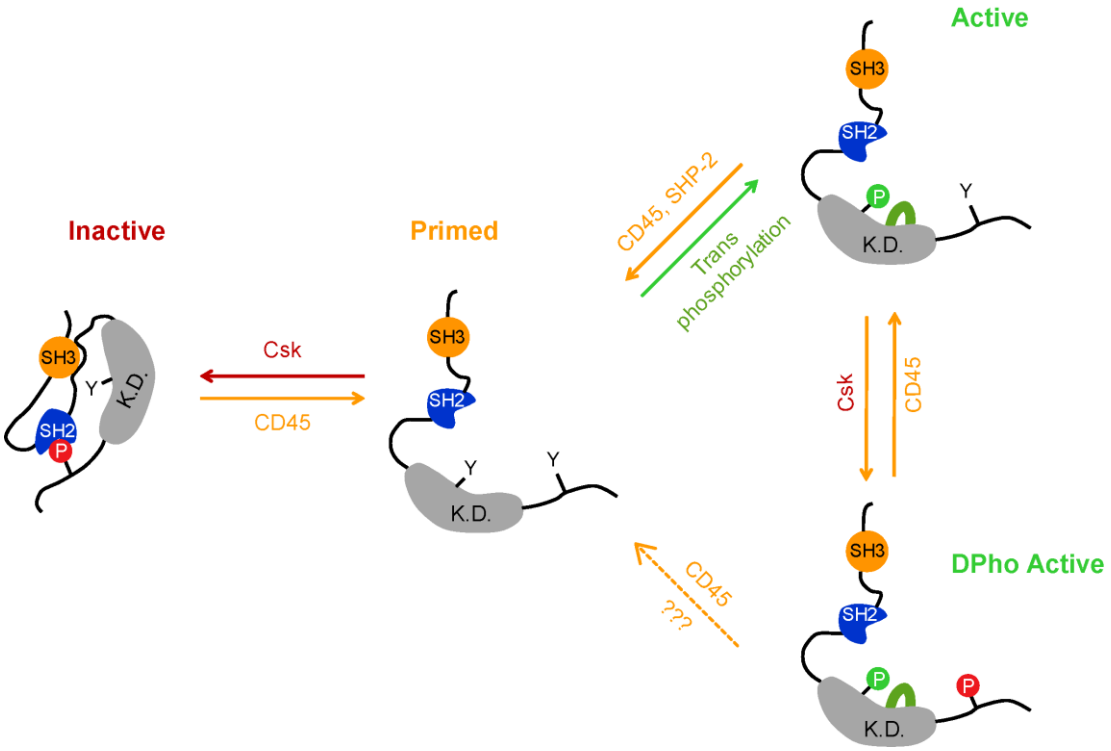

Figure 4: Regulation of Lck activity. *Lck activity is regulated by phosphorylation and dephosphorylation events. In inactive state, Lck is phosphorylated by Csk kinase at C-terminal regulatory tyrosine (Y505) which binds intramolecularly into the pocket of SH2 domain resulting in "closed" inactive Lck conformation with inaccessible substrate binding site. The linker region between SH2 and kinase domains further stabilizes this structure via binding to SH3 domain. On contrary, dephoshorylation of pY505 residue by CD45 phosphatase releases Lck into "open" primed conformation. The full (optimal) Lck activity is achieved by trans-autophosphorylation of Y394 residue, resulting in reposition of activation loop within the catalytic domain and enhancing Lck phosphotransferase activity. CD45 along with PTPN22 and SHP-2 phosphatase can also act as negative regulators by dephophorylating the activatory tyrosine 394. Double phosphorylated form of Lck (DPho Active) where both regulatory tyrosines are concurrently phosphorylated retaining Lck enzymatic activity was also detected.*

Recently, relative levels of enzymatic activity of phosphorylated Lck, measured in membrane reconstitution system under conditions mimicking T-cell activation, revealed ten-fold difference between closed/inactive and opened/fully active Lck (Hui and Vale, 2014). Moreover, enzymatic activity of primed, but non-phosphorylated Lck is considerably decreased after phosphorylation of inhibitory Y505 residue (5-fold), but increased only mildly after trans-autophosphorylation of activatory Y394 (2-fold). This suggests that phosphorylation of Y394 has a protective role against kinase inactivation via phosphorylation of Y505 rather than being important for increasing Lck activity *per se* (Hui and Vale, 2014). To fully interpret these findings in the context of TCR triggering, we have to fully understand the complexity of mechanisms regulating Lck activation.

The main actors controlling phosphorylation status of these critical tyrosines are C-terminal Src kinase (Csk) and CD45 or, to lesser extent, SHP-2 and PTPN22 phosphatases. Csk is the only known kinase responsible for the phosphorylation of Y505 residue and plays a negative regulatory role in Lck activation. The dephosphorylation of pY505, and thus opening of Lck, in T-cells is driven by CD45 phosphatase, which thus counteracts Csk action and having a positive regulatory role in Lck regulation. Nevertheless, it was reported that CD45 can also exhibit a negative regulatory function. Since Y394 residue is phosphorylated by trans-autophosphorylation, the mechanism of its dephosphorylation is less clear. It was shown that increasing amounts of CD45 in T-cells results in decreasing of pY394 as well as pY505 levels (Zikherman et al., 2010), suggesting a possible negative regulatory feedback which restricts the extent of Lck activation. In addition, one recent study showed that rates of pY394 and pY505 dephosphorylation by CD45 are similar, strengthening its dual role in Lck regulation (Hui and Vale, 2014). The absence of either Csk or CD45 greatly impact T-cell activation. Thymocytes from Csk-deficient mice as well as a specific inhibition of Csk leads to increased Lck activity and spontaneous T-cell signalling without TCR engagement (Schmedt et al., 1998; Tan et al., 2014). Similarly, in CD45-deficient T-cells the initiation of TCR signalling was blocked and CD45-deficient thymocytes failed to develop (McNeill et al., 2007; Zikherman et al., 2010).

Besides CD45, there is also PTPN22 phosphatase, a member of PEST family, which plays a negative role in TCR signalling and T-cell activation by dephosphorylating pY394 residue in Lck and other related substrates (Cloutier and Veillette, 1999; Hasegawa et al., 2004).

It is obvious that the equilibrium between Csk and CD45 enzymatic activities is crucial for T-cell function and must be tightly regulated. Their abundance and distribution within a plasma membrane is critical for determination of threshold for activation and inactivation of Lck and other SFKs.

CD45 phosphatase is a very abundant transmembrane protein in T-cells and its localization into distinct plasma membrane structures varies during T-cell activation. Several models describing details of these processes will be discussed later in the chapter "*Models of TCR triggering"*. Opposite to CD45, Csk is cytosolic protein and in order to deliver its function it has to be actively recruited to the plasma membrane – into the vicinity of its substrate, the Lck kinase. Members of transmembrane adaptor proteins family (TRAPs) are responsible for Csk docking. This mostly concerns the phosphoprotein associated with glycosphingolipid-enriched microdomains, PAG (Brdicka et al., 2000; Kawabuchi et al., 2000). This adaptor retains a palmitoyl anchor which targets PAG into the same specific membrane compartments as Lck. The association of PAG and Csk is then regulated by phosphorylation of tyrosine residue in PAG by Fyn kinase resulting in its binding to Csk via SH2 domain (Filby et al., 2007).

Dephosphorylation of this tyrosine upon T-cell activation then causes the release and dislocation of Csk away from Lck, leading to increase of Lck activity. This was confirmed in *in vitro* studies where acute inhibition or dislocation of Csk away from membrane resulted in enhanced T-cell activation and CD3ζ phosphorylation (Hui and Vale, 2014; Schoenborn et al., 2011). Nevertheless, PAG deletion in mice does not have any significant effect on T-cell activation as one would expect, suggesting existence of compensatory mechanisms. Besides PAG, there were recently described other Csk-interacting TRAPs – DOK1/2, LIME or LAT (Brdickova et al., 2003; Schoenborn et al., 2011) which could substitute for PAG action.

Fyn is another important kinase in T-cell signalling belonging to SFK family. Originally it was thought that there is a redundancy between Lck and Fyn in T-cells, nevertheless it was later shown that their roles are distinct and more specific (Filipp and Julius, 2004). Fyn is not a tissue specific kinase as Lck and is expressed in many different cell types. It has been demonstrated that activation of Fyn and Lck in proximal T-cell signalling is sequential, and it is temporarily and spatially uncoupled – Lck first then Fyn (Filipp et al., 2003). Moreover, besides above mentioned involvement in regulation of Csk kinase, Fyn was also implicated in the regulation of integrins and cytoskeleton network (Griffiths and Penninger, 2002). Distinct sets of interaction partners of both Lck and Fyn were identified supporting their essential and independent role in T-cell signalling network (Palacios and Weiss, 2004).

TCR TRIGGERING MODULE

The key event in the initiation of T-cell activation is the engagement of TCR/CD3 complex by a cognate antigen. This complex is responsible for transmission of signal to downstream effectors (Fig. 2, middle part). It is composed of TCRα and TCRβ chains (or TCRγ and TCRδ chains on specific T-cell population which are not part of this study) together with CD3γε and CD3δε heterodimers and CD3ζ homodimer. Specificity of antigen recognition is given by TCRα and TCRβ chains which form heterodimer and contain a combination of variable regions positioned by a process of somatic V(D)J rearrangement during thymocytes development. There are ten immunoreceptor tyrosine-based activation motifs (ITAMs) in cytosolic parts of CD3s which together with ZAP-70 protein tyrosine kinase form the core of TCR triggering module. ITAMs could be phosphorylated and detection of this phosphorylation is considered as the very first biochemical event of proximal T-cell activation. Besides ITAMs and ZAP-70, other auxiliary molecules are taking part in early TCR signalling. Specifically, CD8 and CD4 co-receptors which can bind via their extracellular domains to MHCI and MHCII, respectively, and consequently juxtaposed intracellularly associated Lck to the vicinity of ITAMs. Close proximity of Lck and ITAMs results in the

phosphorylation of the latter and trigger the signalling pathways which can initiate the activation of T-cell.

Phosphorylated ITAMs serve as docking sites for SH2 domains of ZAP-70 kinase. CD3ζ – associated ZAP-70 is then phosphorylated by Lck and is further activated. All these events occur within a few seconds and are crucial for development of downstream responses. It is of note that this is very simplified "text-book" view on the initiation of the proximal TCR signalling. More detail account how Lck is activated, and an assessment of several proposed models will be discussed later in the chapter *"Models of TCR triggering"*.

SIGNAL DIVERSIFICATION AND REGULATION MODULE

The signal emerging from TCR/pMHC recognition is transduced via TCR/CD3 complex resulting in the recruitment and activation of ZAP-70. The main downstream target of activated ZAP-70 is the Linker for activation of T-cells (LAT). Activated ZAP-70 phosphorylates LAT at several intracellularly positioned tyrosine residues and thus forming a signalling hub for branching and diversification of TCR transduction pathway. LAT is a scaffold transmembrane protein anchored to membrane microdomains via palmitoylation. When phosphorylated, it recruits various molecules including PLCγ, Grb2, PI3K or GADS. PLCγ pathway cleaves membrane PIP2 into IP3 and DAG, second messengers, playing a role in calcium release and activation of NFAT and NFκB transcription factors. Together with AP-1 transcription factor, activated by Grb2-Ras/Raf-Erk pathway regulates the proliferation of T-cells through IL-2 gene expression. Additional branch stemming from LAT signalling leads through adaptor protein GADS to another scaffold protein SLP-76 which links signalling elements responsible for actin rearrangement or integrin inside-out responses. Recruitment of PI3K kinase to LAT also activates Akt signalling pathway important for upregulation of survival anti-apoptotic factors. Taken together, TCR triggering generates signals that activate an array of pathway leading to comprehensive and robust changes in gene expression, proliferation and differentiation of T-cells.

NEGATIVE AND POSITIVE REGULATORS

TCR signalling in all its phases employs its own intrinsic regulatory mechanisms and various feedback loops important for maintaining T-cell homeostasis. Positive feedback loops influence mainly sensitivity and speed of signal transmission within the cell, whereas the negative ones control the initiation and amplification of signal transduction. Together, these mechanisms contribute to setting up a signalling threshold for a full T-cell activation (Acuto et al., 2008). PAG-Csk axis can serve as an example of the negative feedback regulatory mechanism

controlling Lck/Fyn activity in very early stages of activation. Similarly, a negative role also exert SHP-1 or SHP-2 phosphatases, which are recruited via immunoreceptor tyrosine-based inhibition motifs to the vicinity of important signalling complexes (e.g. TCR/CD3 complex, ZAP-70, LAT) and contribute to their dephosphorylation and thus inactivation (Lorenz, 2009)

The action of co-stimulatory or co-inhibitory molecules from B7 receptor family is important mainly in later stages of T-cell activation. Two typical members are CD28 and CTLA-4. Whereas CD28 is expressed on naïve T-cells and has a positive effect, CTLA-4 is upregulated in activated lymphocytes and has a negative role (Sansom, 2000). CD28 binds ligands CD80/CD86 presented on activated APCs which provide the second signal resulting in the activation of transcription factors and production of cytokines needed for T-cell activation. CTLA-4 acts in the opposite way. In activated T-cells is upregulated and then competes with CD28 for its ligands. After binding it recruits SHP-2 phosphatase which attenuates TCR signalling pathways.

Many other proteins (reviewed in detail in Acuto et. al, 2008) are involved in regulation of T-cells. They include but are not limited to DOK, HPK1, STS or Cbl (the E3 ubiquitine ligase responsible for degradation of some PTKs) to mention just a few of them.

MEMBRANE MICRODOMAINS

At early 1970's Singer and Nicholson proposed the "fluid mosaic model" of cell membrane, which characterized membrane as a dynamic liquid structure where membrane components can freely move in lateral dimensions (Singer and Nicolson, 1972).

Lipid (micro)domains concept was initially formulated based on experimental observations of membrane heterogeneities. The construction of artificial lipid bilayers containing high amounts of sphingolipids and cholesterol exhibited lipid phase separation into liquid ordered (lo) and liquid disordered (ld) phases. In ld phase, lipids are distributed randomly and display high lipid motility. In contrast, lo phase is characterized by lower lateral lipid motility and increase of membrane thickness due to tight packing of saturated acyl chains of sphingolipids and cholesterol intercalation (Brown and London, 2000). The solubilisation and extraction of cell membranes with specific mild non-ionic detergents e.g. Triton X-100, NP-40 or Brij-series revealed that some membrane components are solubilisation resistant. This insoluble material was enriched with sphingolipids, cholesterol and GPI-anchored proteins, thus indicating similarities with lo phase composition. It was concluded that lipid phase separation also occurs in cell membranes and these insoluble fractions were termed detergent resistant membranes (DRMs) (Brown and Rose, 1992).

The biological importance of DRMs *in vivo* was formulated by Kai Simons in 1997 as "raft hypothesis". It assumes that dynamic clustering of sphingolipids with long saturated acyl chains, cholesterol and certain proteins can form some kind of structurally rigid and biologically active signalling platforms (Simons and Ikonen, 1997). These platforms called "lipid rafts" (LR) were described as relatively small, various in size (10-200 nm), highly mobile structures floating within plasma membrane. They can merge into larger stabilized domains and thus facilitate the organized movement and aggregation of targeted proteins within specific site during membrane trafficking and signal transduction (Pike, 2006).

This definition of LR and their predicted significance cause renewed interest in plasma membrane and initiated intense investigation of these structures in distinct signal transduction pathways, especially in T-cells. Discovery that several crucial T-cell signalling proteins, including adaptor proteins LAT, PAG or kinases Lck and Fyn, reside with LR, fit into the concept of LR-mediated spatial distribution and compartmentalization of these proteins during T-cell activation and this phenomenon has been extensively discussed (Dykstra et al., 2003; Horejsi and Hrdinka, 2014; Klammt and Lillemeier, 2012; Manes and Viola, 2006).

Nevertheless, several doubts and concerns about the existence of LR in physiological conditions were communicated in the literature (Munro, 2003; Sevcsik and Schutz, 2016; Shaw, 2006). DRMs extraction was originally used in all experiments as the only method for LR isolation because it was thought that formation of domains reflects lipid-protein associations in living cells. Unfortunately, it has been reported, that this detergent-employing biochemical approach introduces severe artefacts. For this reason it was argued that the native rafts and DRMs should not be equalized because the outcome from detergent extraction highly depend on a type and concentration of detergent used as well as on temperature or duration of solubilisation (Lichtenberg et al., 2005). On the other hand, the detergent-induced membrane segregation due to local biophysical properties and composition is likely not accidental since it is reproducible and can mirror some biological properties. Thus, even today, DRMs extraction still provides an invaluable and important tool for membrane studies. However, caution with interpretation of such results in biological system must be exercised and results should be complemented and supported by other independent methods.

Nowadays, membrane biologists incline to a model of local membrane microheterogeneities or nanodomains, which are unfortunately very small and very dynamic, thus their study in native membranes is limited by current technologies. However, some data indicates that these nanodomains in response to some stimuli, are able to cluster and stabilize into larger domains, e.g. microclusters or protein islands (Fig. 5) through protein-protein, protein-lipid or lipid-lipid interaction. Under such conditions their dynamics can be visualized by advance microscopy technics (Lillemeier et al., 2010; Owen et al., 2012; Yokosuka et al., 2008).

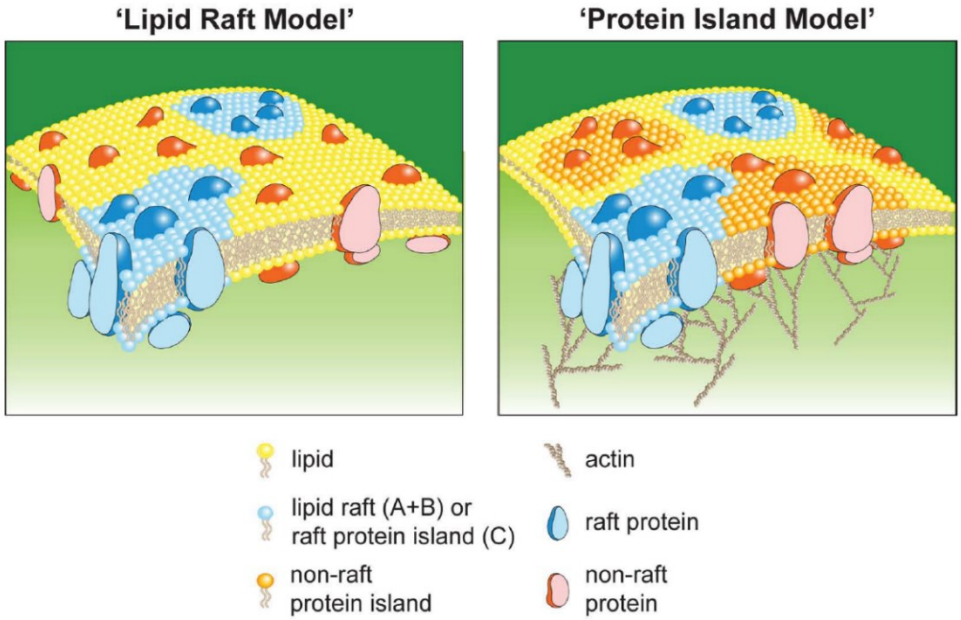

Figure 5: Lipid raft vs Protein island model. *Lipid raft model is based on observations that part of membrane proteins is localized into membrane domains termed LR whereas others are randomly distributed and can move freely. LR localization is determined by specific protein-lipid interactions which in most cases requires a post-translational protein modification (palmitoylation, GPI-anchors). The size and also life-time of LR can vary. In contrast, the protein islands model postulates that all membrane proteins are segregated into domains according their function and nature. LR are then a subpopulation of protein islands. Protein islands are connected to actin cytoskeleton which is responsible for their segregation. Protein free areas then separate distinct protein islands from each other. Adapted from Klammt and Lillemeier, 2012.*

On a whole, while the structure and dynamics of membrane domains is still not fully resolved and their biological function appreciated, the existence of raft-like native membrane structures was confirmed by several distinct advance imaging microscopy techniques enabling the visualization of up to a single molecule lipids and/or protein level in native cell membranes (Eggeling et al., 2009; Gaus et al., 2006; Owen et al., 2012). Moreover, the functional impact of mutated proteins which were targeted to these structures was also reported (Otahal et al., 2011). To better understand their biological properties in physiological conditions, development of new advanced and more sensitive techniques is required.

In general, the potential role of lipid microdomains was implied in various cellular processes. For example in membrane trafficking, where microdomain clustering leads to the formation of carriers which transport lipids and proteins in a different way than classic coat-mediated transporter (Klemm et al., 2009). They were also shown to participate in processes associated with virus budding (Waheed and Freed, 2009). Nevertheless, microdomains are the most studied in T-cell signalling. Overview, discussion and some elementary data about their role,

function, dynamics and distinct types in T-cells are described in detail in next chapters and in review paper: *"Lck, membrane microdomains, and TCR triggering machinery: Defining the new rules of engagement"* which is a part of this thesis (Filipp et al., 2012).

CYTOSKELETON IN T-CELL ACTIVATION

T-cell activation is highly associated with cytoskeleton-dependent processes from cellular polarization, integrin-dependent cell adhesion, formation of IS to sequestration of important signalling molecules (Billadeau et al., 2007; Comrie and Burkhardt, 2016). Cytoskeleton is formed from three major cytoskeletal components – actin microfilaments, microtubules and intermediate filaments. Whereas actin microfilaments are largely responsible for cell morphology and plasticity, microtubular network orchestrates the T-cell cargo transport and polarized secretion of effector molecules. Intermediate filaments then provide some structural supportive functions. As mentioned in the previous chapter, cytoskeletal network has an essential role in shaping of T-cell/APC contact. It is mainly rapid polymerization and depolymerization of filamentous actin (F-actin) after TCR engagement what promotes dynamic T-cell polarization – formation of IS at T-cell/APC interface and distal pole complex (DPC) at the opposite site of cell. This leads to the establishment of SMACs and related sequestration of signalling molecules into their interaction sites. The process of F-actin polymerization is driven by TCR engagement and downstream signalling pathway leading through Lck-ZAP-70-LAT-SLP-76-Vav1 cascade. Vav1 is guanine-nucleotide-exchange factor which activates small Rho GTPases CDC42 and Rac1. They are responsible for the recruitment and regulation of ARP2/3 complex important for nucleation and polymerization of F-actin (Kumari et al., 2014).

There are several currently known functional links implicating actin microfilaments in T-cell activation. It was demonstrated that several negative regulators of T-cell activation including CD43, CD148, SHP-1, EBP50, are during IS formation sequestered at the opposite site of T-cell into DPC (Burkhardt et al., 2008). Here, the CD43 transmembrane protein and EBP50 adaptor protein, which interacts with SFKs negative regulator PAG (Brdickova et al., 2001) can associate with actin linker, ezrin. Ezrin was shown to shuttle between DPC and IS during T-cell activation, suggesting its role as an actin-dependent cargo carrier and T-cell regulator (Shaffer et al., 2009). Last, but not least, is the connection of integrins to F-actin network. Recently, it was shown that actin network generates a mechanical force leading to affinity maturation and clustering of LFA-1 in IS thus supporting T-cell/APC adhesion (Comrie et al., 2015).

Microtubules are mainly implied in reorientation of MTOC towards IS. When the T-cell polarization is accomplished, vesicles containing various lymphokines are delivered along the microtubules into IS. Moreover, dynein – the minus end

directed microtubule motor was shown to be involved in TCR microcluster movement from peripheral to central SMAC (Hashimoto-Tane et al., 2011), thus providing evidence of active microtubules involvement in lymphocyte signalling.

The role of cytoskeleton in dynamics and subcellular distribution of membrane-associated proteins and various membrane structures, including lipid microdomains is not fully understood yet. Several data suggested the involvement of F-actin through interaction of actin binding proteins with microdomain-targeted proteins. One such example – F-actin cross-linking protein filamin A (FLMNa), was shown to associate with CD28 co-stimulatory molecule. Upon TCR engagement FLMNa is in CD28 depending manner recruited to IS, inducing cytoskeleton rearrangements and lipid microdomains accumulation in IS (Tavano et al., 2006). Disruption of cytoskeleton components by pharmacological agents also exhibits a profound effect on the function of lipid microdomain-associated proteins (Chichili et al., 2012).

Taken together both actin cytoskeleton and microtubules are essential for successful T-cell activation – from shaping of immunological synapse to T-cell signalling regulation (Comrie and Burkhardt, 2016; Lasserre and Alcover, 2010).

MODELS OF TCR TRIGGERING

Since the discovery of T-cell receptor, an enormous effort has been put into to find out the precise molecular mechanism leading to full T-cell activation. Nevertheless, the very early biochemical events which trigger this process have not been elucidated with consensual satisfaction. The answer on question what is the signal which precede and is needed for T-cell activation and how this signal is generated and regulated, remains to be resolved. In the course of time, several "TCR triggering" models, which more or less explain scientific findings, were postulated. In next few paragraphs I will highlight the most important features of these models.

AGGREGATION MODEL

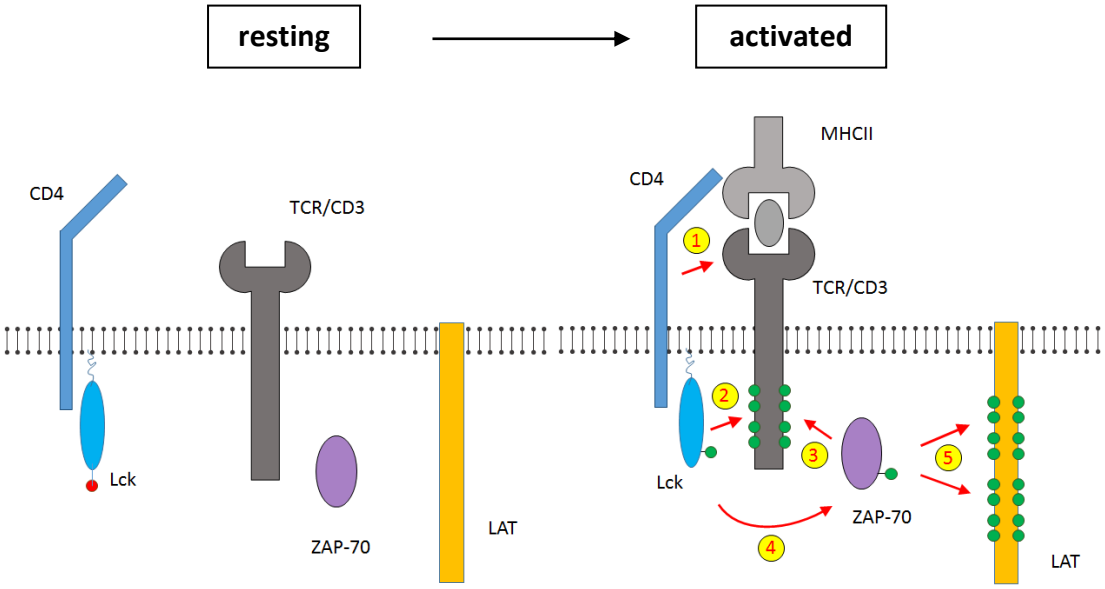

Figure 6: The aggregation model: *In resting CD4⁺ T-cells, TCR/CD3 complex and CD4 co-receptor with inactive Lck are physically segregated. Upon TCR engagement, they aggregate together (1), Lck is activated and phosphorylates ITAM motif in CD3 chains (2), ZAP-70 is recruited to ITAMs (3) and activated by Lck (4). Active ZAP-70 phosphorylates LAT (5), thus initiating downstream signalling pathway.*

The original, and for its simplicity still widely accepted "text-book" model is the "aggregation model". Its mechanistic view of action is based on the redistribution and recruitment of Lck kinase to the proximity of TCR/CD3 complex. The Lck is non-covalently bound to the intracellular part of CD4 or CD8 co-receptor, depending on T-cell subtype (Veillette et al., 1988). Since these co-receptors can bind via their extracellular part to pMHC complex, upon TCR engagement, TCR and CD4/CD8-Lck complexes can aggregate together (Delon et al., 1998; Trautmann and Randriamampita, 2003). Clustering of Lck in these complexes leads to its activation. Active Lck is then recruited to the vicinity of TCR where initiates the TCR signalling pathway by phosphorylation of activatory ITAM motif in CD3 chains (Fig. 6). Unfortunately, this model does not explain some important findings. The major one is concerning how Lck kinase activity is regulated? How and what mechanism initiate Lck trans-autophosphorylation on Y394? Does membrane microenvironment play any role in this event, and/or is the TCR-induced redistribution of Lck and other membrane signalling components accompanying this process? Could it be possible that Lck is in a constitutively activate state or it is rather a question of its molecular conformation or submembrane distribution? These and other related questions are addressed in an alternative models of TCR triggering.

KINETIC-SEGREGATION MODEL

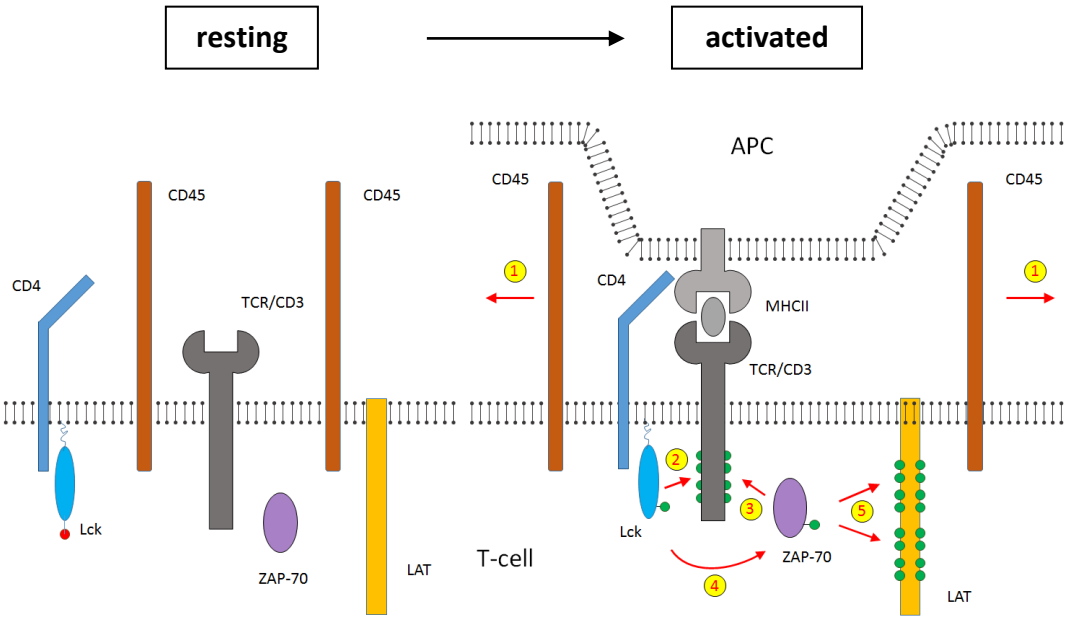

Figure 7: Kinetic-segregation model of TCR triggering. *In resting CD4⁺ T-cells phosphorylation of TCR/CD3 complex is maintained by CD45. CD4 co-receptor with inactive Lck are spatially separated. Upon TCR engagement, due to size exclusion, CD45 is redistributed away from TCR/CD3 (1), activated Lck phosphorylates ITAM motif in CD3 chains (2), ZAP-70 is recruited to ITAMs (3) and activated by Lck (4). Active ZAP-70 phosphorylates LAT (5), thus initiating downstream signalling pathway.*

Nowadays, one of the most discussed and accepted models of TCR triggering is "kinetic-segregation model" (K-S model) (Anton van der Merwe et al., 2000; Davis and van der Merwe, 2006). This model came from the observation of membrane signalling molecules redistribution during T-cell antigen recognition and formation of the IS. (That is in contrast to aggregation model which is based mostly on biochemical data). It was shown that in the IS cell surface molecules segregate into distinct SMACs (Monks et al., 1997). This separation is caused by passive (physical forces) or active mechanisms (e.g. cytoskeleton). Among passive processes we can include size-dependent segregation and exclusion of some molecules from an IS. Molecules differ in the size of their extracellular parts and thus, logically upon the IS formation between APC and T-cell, their membranes are in a close apposition with only a very narrow gap between them. Hence, molecules with larger extracellular parts are pushed away to the edge of such contact area. Efficient T-cell activation is based on TCR-pMHC interaction and because extracellular parts of TCR/MHC are much smaller then LFA-1/ICAM-1, CD43 or CD45, it is suggested that at the beginning of T-cell activation, several close-contact zones between APC and T-cell are formed. Inside these zones, TCRs and other accessories molecules (e.g. CD4/8 co-receptor) with similar size co-accumulate and can spatially communicate with each other.

However, the large molecules such as CD43, CD45, CD148 or integrines are strictly excluded to the edge of a contact area.

As was mentioned previously, equilibrium between phosphorylation and dephosphorylation events protects and keeps TCR signalling off. The key regulator is CD45 phosphatase, probably the most abundantly expressed protein at the T-cell surface. This molecule is also responsible for the dephosphorylation of TCR/CD3 complex, thus dampening TCR signalling. Spatial segregation of CD45 molecules away from TCRs nudges the phosphorylation equilibrium towards tyrosine phosphorylation which, in turn, drives TCR triggering (Cordoba et al., 2013). The basic principles of K-S model are illustrated in Figure 7.

The advantage of this model is that TCR triggering is dependent on a half-life of TCR-pMHC binding within close-contact zones. That corresponds with the affinity of TCR to peptide presented on MHCs. The stronger the affinity, the longer time for sequential phosphorylation of CD3 ITAMs and more of them get phosphorylated leading to more sustained progression of signalling and its persistent to later stages of T-cell activation and maturation of IS. On the other hand, when affinity of antigen is low, and thus TCR-pMHC binding is weak, the close-contact zone is rapidly dissolved, TCR is dephosphorylated by CD45 phosphatase and signalling is attenuated. To conclude, this model has two key elements – the molecular segregation and TCR-pMHC binding kinetics and provides a simple mechanistic explanation how TCR triggering could be regulated (Davis and van der Merwe, 2006).

STAND-BY MODEL

However, the K-S model does not provide any explanation concerning the regulation of Lck activity at the very beginning of IS formation. Is Lck already active or what causes its initial activation (in sense of phosphorylation on the activatory Y394 tyrosine)? Conventional views suggest that pMHC binding activates Lck, however some experimental evidence suggest the opposite. In the work of Nika et al., levels of $pY394^{Lck}$ in naïve as well as in activated T-cells were analysed and quantified (Nika et al., 2010). It was shown that more than 40% of total Lck in human naïve T-cells is phosphorylated at Y394 and these levels does not change upon T-cell activation. This is quite surprising, because previous models suggested that amplification of Lck activity is needed in order to trigger TCR signalling (Filipp et al., 2003; Holdorf et al., 2002; Philipsen et al., 2017; Wang et al., 2011) . According to their findings, Nika et al. postulated the "Lck stand-by model" of TCR triggering. This model assumes that a relatively high level of constitutively active Lck present in naïve T-cells is in ready-to-go status and waiting for a stimulus (e.g. pMHC binding). TCR engagement than results in the redistribution of already kinase active Lck to the vicinity and a very

fast phosphorylation of ITAM motifs in TCR/CD3 complex, thus triggering the signalling pathway.

However, stand-by model does not solve the potential problem of the presence of such high amounts of active Lck which would results in harmful spontaneous activation of T-cells. Thus, the authors suggested that the TCR must be somehow shielded from Lck – e.g. by conformational changes in TCR or by distinct distribution of membrane signalling components. These suggestions are moreover in agreement with models concerning conformational changes in TCRs as the main regulatory mechanism of TCR signalling.

CONFORMATION CHANGE MODEL

One such model provides biophysical data showing that intracellular domains of CD3s, which are positively charged, in naïve T-cells can bind to the inner leaflet of plasma membrane, thus making ITAMs inaccessible to Lck phosphorylation (Xu et al., 2008). When T-cells are activated, these portions of CD3 chains are released from the membrane, Lck phosphorylates ITAMs a TCR signalling pathway can be triggered. Nevertheless, while the precise mechanism of CD3 chain dislodging is not currently known, some kind of transmission of mechanical force after ligand binding or change in local lipid microenvironment after TCR engagement could be envisioned. There are some data supporting this model. Previously, it has been already shown that cytoplasmic domain of CD3ζ can bind phospholipids and this interaction prevents phosphorylation of ITAMs in CD3ζ by Lck (Aivazian and Stern, 2000). Moreover, the role of conformational changes in CD3ζ structure upon pMHC binding was also reported, revealing proline rich sequences which, in turn, allowed binding of Nck adaptor protein to CD3 (Gil et al., 2002; Minguet et al., 2007). Of course, there are more examples, but also there are concerns about the artificiality of this model too and further experimentation is needed (Fernandes et al., 2010; van der Merwe et al., 2012; Zhang et al., 2011).

LIPID-BASED SEGREGATION MODEL

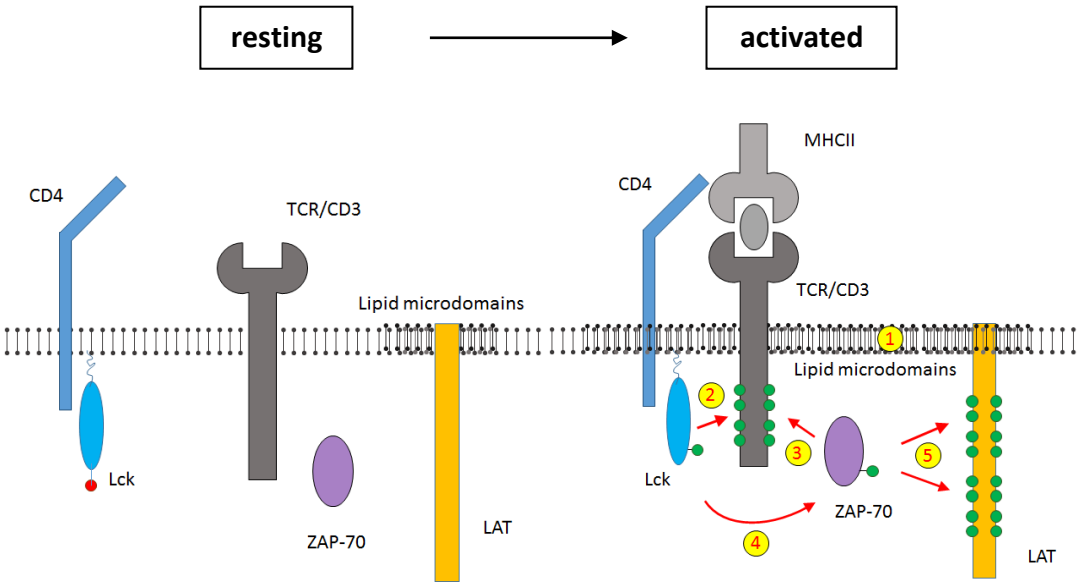

Figure 8: Lipid-based segregation model. *In resting CD4⁺ T-cells, TCR/CD3 complex and CD4 co-receptor with inactive Lck and LAT are segregated by distinct lipid microenvironment. Upon TCR engagement, they aggregate together into one lipid-based platform (1), Lck is activated and redistributed to lipid microdomains, phosphorylates ITAM motif in CD3 chains (2), ZAP-70 is recruited to ITAMs (3) and activated by Lck (4). Active ZAP-70 phosphorylates microdomain-associated LAT (5), thus initiating downstream signalling pathway.*

This model proposes that another level of regulation to TCR triggering is provided by lipid-dependent spatial distribution of signalling proteins within the plasma membrane. The details are extensively discussed and reviewed in our work "*Lck, membrane microdomains, and TCR triggering machinery: Defining the new rules of engagement*", which is a part of this study, thus I will touch only upon the key points. Since the discovery of lipid microdomains or LR, a lot of effort was made to characterize the distribution of important TCR signalling molecules into "non-raft" or "raft" fractions prior and after T-cell activation (Dykstra et al., 2003; Horejsi and Hrdinka, 2014; Ventimiglia and Alonso, 2013). According to these data, several models of membrane microdomain involvement in TCR triggering were proposed (Fig. 8).

The original LR theory implicated in TCR signalling suggested that in resting T-cell the important membrane signalling molecules like SFK kinases, TCR/CD3 complex or adaptor proteins are segregated from each other by virtue of forming small clusters or lipid islets protecting their spontaneous activation. Upon TCR engagement these clusters merge into larger signalling platforms allowing their interactions and thus initiate the signalling pathways (Lillemeier et al., 2010).

Particular example is the model of Lck-dependent Fyn activation (Filipp et al., 2004; Filipp et al., 2003). It was shown that these two SFK kinases are in naïve T-

cells associated with distinct membrane compartments. Whereas Fyn is constitutively localized to LR fraction, Lck resides in non-LR fractions. Upon TCR engagement Lck redistribute from “non-raft” to “raft” fractions, where it triggers Fyn activation and subsequently TCR signalling. The exact mechanism responsible for the Lck translocation has not been not resolved so far, nevertheless, the functional C-terminal part of Lck is the prerequisite for such mode of action (Filipp et al., 2008). This model illustrates that Lck can act as a mobile element and that spatial-temporal regulation via lipid microdomains could be an important early step in TCR triggering.

Along with all these experimentations, several doubts and concerns about the existence of LR were raised, pointing at the fact that biophysical properties obtained by detergent-mediated cell lysis should not be equalized with the native LR and that results should be interpreted with caution (Jacobson et al., 2007; Munro, 2003; Sevcsik and Schutz, 2016; Shaw, 2006). Nowadays, with improving technical resources, membrane heterogeneities are classified as protein islands, nanodomains or nanoclusters and could be visualized in living cells (Lillemeier et al., 2010; Owen et al., 2012). It has been shown that these membrane structures are very short-lived, dynamic and might be as small as less than 20 nm (Eggeling et al., 2009). In addition, these new techniques are allowing to examine their properties and behaviour during early stages of T-cell signalling in better resolution, arising both new conclusions and questions. (Kapoor-Kaushik et al., 2016; Pageon et al., 2016; Rossy et al., 2013a). For example the work of Rossy et al. who studied the conformation-driven Lck clustering, proposed a model, whereby in naïve T-cells, Lck localizes in nanodomains which upon TCR engagement recruit more Lck molecules. This process is independent of Lck association with protein network or even with lipid microdomains. The authors concluded that the major “localization” determinant is an open Lck conformation (i.e. kinase active state). This conformation induces self-association and clustering of Lck, whereas the closed one prevents it. Moreover, CD45 phosphatase was excluded from clusters containing Lck and TCR, thus supporting bias towards TCR phosphorylation. In conclusion, this model introduces a fresh look at the mechanism how could TCR signalling be regulated by conformational states of Lck and its clustering in membrane. Nevertheless, how this clustering mechanism is regulated and if it contributes to early TCR triggering or later responses have to be further investigated.

OTHER MODELS OF TCR TRIGGERING

There are additional models of how TCR signalling is triggered (Chakraborty and Weiss, 2014; Malissen et al., 2014; van der Merwe and Dushek, 2011). Among them it is worth to mention a model concerning the phosphorylation of ZAP-70 kinase by Lck as the key triggering regulatory element (Chakraborty and Weiss, 2014; Thill et al., 2016). This model assumes that Lck is in a permanent dynamic

regulation of its activity by phosphorylation and dephosphorylation events. Thus there is always some basal level of Lck activity. This dynamic state is not sufficient to enhance Lck activity *per se*, but it is sufficient to phosphorylate ITAM motifs when transient and weak interaction between TCR and pMHC occurs. In this circumstances, ZAP-70 could bind to ITAMs but is not activated. Other situation arise when the TCR is engaged with a stronger pMHC and this interaction is assisted with engagement of CD4/8 co-receptors bound Lck. They provide some extra time for Lck action, thus Lck can phosphorylate ZAP-70 and activate it. Phosphorylated ZAP-70 then in turn binds Lck and stabilize its active conformation what helps Lck to overcome negative regulatory mechanisms imposed by CD45. ZAP-70 then triggers downstream signalling pathways.

LCK SIGNALLING NETWORK

In connection to our previous results identifying activation-induced Lck membrane redistribution machinery (Filipp et al., 2008; Filipp et al., 2003), one of the objective of our research presented in this study was to find out the best candidate protein which could participate in Lck membrane redistribution mechanism. We looked for candidates among previously described molecules that have following attributes: (i) regulation of intracellular translocation of kinases, (ii) ability to interact with SFKs and modulate their kinase activity; (iii) capacity to associate with elements of the cytoskeletal network and (iv) capacity to interact with multiple partners. As the best option we decided to consider for further investigation adaptor protein Receptor for activated C kinase 1 (RACK1) which fulfilled all these criteria. This protein and his selected interaction partners which we identified in follow up experiments seem to be involved in this redistribution process and will be shortly reviewed below.

RACK1

RACK1 (Receptor for activated C-kinase) is a 36 kDa adaptor protein which is highly conserved among all eukaryotes from yeast, through plants to mammals and is abundantly expressed in almost all cell types. It belongs to a family of so-called RACKs – proteins which are able to interact with activated protein kinases C (PKC) (Mochly-Rosen et al., 1991). RACK1 was originally described as PKCβII – interacting protein, which exhibits more than 47% homology with beta subunit of trimeric G-proteins (Ron et al., 1994). Up today, more than 150 other interaction partners have been described (BioGRID database; *thebiogrid.org*) implicating and connecting RACK1 to various signalling pathways and processes. Its structure consist of seven WD40 repeats (Neer et al., 1994) organized into seven-blade propeller structure (Fig. 9) (Coyle et al., 2009; Ruiz Carrillo et al., 2012). RACK1 has no catalytic activity *per se*, arguing for its dedication to act as a scaffold protein.

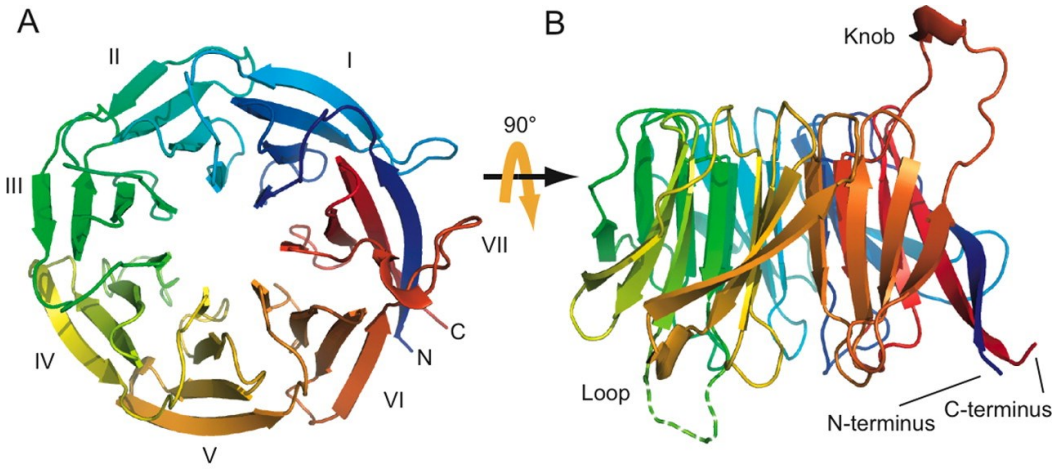

Figure 9: Crystal structure of RACK1. *Top (A) and side (B) view at RACK1 structure illustrates seven-bladed β-propeller protein architecture. Each blade corresponds to WD40 domain and consist of three β-sheets and two α-loops. Adopted from Coyle et al., 2009.*

Present view at this protein suggests that there are at least two pools of RACK1 in the cell (Gibson, 2012; Ron et al., 2013). First cytoplasmic one, which orchestrates various signalling events, and the second, ribosomal pool, which associates with small ribosomal 40S subunit (Nilsson et al., 2004; Sengupta et al., 2004). Thus, besides acting as a signalling hub, it also behaves as a ribosomal structural protein essential for efficient translation from the initiation to quality control of nascent polypeptides (Kuroha et al., 2010; Sharma et al., 2013).

The RACK1's critical role is further underlined by its loss of function mutations. In mammals, embryonic stem cells with a complete knockout of RACK1 gene are not viable; same applies to mouse homozygotes expressing RACK1 hypomorphic alleles which are lethal during embryonic development (Volta et al., 2013). These data confirm the importance of this protein.

RACK1 – our premier candidate

Since original discovery of RACK1-PKC interaction, a dozen of other RACK1 – binding partners were identified and annotated (Gandin et al., 2013; McCahill et al., 2002). They play a role in cell growth, tumorigenesis, apoptosis, survival, migration, translation and many other biological processes (Adams et al., 2011; Gandin et al., 2013). In context of this study, only some of them will be highlighted.

RACK1 was chosen as the most suitable candidate protein in our study because matched several criteria. One of such conditions was interaction with SFK family kinases. RACK1 was indeed shown to associate with Src as well as with Lck and Fyn in *in vitro* translation assay (Chang et al., 1998). Moreover, structure-function analyses revealed that this interaction is mediated by binding of phosphotyrosines in sixth WD40 domain of RACK1 with SH2 domain of Src (Chang et al., 2001).

Distinct biological role of RACK1 on Src function was reported, suggesting that RACK1 exhibits a cell specific effect. It was shown that RACK1 acts as an inhibitor of Src activity, cell grown and tumorigenesis (Chang et al., 1998; Mamidipudi et al., 2004). Similarly, RACK1 was identified as a pro-apoptotic regulator acting via suppressing Src activity (Mamidipudi and Cartwright, 2009) or by activation of stress-induced MAPK signalling (Arimoto et al., 2008). Role of RACK1 in regulation of Src activity is often studied in the context of cancer (Li and Xie, 2015).

Our other assumption was that our candidate should participate in the intracellular protein transport. In this context RACK1 was shown to interact with PKCβII and in activation-dependant manner this complex redistributes from cytoplasm to plasma membrane (Ron et al., 1999). Another example is provided by association of RACK1 with 14-3-3 scaffold protein in response to activation of cAMP/PKA signalling pathway which is necessary for shuttling of RACK1 to the nucleus, where it activates transcription (Neasta et al., 2012).

RACK1 also fulfilled the condition concerning its association with elements of cytoskeleton. It was shown that RACK1 is important regulator of cell adhesion and migration (Cox et al., 2003) especially in the process of focal adhesion assembly. Direct interaction of RACK1 with β-integrins, focal adhesion kinase and cytoskeleton linker paxillin and talin was reported (Doan and Huttenlocher, 2007; Serrels et al., 2010), making RACK1 the main integrating and core component of this multiprotein assembly. Moreover, all these interactors provide direct links to cytoskeleton assembly and thus regulate their reorganization. RACK1 was also identified as plectin interactor. Plectin is another cytoskeleton linker dynamically associating with all major cytoskeleton components (Svitkina et al., 1996). It also serves as a scaffold for various signalling proteins and components of cell adhesion machinery. In this context, plectin-RACK1 interaction was shown to be important in recruiting of PKCδ to the site of its action (Osmanagic-Myers and Wiche, 2004). RACK1 is also suggested to localize to plasma membrane through association with proteins containing plextrin homology domains. These proteins including cytoskeletal binding proteins β-spectrin or dynamin-1 are PKCβ substrates (Myklebust et al., 2015; Rodriguez et al., 1999). Actin – spectrin meshwork then serves as a docking site for various cellular components, thus supporting the role of RACK1 in signal networking.

Taken together these and other RACK1 properties makes from this protein very interesting and promising component of numerous cellular processes. Its ability to interact with so many proteins including cytoskeletal components provides evidence for its essential role in the physiology of relevant cells.

THESIS AIMS

The general aim of this study was to investigate and characterize the role of compartmentalization and redistribution of plasma membrane associated proteins in T-cells within early stages of T-cell activation. Particular aims were as follows:

1. Characterize the spatial-temporal organization of critical signalling molecules before and after TCR engagement within the context of lipid microdomains with focus on Lck kinase.

2. Investigate the role of pre-activated Lck in the initiation of T-cell signalling.

3. Elucidate the molecular mechanism of Lck redistribution process within plasma membrane upon T-cell activation.

4. Based on our new data provide an updated version of TCR triggering models.

RESULTS

The list of publications directly related to this thesis:

Ballek O, Valečka J, Dobešová M, Broučková A, Manning J, Řehulka P, Stulík J, Filipp D. TCR triggering induces the formation of Lck-RACK1-Actinin-1 multiprotein network affecting Lck redistribution. *Front. Immunol. 2016 Oct 27;7:449*. (IF_{2016}=5.695)

Ballek O, Valečka J, Manning J, Filipp D. The pool of preactivated Lck in the initiation of T-cell signaling: a critical re-evaluation of the Lck standby model. *Immunol Cell Biol. 2015 Apr; 93(4): 384-95*. (IF_{2016}=4.473)

Filipp D, **Ballek O**, Manning J. Lck, Membrane Microdomains, and TCR Triggering Machinery: Defining the New Rules of Engagement. *Front Immunol. 2012 Jun 12; 3:155*. (IF_{2016}=5.695)

Ballek O, Broučková A, Manning J, Filipp D. A specific type of membrane microdomains is involved in the maintenance and translocation of kinase active Lck to lipid rafts. *Immunol Lett. 2012 Feb 29;142(1-2):64-74*. (IF_{2016}=2.483)

The list of publication, where applicant contributed as a co-author mostly by technical expertise and advices related to various microscopy techniques and biochemical procedures:

Dobeš J, Neuwirth A, Dobešová M, Vobořil M, Balounová J, **Ballek O**, Lebl J, Meloni A, Krohn K, Kluger N, Ranki A, Filipp D. Gastrointestinal Autoimmunity Associated With Loss of Central Tolerance to Enteric α-Defensins. *Gastroenterology. 2015 Jul;149(1):139-50.* (IF_{2016}=18.187)

Balounová J, Vavrochová T, Benešová M, **Ballek O**, Kolář M, Filipp D. Toll-like receptors expressed on embryonic macrophages couple inflammatory signals to iron metabolism during early ontogenesis. *Eur J Immunol. 2014 May;44(5):1491-502.* (IF_{2016}=4.179)

Neuwirth A, Dobeš J, Oujezdská J, **Ballek O**, Benešová M, Sumník Z, Včeláková J, Koloušková S, Obermannová B, Kolář M, Stechová K, Filipp D. Eosinophils from patients with type 1 diabetes mellitus express high level of myeloid alpha-defensins and myeloperoxidase. *Cell Immunol. 2012;273(2):158-63*. (IF_{2016}=2.399)

A SPECIFIC TYPE OF MEMBRANE MICRODOMAINS IS INVOLVED IN THE MAINTENANCE AND TRANSLOCATION OF KINASE ACTIVE LCK TO LIPID RAFTS.

The membrane microdomains or lipid rafts were suggested as important dynamic platforms for controlling redistribution of signalling proteins in plasma membrane (Simons and Ikonen, 1997). Their role in T-cell signalling was implicated as a regulators of spatial-temporal organization of critical T-cell signalling proteins (Dykstra et al., 2003). Even though their existence is challenged due to possible artificiality arising from their biochemical isolation by non-ionic detergents, they still provide an invaluable biochemical tool for the assessment of membrane distribution of the protein of interest. Recently it has been shown that there are at least two kinds of lipid rafts – more accurately DRMs, clearly distinguishable by a different density and protein to lipid ratio (Otahal et al., 2010).

Here, in this study we described the spatial-temporal distribution of critical T-cell signalling proteins, Lck kinase in particular, in the context of these DRMs which we called “heavy” and “light” DRMs. The “heavy” DRMs are characterized as high molecular weight (HMW) complexes with higher density and lower lipid-to-protein ratio compare to “light” DRMs.

We showed that only Lck from HMW fractions redistribute after TCR engagement, in microtubules dependent fashion, into “light” DRMs thus partially characterizing the mechanism of delivery of Lck function during proximal T-cell signalling. In addition, for the first time, we identify that TCR/CD3 complex together with active Lck and CD45 resides in “heavy” DRMs in unstimulated T-cells thus forming the platform for maintaining their enzymatic activity. Taking together we demonstrated the microdomain-based mechanisms of regulation of T-cell activation via spatial-temporal segregation of critical signalling proteins.

Immunology Letters 142 (2012) 64–74

Contents lists available at SciVerse ScienceDirect

Immunology Letters

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

A specific type of membrane microdomains is involved in the maintenance and translocation of kinase active Lck to lipid rafts

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ARTICLE INFO

Article history:
Received 4 January 2012
Received in revised form 10 January 2012
Accepted 10 January 2012
Available online 18 January 2012

Keywords:
pY394Lck
T-cell proximal signaling
Membrane microdomains
Heavy DRMs
CD45
Microbutular cytoskeleton

ABSTRACT

Lck is the principal signal-generating tyrosine kinase of the T cell activation mechanism. We have previously demonstrated that induced Lck activation outside of lipid rafts (LR) results in the rapid translocation of a fraction of Lck to LR. While this translocation predicates the subsequent production of IL-2, the mechanism underpinning this process is unknown. Here, we describe the main attributes of this translocating pool of Lck. Using fractionation of Brij58 lysates, derived from primary naive non-activated CD4$^+$ T cells, we show that a significant portion of Lck is associated with high molecular weight complexes representing a special type of detergent-resistant membranes (DRMs) of relatively high density and sensitivity to laurylmaltoside, thus called heavy DRMs. TcR/CD4 coaggregation-mediated activation resulted in the redistribution of more than 50% of heavy DRM-associated Lck to LR in a microtubular network-dependent fashion. Remarkably, in non-activated CD4$^+$ T-cells, only heavy DRM-associated Lck is phosphorylated on its activatory tyrosine 394 and this pool of Lck is found to be membrane confined with CD45 phosphatase. These data are the first to illustrate a lipid microdomain-based mechanism concentrating the preactivated pool of cellular Lck and supporting its high stoichiometry of colocalization with CD45 in CD4$^+$ T cells. They also provide a new structural framework to assess the mechanism underpinning the compartmentalization of critical signaling elements and regulation of spatio-temporal delivery of Lck function during the T cell proximal signaling.

1. Introduction

In spite of a relatively detailed understanding of the events that occur following TCR engagement, there is still a gap in our understanding of how the signal is transduced across the membrane and how the very first biochemical signals are generated [1,2]. The traditional view is that Lck, which is primarily responsible for ITAM phosphorylation [3,4] exists in resting non-stimulated T-cells in an enzymatically inactive state. This is due to the negative regulatory tyrosine residue in C-terminal end of Lck at position 505 (Y505). When phosphorylated (pY505) by C-terminal Src kinase (Csk), it forms an intramolecular bond with its own SH2 domain, locking the kinase domain in a substrate inaccessible, closed conformation [5]. CD45 can dephosphorylate pY505, leaving Lck structure in an open, primed conformation displaying a relatively low kinase activity. To achieve full kinase activity, the phosphorylation of the positive regulatory tyrosine located in the kinase domain at position 394 must be achieved (pY394) [6]. Stimulation by the peptide-MHC complex leads to the coaggregation of TCR with CD4 or CD8 proteins, activation of associated Lck by auto/transphosphorylation and its juxtaposition to and subsequent phosphorylation of ITAMs of CD3 chains [7]. This so called aggregation model of Lck activation relies on regulatory proteins CD45 and Csk that can rapidly and reversibly turn Lck on and off, respectively. A caveat to this model is that these molecules, notably CD45, would have to preferentially target those CD4-associated Lck molecules that are involved in clustering with TCR. While structural determinants underpinning the interaction between CD45 and CD4–Lck complexes have been described [8–10] the mechanism coupling TCR triggering with CD45-mediated dephosphorylation of pY505 Lck remains unclear.

Recent data has provided new insight into the source of pY394Lck used for the initiation of TCR signaling [11]. Using phospho-specific antibodies against pY394 and pY505, the authors showed that the primary naive CD4$^+$ T cells contain a sizeable pool (~40%) of preactivated pY394Lck and this amount remained unchanged on the global cellular level after antibody as well as antigen mediated TCR stimulation. These findings corroborated results from a previous study, where the lack of evidence for anti-CD3 activation-mediated acute enrichment of the cellular pool in Lck

Abbreviations: DRM, detergent resistent membranes; LR, lipid rafts; HMW, high molecular weight fraction; SDGC, sucrose density gradient centrifugation.

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doi:10.1016/j.imlet.2012.01.001

with open and thus a more active conformation in Jurkat T cell line using FRET-based Lck biosensor construct led to similar conclusions [12]. The authors further suggested that the maintenance of steady levels of pY394Lck is dependent on its interaction with HSP90–Cdc37 chaperone complex [11]. These studies thus pose the question concerning the mechanism underpinning a spatio-temporal coupling of TCR engagement with phosphorylation of CD3 chains by preactivated Lck. That includes the elucidation of the subcellular distribution of pY394Lck and its regulatory molecules, their transient interactions and the organization of membrane structures that support the redistribution of preactivated Lck to access its physiological substrates upon TCR/CD4 ligation.

It has been postulated that certain types of signaling proteins are confined to functional membrane microdomains, called lipid rafts (LR), which are enriched in cholesterol and sphingolipids [13,14]. Due to their highly ordered structure of long saturated acyl chains stabilized by intercalated cholesterol, LR resist solubilization by mild detergents and can be isolated as detergent-resistant membranes (DRMs). Light DRMs, corresponding to classical LR, fractionate as low density, light fractions by discontinued sucrose density gradient centrifugation (SDGC) [15]. Notwithstanding controversies surrounding the existence of LR in an *in vivo* setting [16,17], including the question to what extent DRMs correspond to bona-fide native LR [18], recent experiments targeting critical signaling molecules to distinct membrane compartments [19–22], together with electron, fluorescent microscopy and nanoscopy (reviewed [23]) support the notion of a critical role of LR in the initiation of T cell signaling. Recently, a distinct type of microdomains, called high-density or heavy DRMs, was proposed to be involved in signaling in immune cells [21,22,24]. Both light and heavy DRMs share their resistance to solubilization illustrated by the use of a polyoxyethylene type of detergent (Brij58, Brij98), as opposed to their sensitivity to treatment with laurylmaltoside (LM), which disrupts protein–lipid and preserve protein–protein interactions. Due to their lower and higher protein-to-lipid ratio, light and heavy DRMs can be separated from each other by SDGC where they sediment at the top and bottom of the gradient, respectively [21]. Interestingly, as many signaling molecules reside in heavy DRMs, these non-conventional, and so far largely overlooked type of microdomains could represent an important membrane structure that is able to support T cell signaling [21,22].

Our previous work has demonstrated an activation-dependent redistribution of pY394Lck from a soluble into light DRM fractions [25]. While the enrichment of Lck in these DRMs predicates the subsequent production of IL-2 [26], the molecular mechanism and structural and functional elements regulating this translocation process remain obscure. The main goal of this study was to characterize the property of the translocating pool of kinase active Lck and reconcile our previous and current data with a newly proposed model of the proximal T cell signaling postulated to be dependent on a pool of pY394Lck preexisting in T cells prior to TCR engagement [11].

2. Materials and methods

2.1. Mice

5–8 week old females of C57BL/6 mice were used. They were maintained in a pathogen-free animal facility at the Institute of Molecular Genetics, Prague, Czech Republic.

2.2. Antibodies and reagents

Rabbit anti-mouse pY505 Lck was purchased from Biosource International (Invitrogen). Antibody specific for pY394Lck, mouse CD3ζ and Csk were obtained from SantaCruz. Antibodies directed against H-ras and anti-CD45 were obtained from Exbio (Prague, Czech Republic). Anti-CD4 antibody (H-129) used for the immunoprecipitation was obtained from BD Biosciences. Biotinylated anti-mouse CD4 specific mAb (GK1.5) and biotinylated anti-mouse TCRCβ-specific mAb (H57) were purchased from eBiosciences (USA). Anti-Lck antibody (3A5) and phosphotyrosine-specific mAb, platinum 4G10, directly conjugated to HRP, were purchased from Millipore. Anti-LAT antibody was a gift from Dr. P. Dráber, Institute of Molecular Genetics, Prague, Czech Republic. Phosphotyrosine-specific anti-pY171LAT antibody was purchased from Cell Signaling. Polyclonal rabbit anti-mouse Fyn specific antibody was a gift from Dr. Andre Veillete, IRCM, Montreal, Canada. For immunoprecipitation purposes, the desired antibodies were coupled to Protein A or G magnetic beads (Millipore). Cholera toxin B subunit-HRP (CTB-HRP), anti-actin antibody, streptavidin, Brij58 (polyoxyethylene 20 cetyl ether) and nocodazole were purchased from Sigma–Aldrich. Anti-Hsp90 antibody (EMD-17D7), latrunculin B, laurylmaltoside (n-dodecyl-β-D-maltoside, LM) and Src-family kinase inhibitor PP2 were obtained from Calbiochem (Merck). Alexa-488-conjugated phalloidin (Invitrogen) and anti-tubulin (GeneTex) visualized with goat anti-rabbit Alexa-555 (Invitrogen) were used for confocal microscopy of actin and tubulin, respectively. Ultra pure grade paraformaldehyde (PFA) was obtained from Polysciences.

2.3. Isolation and activation of primary CD4$^+$ T cells

The procedure was performed as previously described [25]. Briefly, the primary CD4$^+$ T-cells (∼95% purity) obtained by using MACS CD4$^+$ T-cell isolation kit II (AutoMACS, Miltenyi Biotec) were pre-coated with biotinylated anti-TCR (1 μg/ml) and anti-CD4 (0.3 μg/ml) in 500 μl of PBS + 3% FCS for 30 min at 4 °C, washed and resuspended in 20 μl of PBS + 3% FCS per tube. The cells were then pre-warmed for 1 min in 37 °C and TCR/CD4 coaggregation-mediated activation was achieved by addition of streptavidin to the final concentration of 50 μg/ml. Cells were briefly vortexed and incubated at 37 °C for the indicated period of time. Activation was terminated by adding either ice-cold TKM–Brij58 lysis buffer (isolation of DRMs or gel filtration) or a hot-boiled Laemli loading sample buffer (Western blotting).

2.4. Isolation of detergent resistant membranes (DRMs)

The isolation of DRMs was performed as described previously [27]. When assessing the distribution of proteins present in fractions obtained by gel filtration, pooled fractions were mixed 1:1 with 80% sucrose and analyzed using the same protocol. Light DRMs, corresponding to classical lipid rafts, are enriched in top fractions (#1–3), while the bottom fractions (#8–10) concentrate heavy DRMs together with soluble proteins.

2.5. SDS PAGE gel electrophoresis and Western blotting

Proteins resolved on polyacrylamide gels were transferred onto PVDF membrane (Millipore) and blocked for 1 h in 5% non-fat milk at RT or in 3% gelatine in TBS-T at 37 °C (phospho-specific blots). Blots were then incubated for 1 h with primary antibodies diluted in blocking buffer (5% milk or 1% gelatine, respectively), washed and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for 1 h. Blots were developed by incubation with SuperSignal ECL substrate (Thermo Scientific) and visualized by exposure to X-ray films. Where indicated, densitometric analysis was performed using a GS710 densitometer (BioRad). Values were obtained only from nonsaturated signals.

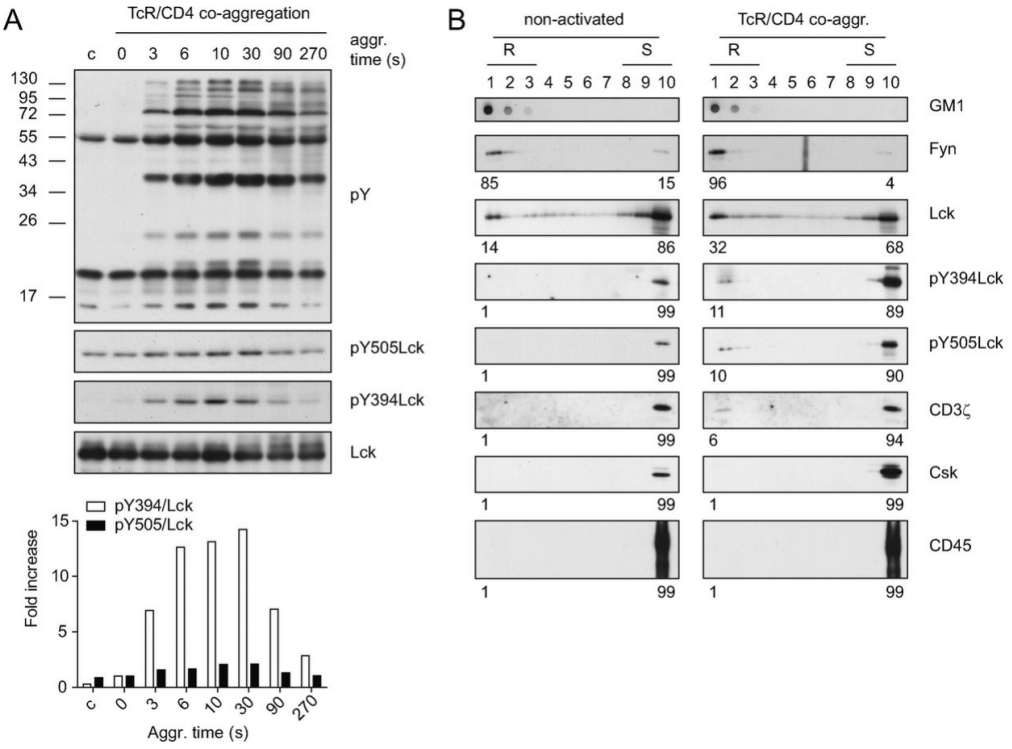

Fig. 1. Activation of CD4⁺ T cells induces a robust tyrosine phosphorylation and the redistribution of kinase active Lck and CD3ζ to light DRMs. (A) A rapid and dramatic increase in levels of kinase active Lck upon TCR/CD4 coaggregation. Freshly isolated primary CD4⁺ lymph node T cells were pre-coated, or not (c), with biotinylated anti-TCR ($H57^b$) and biotinylated anti-CD4 ($GK1.5^b$) mAbs and co-aggregated, or not (0 s) with streptavidin for the indicated time. Phosphotyrosine content of cell lysates derived from 0.5×10^6 cells/sample was probed with mAb 4G10. One representative blot from several independent experiments is shown. The histogram shows the quantification of the kinetics of pY394 and pY505 phosphorylation. Signals were normalized to total Lck. (B) CD4⁺ T-cells prepared as described in (A) and activated or not for 30 s, were lysed in TKM + 0.5% Brij58 buffer and subjected to sucrose density gradient centrifugation (SDGC) assay. Fractions were probed with CTB-HRP detecting light DRM marker GM1, and antibodies specific for pY394-, pY505- and total Lck, CD3ζ, Csk and CD45. Fyn was used as a marker of light DRMs. The numbers represent the relative distribution of proteins in the light DRMs (R) and soluble fraction (S). Number "1" indicates ≥1% of the total protein.

2.6. Gel filtration

The procedure is based on a previously described protocol [28] with slight modifications. Briefly, 5 ml pipette tip plugged with a small piece of glass wool and filled with 2 ml bed volume of Sepharose 4B beads (Sigma–Aldrich) was used as the column. 10^7 cells were lysed in TKM–Brij58 lysis buffer (50 mM Tris–HCl pH 8.0, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA pH 8.0, 0.5% Brij58), complete cocktails of protease and phosphatase inhibitors (Roche) supplemented with 10 μM PP2 for 30 min and spun down for 2 min at $800 \times g$. Supernatant in a total volume of 1/10 of stationary bead volume (200 μl) was loaded on the top of the column and eluted with cell lysis buffer. All steps were performed at 4 °C. In this setting, the fraction #4 contains complexes of $>10^7$ Da; most of the IgM and IgG standards eluted in fractions 7 and 9, respectively [28]. For assessing the involvement of cytoskeletal components in the redistribution of Lck after TCR/CD4 aggregation, CD4⁺ T cells were resuspended in PBS + 3% FCS and treated with either 2 μg/ml of latrunculin B or 10 μM of nocodazole for 30 min on ice. Subsequent pre-coating of the cells and TCR/CD4 coaggregation were also performed in the presence of these inhibitors. Before activation, small aliquots of latrunculin B and nocodazole treated as well as untreated control sample were taken for analysis by confocal microscopy.

2.7. Immunoprecipitation

High molecular weight fractions (fr#4–5) obtained by the gel filtration of Brij58 cellular lysates were pooled, transferred to antibody-pre-coated beads and incubated overnight at 4 °C on a rotational wheel (unless stated otherwise). Beads were washed, boiled in Laemmli sample buffer at 100 °C for 5–10 min and used for SDS-PAGE analysis.

2.8. Immunofluorescence microscopy

To assess the effect of cytoskeletal inhibitors on Lck distribution, treated and untreated CD4⁺ T cells (Section 2.6) were immobilized on a glass cover slip using cytospin centrifugation. After fixations with 4% PFA, the cells were washed with PBS and permeabilized with ice-cold methanol for 10 min at −20 °C (methanol step was omitted when phalloidine was used to stain actin cytoskeleton). The cells were blocked for 1 h with PBS containing 0.3% Triton X-100 (PBT) with the addition of 2.5% FCS and 2.5% BSA and incubated with primary antibody diluted in PBT for 1 h. Samples were washed and incubated with secondary antibody for 1 h. The coverslips were mounted using Vectashield containing DAPI (Vector). Samples were analyzed by confocal microscopy using Leica DMI6000.

3. Results

3.1. Coaggregation of TCR and CD4 in primary CD4+ T cells results in significant increase of pY394Lck and the redistribution of kinase active Lck and TCR/CD3 to light DRMs

We have previously demonstrated the utility of T cell activation model where the very early changes in the phosphorylation status of Lck are measured after streptavidin-mediated coaggregation of biotin-conjugated antibodies against TCRCβ and CD4 [25]. As illustrated in Fig. 1A, top panel, a few seconds after TCR/CD4 engagement, a pattern of significant tyrosine phosphorylation of several cellular substrates is readily observable. Concomitantly, using phospho-specific antibodies against positive and negative regulatory tyrosine residue Y394 and Y505 of Lck, respectively, we demonstrated the distinct magnitude of their phosphorylation levels (Fig. 1A, two middle panels). Specifically, while pY505 levels increased only slightly compared to a non-aggregated control sample (Fig. 1A, lane 0), up to a 15-fold increase in the pY394Lck levels was observed between 6 and 30 s following TCR/CD4 coaggregation. Then, over the ensuing 4 min, pY394Lck signal diminished nearly to background levels. An even more dramatic difference was observed when the maximal post-activation pY394Lck levels (Fig. 1A, lane 30) were compared to those detected in antibody non-precoated control CD4+ T cells (Fig. 1A, lane c), which were 3.3 times lower than those in the precoated but, non-aggregated cells (Fig. 1A, lane 0). This small but reproducible difference between nonprecoated and precoated cells was likely due to the limited antibody-mediated forced dimerization of CD4–Lck complexes. Thus, more than a 50-fold increase in pY394Lck levels after TCR/CD4 aggregation compared to the background levels found in unmanipulated cells indicated that the pool of preactivated Lck in primary naive CD4+ T cells represents ~2% fraction of the total Lck, at best.

The activation-induced upregulation of pY394Lck was also accompanied by the subcellular co-redistribution of signaling molecules. Notably, both pY394 and pY505 forms of Lck and CD3ζ were readily detected in light DRMs 30 s after TCR/CD4 coaggregation. In contrast, Csk and CD45 were reproducibly found outside of DRMs and the presence of neither molecule followed the pattern of Lck and CD3 redistribution after TCR/CD4 activation (Fig. 1B). These results recapitulated our previously published data demonstrating TCR/CD4 aggregation-induced rapid activation of Lck followed by its redistribution to light DRMs [25]. In addition, they showed that the global activation of Lck measured by pY394-specific antibody is independent of concomitant stochiometric dephosphorylation of negative regulatory tyrosine by CD45 as pY505 Lck levels were also, albeit marginally, increased.

3.2. Separation of heavy and light DRMs complexes containing Lck

Isolation of light DRMs with the subsequent analysis of their protein constituents provides a limited view on the composition and structural heterogeneity of various membrane microdomains. To gain insight into the nature of the translocating pool of Lck, we employed a two-step fractionation procedure which increased our ability to isolate distinct pools of Lck complexes. In the first step, the naive CD4+ T cells were treated with 0.5% Brij58 detergent and lysates were fractionated by gel filtration chromatography. As both heavy and light DRMs are insensitive to solubilization by Brij58, they are easily separated from solubilized cellular content by filtration using macroporous Sepharose 4B matrix where they co-elute in the void volume fractions of the column with high molecular weight (HMW) complexes [29]. As shown in Fig. 2A, GM1, a surrogate marker of DRM fractions as well as the light DRMs marker Fyn [25] eluted in fraction 4–5 and 5, respectively, that corresponded to large >10^6 Da complexes resistant to Brij58 mediated lysis. In contrast, Lck displays a continuous distribution along eluted fractions with approximately 25–30% of total Lck present in HMW fractions 4 and 5 that correspond to DRMs (Fig. 2A, bottom panel). When these two fractions were pooled and subjected to SDGC, nearly all (>90%) of HMW fraction-associated Lck was found at the bottom of the gradient, *i.e.* in the heavy DRMs fraction (Fig. 2B). Importantly, the integrity of these Lck-containing DRM fractions is sensitive to treatment with LM detergent (Fig. 5, top panel). The remaining Lck gel filtration-derived fractions with lower MW were found at the bottom of the SDGC gradient as they likely represent soluble complexes of different sizes. In contrast, virtually all Fyn from HMW fractions localized to light DRMs on the top of the gradient (Fig. 2B).

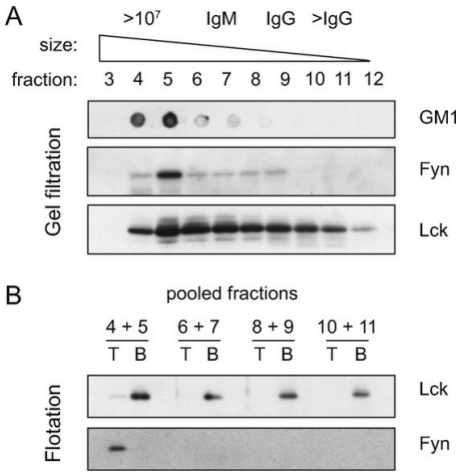

Fig. 2. Distribution of high molecular weight complexes-associated Lck in lipid rafts. (A) The fractionation of CD4+ T-cells lysed in TKM + 0.5% Brij58 buffer using a Sepharose 4B gel filtration chromatography. Fractions (3–12) were probed with CTB-HRP detecting GM1 (upper panel), anti-Fyn (middle panel) and anti-Lck (bottom panel). Approximate molecular weight size marker is shown on the top. (B) SDGC (flotation) assay of pooled fractions (as indicated) obtained by gel filtration chromatography in (A). Light DRM fractions 1 and 2 from the top of the gradient (T) and soluble protein enriched fraction 9 and 10 from the bottom (B) were probed with anti-Lck and anti-Fyn.

3.3. Heavy DRM-associated Lck redistributes to light DRMs after T cell stimulation

To evaluate which Lck fractions participate in its activation-induced translocation to light DRMs, we assessed the redistribution of Lck in the context of pooled fractions derived from gel filtration before and 30 s after TCR/CD4 coaggregation. As illustrated in Fig. 3A, under these conditions more than half of the heavy DRM fraction-associated Lck (pooled fraction #4–5) was redistributed to light DRMs. The kinetics of this redistribution was relatively rapid, reaching its maximum between 10 and 30 s following the TCR/CD4 engagement (Fig. 3B and C). Together, these experiments (Figs. 2 and 3) revealed that in unstimulated primary CD4+ T cells, different types of DRMs physically segregate Lck and Fyn. Notably, while Fyn localizes to light DRMs corresponding to classical LR, ~25% of total cellular Lck is detected in heavy DRMs. The significant redistribution of Lck from this membrane fraction to light DRMs is

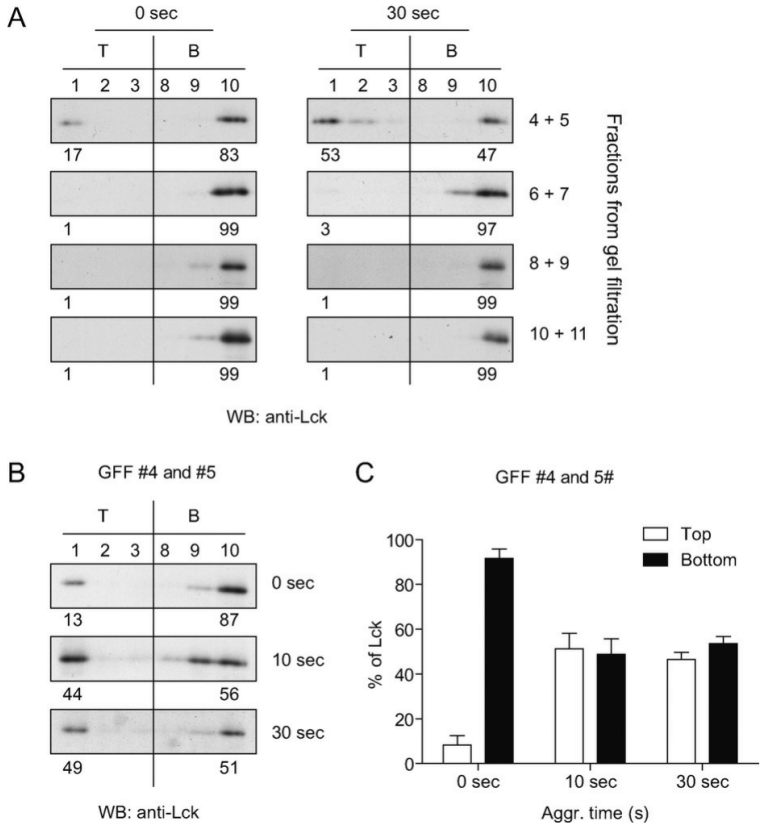

Fig. 3. TCR/CD4 coaggregation resulted in the redistribution of high molecular weight complexes-associated Lck to light DRMs. (A) The primary CD4$^+$ T cells were pre-coated with H57^b and GK1.5^b and co-aggregated, or not (0 s) with streptavidin for 30 s. Pooled fractions from lysates derived from non-aggregated or aggregated cells obtained as described in Fig. 2A were subjected to SDGC. The relative distribution of Lck to the top fractions with light DRMs (T) and bottom heavy fractions (B) is indicated under each panel. (B) The kinetics of Lck redistribution to light DRMs (T). The cells were prepared as described in (A) and activated or not (0 s) for 10 s and 30 s. The relative distribution of Lck in gel filtration fractions (GFFs) #4–5 was assessed by immunoprobing with anti-Lck and is indicated under each panel. (C) Quantification (±s.d.) of enrichment of HMW complexes-associated Lck in light DRMs (T) as shown in (B) from three independent experiments.

indicative of a possible functional involvement of heavy DRMs in the initiation of TCR signaling.

3.4. Microtubular inhibitor profoundly affects the outcome of activation-induced Lck redistribution

As shown in Fig. 3A, only the pool of heavy DRM-associated Lck is "licensed" to redistribute to light DRMs after TCR/CD4 cell stimulation. Previous and current data implicated both actin and microtubular cytoskeletal networks in the initiation of T cell signaling [30,31]. To distinguish between these two possibilities, we pretreated non-stimulated CD4$^+$ T cells with either latrunculin B or nocodazole, pharmacological agents inhibiting polymerization of actin or tubulin, respectively (Fig. 4A), and assessed their effect on activation-induced Lck redistribution. As illustrated in Fig. 4B, nocodazole, but not latrunculin B, completely blocked the activation-induced enrichment of Lck in light DRMs. This suggests that the microtubular network is essential for the integrity of redistribution processes occurring upon TCR/CD4 engagement.

3.5. Preactivated pool of kinase active pY394Lck localizes to heavy DRMs

To gain insight into a possible role of heavy DRMs in the regulation of Lck physiology, we first assessed the phosphorylation pattern of Lck residing in these microdomains (Fig. 5). Surprisingly, pY394Lck signal was almost exclusively localized to fractions representing heavy DRMs as the treatment with LM resulted in its complete shift to lower MW fractions. In contrast, pY505 Lck signal displayed a more balanced distribution with some diminishment in fractions with medium sized complexes. Among other proteins that positively or negatively regulate Lck activity or are important for initiation of TCR signaling, almost the entire pool of CD3ζ, a tangible fraction of CD45 (up to 20%) and a small fraction of actin (<2%) were found to be associated with heavy DRMs. On the other hand, Csk, Hsp90 and tubulin were completely absent in these fractions and treatment with LM failed to change this distribution pattern. LAT and H-ras, residing in light and heavy DRMs [21], respectively, were used as distribution markers. These data together with those presented in Fig. 1B demonstrate that in unstimulated primary CD4$^+$ T cells, most of the constitutively active pool of

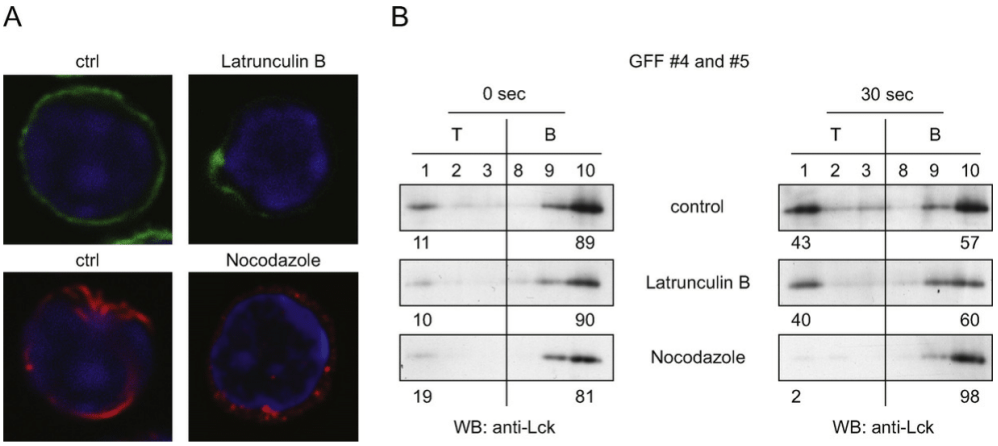

Fig. 4. The destabilization of microtubular network profoundly affect the outcome of activation-induced Lck redistribution to light DRMs. (A) Confocal microscopy images of primary CD4⁺ T cells pretreated with cytoskeletal inhibitors show that both latrunculin B and nocodazole efficiently destabilize actin (green) and microtubular networks (red), respectively. Nuclei are stained with DAPI (blue). (B) Primary CD4⁺ T cells were pretreated with latrunculin B or nocodazole and activated for 30 s as described in Fig. 3A. The relative distribution of Lck in gel filtration fractions (GFFs) #4–5 was assessed by immunoprobing with anti-Lck and is indicated under each panel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

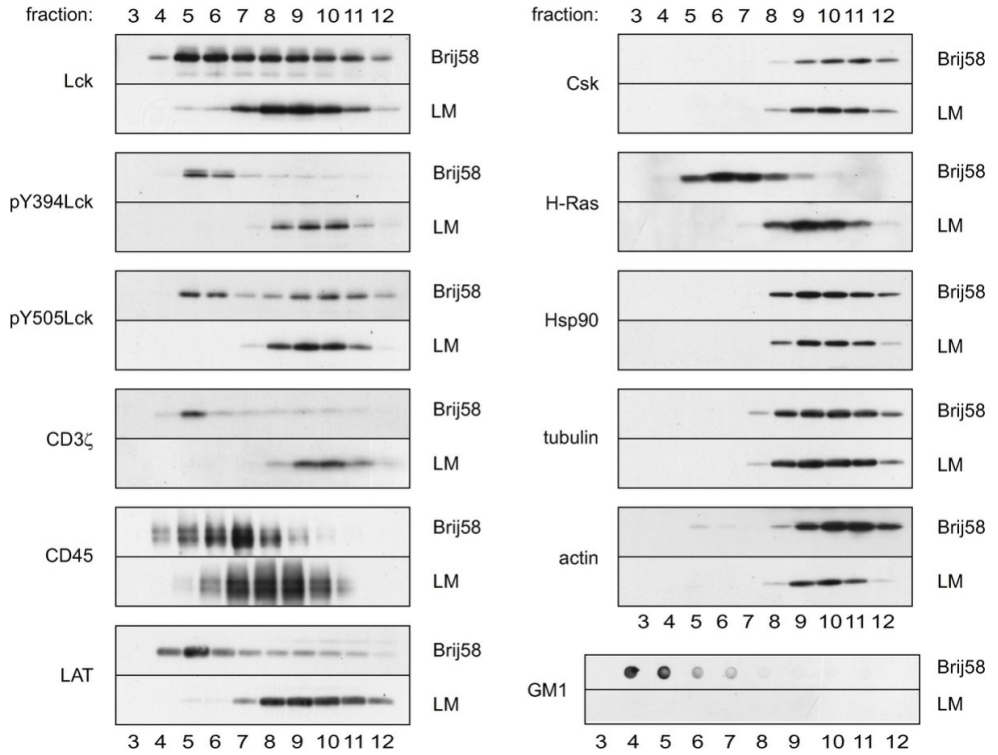

Fig. 5. In primary naive CD4⁺ T cells pY394Lck associates with LM-sensitive type of membrane microdomains, heavy DRMs. CD4⁺ T-cells were lysed in TKM + 0.5% Brij58 buffer (left panels) or in TKM + 1% laurylmaltoside buffer (right panels) and fractionated using a Sepharose 4B gel filtration chromatography. Fractions (3–12) from each of these two lysates were probed with antibody directed against indicated protein or its particular phosphorylated form. The same fractions were also probed with CTB-HRP to reveal GM1, the surrogate marker of light DRMs.

Lck and CD3ζ are sequestered in the membrane microdomains that upon solubilization with Brij58 yielded heavy DRMs. They also suggests that CD45 phosphatase localized to heavy DRMs could contribute to the maintenance of pY394Lck levels in these microdomains.

3.6. pY394Lck but not the inactive Lck colocalizes with CD45 to heavy DRMs

The physical association of CD4–Lck and CD45 has been previously documented [8,10]. It has been proposed that the essential function of CD45 in these complexes is to keep CD4-associated Lck primed for rapid activation upon TCR triggering [8]. As illustrated in Fig. 6A, TCR/CD4 coaggregation for 30 s significantly increased the pY394Lck signal in gel filtration fractions (GFFs) corresponding to heavy DRMs. Specifically, from a total cellular >5-fold increase in pY394Lck content, the fraction #5 alone contributed by up to 70%. The increase in pY171LAT level served as a control of activation. Since the proximity of CD45 and pY394Lck may be critical for this effect, their direct interaction in this fraction was examined. The immunoprecipitation of CD4 or Lck from the total cellular or heavy DRM NP-40 lysates failed to reveal co-immunoprecipitated CD45 (data not shown). However, it has been previously suggested that membrane confinement of proteins can dramatically increased their association rates [32]. In this respect, we took advantage of the previously documented fact that the solubilization of cells with Brij98 allows the coimmunoprecipitation of several TCR signaling molecules associated by the virtue of their co-residency in a certain subset of DRMs [33]. Thus, testing this possibility, we first assessed whether CD4–Lck and CD45 are juxtaposed by the virtue of their membrane confinement. Using anti-CD4 immunoprecipitation from DRM fractions #4–5 prepared from Brij58 lysates in which the integrity of DRMs is preserved, the colocalization of CD4–Lck with CD45 was readily detectable (Fig. 6B). Since both TCR/CD4 coaggregation-mediated increase of pY394Lck signal (Figs. 6A and 1B) and the source of translocating Lck (Fig. 3) are largely limited to the pool of Lck associated with heavy DRMs, we assessed the effect of TCR activation on the stochiometry of Lck colocalization with CD45 and changes in the pY394 status of Lck. As shown in Fig. 6C (top two panels and the bar graph on the left), TCR/CD4 coaggregation resulted in a rapid, >5 fold increase in pY394Lck signals. Concomitantly, the amount of CD45 colocalized with anti-Lck was gradually reduced to approximately 30% of that observed in unstimulated $CD4^+$ T cells (Fig. 6C, bottom panel and the bar graph on the right). These dynamic changes in the stochiometry of CD45–CD4–Lck colocalization point to a functional link between heavy DRMs and the initiation of TCR signaling.

While the above described results support the notion about the co-partitioning of critical signaling molecules to membrane microdomains, they failed to provide resolution to the question of whether the pool of heavy DRM-associated Lck is colocalized with CD45 independently of its activation status and whether T cell activation-mediated increase in pY394Lck levels can impact this interaction. Since both kinase active pY394- and non-phosphorylated Y394-Lck can be colocalized with CD45, sequential precipitations of these two forms of Lck followed by the visualization of associated CD45 were performed. As illustrated in Fig. 6D (left panels), sequential precipitation from pooled heavy DRM fractions (fr#4–5) derived from non-activated primary $CD4^+$ T cells with anti-pY394Lck followed by anti-Lck revealed that only about 20% of Lck in these fractions were present in its active form (top left panel). Unexpectedly, while the preactivated form of Lck effectively co-precipitated with CD45, the amount of co-precipitated phosphatase was vastly reduced upon the clearing of remaining non-phosphorylated Y394Lck with anti-Lck (middle and bottom left panels). Thus, because close to 80% of heavy DRM-associated Lck is present in non-phosphorylated Y394 form and is not colocalized with CD45 (Fig. 6D, left panels), it suggests that two distinct heavy DRMs exist in non-activated $CD4^+$ T cells: one type containing pY394Lck and CD45 and the other accumulating Y394Lck largely deprived of CD45. TCR/CD4 coaggregation significantly increased the ratio between pY394- and Y394-Lck in heavy DRM fractions (upper right panel). However, similar to non-activated T cells, only kinase active pY394Lck, but not Y394Lck, co-precipitated a considerable amount of CD45. It is of note that despite their very low stoichiometry of association, Y394Lck predominantly precipitated the HMW isotype of CD45. These findings suggest that the colocalization of heavy DRM-associated CD45 with active pY394Lck is imposed by the mechanism of membrane confinement.

4. Discussion

In this report, we provide evidence that the pool of preactivated cellular pY394Lck is associated with high molecular weight complexes that are sensitive to treatment with LM, identifying them as heavy DRMs. We also show that heavy DRM-associated pY394Lck is colocalized with CD4 and CD45 proteins and that upon TCR/CD4-mediated activation only this pool of Lck increased its kinase activity and was redistributed to light DRMs in a microtubular network dependent fashion. These data are first to describe a lipid microdomain-based mechanism concentrating the preactivated pool of cellular Lck and supporting its high stoichiometry of colocalization with CD45 in $CD4^+$ T cells.

Before discussing our findings in the context of their coherence with previously published data, we feel obliged to briefly comment on the credibility of our methodological approach which relies on membrane solubilization and fractionation protocols. We are fully aware of potential artifactuality of detergent-mediated cellular solubilization in respect to the content resemblance of various types of DRMs with different lipid rafts in cells. In this regard, and highly relevant to data presented herein, a relatively high degree of specificity of Brij98 detergent for assorted lipid rafts translated to its capacity to preserve distinct subpopulations of DRMs has been recently demonstrated [23,33,34]. We show here that Brij58 detergent which displays a very similar solubilization pattern in T cells [21,28,29] reproducibly separates at least two different types of DRMs that are separable by SDGC: light (corresponding to classical LR) and heavy (newly described type of microdomains in T cells). A strongly biased distribution of Fyn to light DRMs and Lck and CD3ζ to heavy DRMs, correlated with uncoupled kinetics of Fyn and Lck activation upon TCR/CD4 aggregation [25] attests to the suitability of Brij58 to yield structurally and functionally separable fractions that likely concentrate a distinct lipid raft membrane microenvironment.

How do our results fit with the current models of T cell activation? Recently published data suggested that more than 40% of cellular Lck is present in an active pY394 phosphorylated state prior to the TCR/CD4 engagement and this level stays unchanged during the first few minutes after the initiation of T cell signaling [11]. In agreement with this statement, Lck conformational changes were not observed [12]. Others also observed the existence of pY394Lck in non-activated T cells [25,29,35–37], but a diligent quantification of this pool of Lck was not reported in those studies. Our data demonstrate that TCR/CD4 activation results in ~15–50 fold increase in pY394Lck levels, depending on the treatment of cells from which background levels are taken into consideration (Fig. 1A). This result strongly argues that the pool of preactivated pY394Lck represents ≤2% of the total cellular Lck. Moreover, we show that the increase in pY394Lck levels is largely restricted to the pool of Lck residing in lipid microdomains yielding heavy DRMs. Since the reason for this discrepancy with a previously published

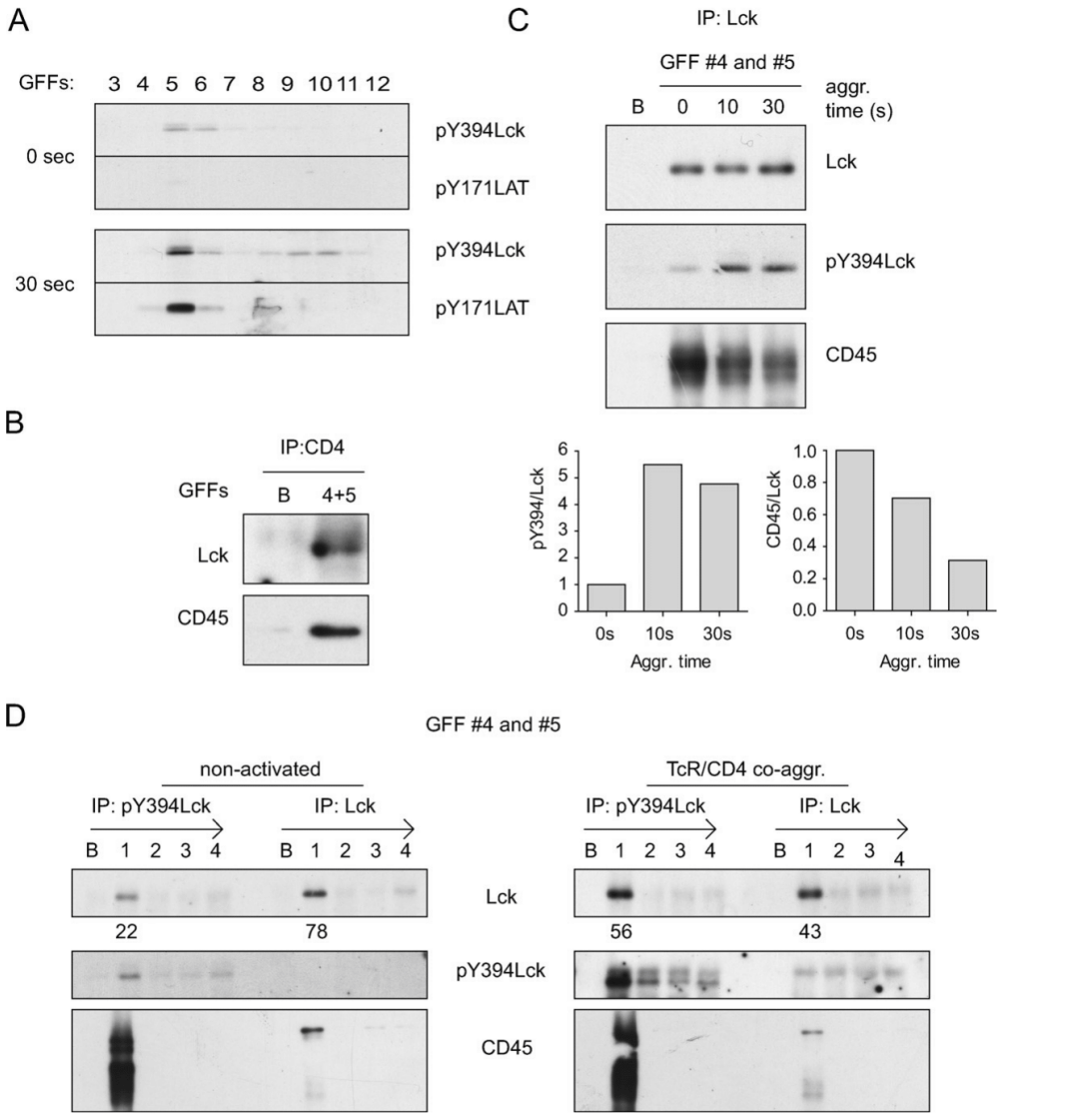

Fig. 6. pY394Lck colocalizes with CD45 to heavy DRMs distinct to those of inactive Y394Lck. (A) TCR/CD4 coaggregation-mediated increase in pY394Lck levels localizes predominantly to heavy DRM fractions. The primary $CD4^+$ T cells were pre-coated with $H57^b$ and $GK1.5^b$ and co-aggregated, or not (0 s) with streptavidin for 30 s. All fractions (3–12) from TKM + Brij58 lysates derived from either non-activated (upper panels) or activated cells (bottom panels), obtained by gel filtration, were probed with anti-pY394Lck and anti-pY171LAT. (B) CD4–Lck and CD45 colocalize in the heavy DRMs. Pooled heavy DRM gel filtration fractions (GFFs#4–5) obtained from unmanipulated primary $CD4^+$ T cells lysed in TKM + Brij58 were immunoprecipitated with anti-CD4. The immunoprecipitate was split and immunoprobed for Lck and CD45. Unprecoated beads (B) served as a control. (C) TCR/CD4 coaggregation affects the levels of pY394Lck and the stoichiometry of colocalization of Lck and CD45 in heavy DRM. The cells were prepared as described in Fig. 3A and then activated or not (0) for 10 s and 30 s. Gel filtration fractions (GFFs) #4–5 were pooled and immunoprecipitated with anti-Lck. The immunoprecipitates were split and immunoprobed with anti-Lck (top panel), anti-pY394Lck (middle panel) and anti-CD45 (bottom panel). Kinetics of changes in pY394Lck and total CD45 levels normalized to total Lck content is presented under panels. (D) The colocalization of Lck with CD45 in heavy DRMs depends on activation status of the kinase. The cells were prepared as described in Fig. 3A and then activated or not (0) for 30 s. Gel filtration fractions (GFFs) #4–5 were pooled and subjected to four sequential precipitations initiated with anti-pY394Lck (4 times, 2 h each) and followed with anti-Lck. Immunoprecipitates were revealed by immunoblotting with anti-Lck, anti-pY394Lck and CD45.

data is uncertain, further experiments must be done before re-addressing this issue. That controversy also concerns the previously reported fact that aggregation of TCR alone or TCR with CD4 results in a detectable increase in cellular levels of pY394Lck [9,25,38,39]. Another finding that is inconsistent with previous reports is the absence of the co-distribution of Hsp90 with pY394Lck in heavy DRMs (Fig. 5). Since it was postulated that Hsp90 and its kinase-specific co-chaperone Cdc37 interact with Lck *via* its catalytic domain [40] which is critical for the stability of the active form of Lck [11,41,42], the specific role of these chaperones in the maintenance of the pool of preactivated Lck residing in heavy DRMs awaits further investigation.

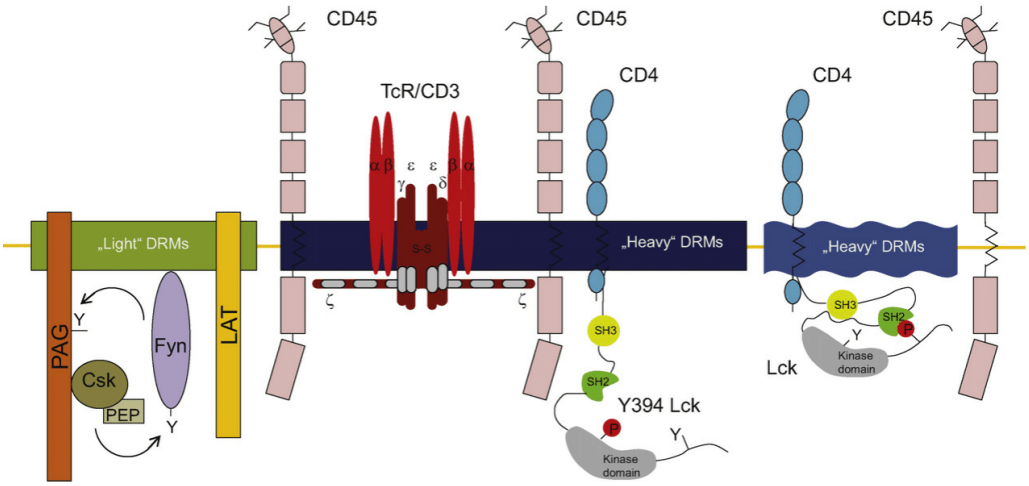

Fig. 7. The incorporation of heavy DRMs into a current model of TCR triggering. In non-activated CD4⁺ T cells only a small fraction (<5%) of Lck exists in activated pY394 form. This preactivated pool of Lck is localized to a specific type of microdomains that upon solubilization with Brij58 or Brij98 detergents yield heavy DRMs. TCR/CD3 complex together with CD45 are colocalized with pY394Lck to heavy DRMs (dark blue rectangle) by a virtue of membrane confinement. At least two mechanisms suppressing tyrosine phosphorylation (pY) driven triggering of TCR signaling could operate in these microdomains: (i) high abundance and the enzymatic activity of the membrane phosphatase CD45 which is able to dephosphorylate pY505 and pY394 residues on Lck as well as pY-ITAMs on CD3 chains thus keeping the net rate of pY very low; and (ii) CD3ζ and CD3ε (not depicted here) intracellular chains could be buried in the lipid-rich inner leaflet of plasma membrane and thus shielded from active Lck (illustrated as a close apposition of CD3ζ with the heavy DRMs) [59]. The novelty of this model is that the enzymatic action and mutual random interactions between pY394Lck, CD45 and CD3 chains are spatially restricted to a confined environment rendering other potential substrates outside these microdomains and thus inaccessible to tyrosine phosphorylation. A vast majority of heavy DRM-associated Lck, which is not activated, likely resides in a distinct type of heavy DRMs largely deprived of CD45 (light blue wavy rectangle). In the third type of microdomains, light DRMs, Fyn kinase activity is likely regulated by a negative regulatory feedback mechanism employing adaptor protein PAG, Csk and PEP phosphatase [46,60]. Critical adaptor protein LAT, the phosphorylation of which initiates TCR downstream signaling, is also localized to light DRMs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Certainly, some of the above described discrepancies could relate to the usage of distinct type of T cells, mode of activation and detection limit of methods used. Just a few examples: (i) primary CD4⁺ T cells and Jurkat cells display significant quantitative differences in the expression of critical signaling molecule such as TCR, CD4, Lck and Fyn as well as in their subcellular distribution [43–47]; (ii) it has been documented that CD3ε- and TCR/CD4-mediated activation preferentially target distinct kinases, Fyn and Lck, respectively [25,36,48–51]; (iii) FRET approach could be less sensitive when the detection of conformational changes is limited to only a small fraction of Lck measured on a high background level of FRET [52]. However, notwithstanding the fact that distinct modes of T cell activation could be operational in various types of T cells, fundamental principles explaining the contribution, interdependence and spatio-temporal relationships between signaling molecules that guide and coordinate the complex process of T cell activation must be formulated.

Low basal levels of pY394 in CD4⁺ T cells described in this report are consistent with basic assumptions of Davis and van der Merwe's kinetic-segregation (KS) model of T cell activation [53]. This model predicts that random interactions between Lck itself, with tyrosine phosphatases CD45 and CD148 and with Csk, in resting state, establish the balance between various forms of Lck. One of them, kinase active pY394Lck can in turn phosphorylate ITAMs of CD3 chains making any T cell vulnerable to spontaneous activation However, this scenario is prevented by a high abundance and the enzymatic activity of the membrane phosphatase CD45 which is able to dephosphorylate pY505 and pY394 residues on Lck as well as pY-ITAMs on CD3 chains [54]. CD45 is thus regarded as the central suppressor of tyrosine phosphorylation-driven T cell activation processes [1,53].

The presence in T cells of a preactivated pool of Lck aided the KS model, as the need for an explanation of the mechanism of Lck activation is obviated [55,56]. However, our estimation of approximately 20-fold lower amount of the fraction of pY394Lck persisting in resting unmanipulated T cells in comparison with a previous report [11] could raise significant discrepancies in the consideration of quantitative aspects of biochemical reactions underpinning T cell activation [55]. Moreover, rather than being freely diffusable in the plasma membrane, our results suggest that pY394Lck and a sizeable pool of CD45 are co-localized to heavy DRMs (Fig. 6D). That suggests that the pool of pY394Lck is not generated at random but is spatially controlled by its confinement to heavy DRMs where it colocalizes with CD45. In the support of this notion, it has been recently documented that, in the native non-adherent state, a considerable fractions of CD45 and CD4 are co-localized by a virtue of membrane confinement that could account for their enhanced association rate [57]. This finding is important as it suggests that Brij58 heavy DRMs selectively concentrate molecules shown to be co-embedded into a certain type of membrane environment *in vivo*, thus attesting to the suitability of Brij58-mediated solubilization approach in characterization these types of membrane microdomains.

Also intriguing is the presence of most of CD3ζ in heavy DRMs and its timely coordinated redistribution with active Lck to light DRMs. While it suggests that heavy DRMs could represent a membrane structural component supporting the communication between the Lck regulatory and TCR triggering modules [58], a better understanding of the structural heterogeneity and functional diversity of heavy DRMs is needed to support such a conclusion. Nevertheless, based on data presented in this study, a simplified model of the distribution of essential TCR signaling molecules to various membrane microdomains can be drawn (Fig. 7). This model is consistent with the suggestion that TCR/CD4 coaggregation increases the affinity of heavy DRMs to coalesce with light DRMs in a tubulin-dependent manner, and thus juxtaposes the

pY394Lck/pYCD3ζ/ZAP70/complex to the vicinity of the LAT adaptor protein residing in light DRMs [21] allowing the amplification of the activation signal.

5. Conclusions

Data presented in this study revealed an unappreciated complexity of mechanism controlling Lck involvement in TCR proximal signaling. The novelty of this work is in providing a framework for the more accurate assessment of subcellular compartmentalization of critical molecules prior to the initiation of TCR signaling. That especially relates to the pool of preexisting kinase active Lck, TCR complex and a tangible fraction of CD45 segregated from other membrane components and potential Lck substrates. This study also indicates that the full understanding of molecular events driving TCR mediated signaling is possible only when the organization of a cell surface protein is put in the context of membrane structures that support it.

Acknowledgments

We thank Z. Cimburek from Flow Cytometry Facility at the Institute of Molecular Genetics for expert technical assistance. This work was supported by Grant 310/09/2084 from Grant Agency of Czech Republic (GA ČR) and Grant AVOZ50520514 from the Academy of Sciences of the Czech Republic.

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THE POOL OF PRE-ACTIVATED LCK IN THE INITIATION OF T-CELL SIGNALLING: A CRITICAL RE-EVALUATION OF THE LCK STANDBY MODEL

How Lck activity is regulated during initiation of T-cell signalling is not fully understood. Recently proposed "standby" model of TCR triggering suggest that in unstimulated T-cells there is up to 50% of constitutively active Lck (Nika et al., 2010). They showed that during the T-cell activation, the levels of $pY394^{Lck}$ would not increase, the result which contrast sharply with our data. We showed that in immediately boiled samples, the $pY394^{Lck}$ levels increase after TCR engagement up to fifty times (Ballek et al., 2012).

In this study we provided the likely explanation what causes such experimental discrepancy. We showed that technical procedure of cellular solubilisation results in a spontaneous increase of phosphorylation levels in proteins. This could severely compromise the quantification outcomes. We suggested to add the kinase inhibitor PP2 into lysis buffer which largely overcomes this problem. In conclusion, the amount of $pY394^{Lck}$ levels is much more limited than showed before, thus challenging the standby model and calling for critical re-evaluation of role of this pre-activated Lck during TCR triggering.

Immunology and Cell Biology (2015) 93, 384–395

www.nature.com/icb

ORIGINAL ARTICLE

The pool of preactivated Lck in the initiation of T-cell signaling: a critical re-evaluation of the Lck standby model

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The initiation of T-cell receptor (TCR) signaling, based on the cobinding of TCR and CD4-Lck heterodimer to a peptide–major histocompatibility complex II on antigen presenting cells, represents a classical model of T-cell signaling. What is less clear however, is the mechanism which translates TCR engagement to the phosphorylation of immunoreceptor tyrosine-based activation motifs on CD3 chains and how this event is coupled to the delivery of Lck function. Recently proposed 'standby model of Lck' posits that resting T-cells contain an abundant pool of constitutively active Lck (pY394^{Lck}) required for TCR triggering, and this amount, upon TCR engagement, remains constant. Here, we show that although maintenance of the limited pool of pY394^{Lck} is necessary for the generation of TCR proximal signals in a time-restricted fashion, the total amount of this pool, ~2%, is much smaller than previously reported (~40%). We provide evidence that this dramatic discrepancy in the content of pY394^{Lck}is likely the consequence of spontaneous phosphorylation of Lck that occurred after cell solubilization. Additional discrepancies can be accounted for by the sensitivity of different pY394^{Lck}-specific antibodies and the type of detergents used. These data suggest that reagents and conditions used for the quantification of signaling parameters must be carefully validated and interpreted. Thus, the limited size of pY394^{Lck} pool in primary T-cells invites a discussion regarding the adjustment of the quantitative parameters of the standby model of Lck and reevaluation of the mechanism by which this pool contributes to the generation of proximal TCR signaling.
Immunology and Cell Biology (2015) **93,** 384–395; doi:10.1038/icb.2014.100; published online 25 November 2014

The classical model of T-cell receptor (TCR) signaling proposes that the initiation of this event depends on the cobinding of TCR and CD4-Lck heterodimer to a peptide-major histocompatibility complex II (MHCII) on an antigen presenting cell. In this process, a non-receptor lymphocyte-specific protein tyrosine kinase Lck has a crucial role in the generation of the most proximal signals.[1] The juxtaposition of activated Lck to the vicinity of immunoreceptor tyrosine-based activation motifs (ITAMs) on TCR-associated CD3 chains leads to their tyrosine phosphorylation. In this process, which is generally referred to as TCR triggering,[2] phosphorylated ITAMs serve as recruitment sites for ZAP70 kinase that drives downstream signaling by phosphorylating the transmembrane adapter protein linker for activated T-cells (LAT) on multiple tyrosine residues and thus nucleating the signalosome.[3] As, in this classical scenario, the basal kinase activity of Lck in resting T cells has long been considered to be negligible, it was originally proposed that its activation is the consequence of TCR–peptide–MHCII complex binding.

The catalytic activity of Lck is controlled via conformation changes in Lck structure which is principally regulated by two critical tyrosine residues, Y505 and Y394, localized at the C-terminal segment and catalytic domain of Lck, respectively.[4] When Y505 is phosphorylated, it binds to its own SH2 domain, thus locking Lck into an inactive, 'closed' conformation. Although the phosphorylation of Y505 residue is under the control of the C-terminal Src kinase, this action can be counteracted by CD45 phosphatase resulting in an open or 'primed' conformation that possesses catalytic activity. The full kinase activity of Lck is achieved via transphosphorylation on the positive regulatory tyrosine residue Y394.[5]

Three distinct, but not mutually exclusive, mechanisms have been proposed to explain how TCR–peptide–MHCII complex interaction translates into TCR triggering, namely TCR aggregation, conformational change and spatial segregation.[6] An important aspect of these models is that while each of them acknowledges the necessity for Lck enzymatic activity, they are not always able to conceptually explain the molecular infrastructure and its spatio-temporal dynamics that couples TCR engagement to the acute activation of Lck.

Recently, the TCR-induced Lck-activation paradigm was challenged by Nika *et al.*[7] in a study in which they proposed an alternative 'standby model of Lck/Fyn'. It posits that (i) in T-cells, ~40% of Lck persists in a constitutively active state (pY394^{Lck}); (ii) the pY394^{Lck} pool does not increase after anti-CD3ε or antigen-mediated TCR stimulation; and (iii) pY394^{Lck}, maintained in resting T-cells by a

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Received 1 July 2014; revised 23 October 2014; accepted 24 October 2014; published online 25 November 2014

geldanamycin-sensitive HSP90-CDC37 complex, is required for TCR signaling. Together, these data argue that the persistence of an abundant pool of pY394^{Lck} in resting T-cells obviates the need for a specific mechanism linking TCR engagement to activation of Lck required for TCR triggering.[8] In our attempt to reproduce these results, we observed that in primary resting CD4$^+$ T-cells, pY394^{Lck} represented $<2\%$ of the total Lck.[9,10] Here, we present data from our investigation that provide possible explanations to account for such a dramatic discrepancy in pY394^{Lck} content and that suggest that the original data may have been incomplete. In addition, we describe experiments that argue that the existence of limited pool of pY394^{Lck} in the primary T-cells seems to be critical for the generation of timely regulated TCR proximal signals.

RESULTS

Experimental conditions and the type of reagents affect the quantification of phosphorylation status of Lck in T-cells

The key for accurate quantification of *in vivo* pY394^{Lck} levels is their preservation during the process of cell solubilization. However, while tyrosine dephosphorylation is routinely prevented by the addition of a protease inhibitor cocktail, the level of spontaneous tyrosine phosphorylation is usually left uncontrolled. Figure 1a demonstrates a marked difference in the pY394^{Lck} levels in non-activated cells that were lysed in regular detergent buffer (PP2$^-$) compared with those instantly solubilized in boiled Laemmli buffer (two left middle panels with the relative amount of pY394^{Lck} indicated). The inclusion of 20 μM of the Src-family kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine)[11] which was omitted in previous studies, lowered pY394^{Lck} levels to that observed in boiled samples (Figure 1a, middle panels, PP2$^+$; for the titration of PP2 see Supplementary Figure 1). Overall, the statistical analysis showed a significant difference in the pY394^{Lck} content between the non-activated and activated samples irrespective of their mode of solubilization. However, a noticeable diminishment of this difference in PP2$^-$ conditions is a direct consequence of much higher pY394^{Lck} levels in non-activated samples (Figure 1a).

Differences in pY Lck patterns were also observed among various detergents used for cell solubilization (Figure 1b). Importantly, the addition of PP2 to these buffers significantly and reproducibly diminished pY levels to values approaching those found in directly boiled samples. We also observed tangible differences between patterns revealed by anti-pY394^{Lck} and anti-pY418^{Src} antibodies, both targeting pY394^{Lck} (Figures 1a and b). Together, these results demonstrate that high levels of pY394^{Lck} found in the original study were likely the consequence of spontaneous phosphorylation of Lck that occurred after cell solubilization[7] and that quantification of these levels is affected by different pY394^{Lck}-specific antibodies as well as the type of detergent used (Figures 1a and b).

Antigen and TCR/CD4- but not CD3ε-mediated activation of primary CD4$^+$ T cells induces the phosphorylation of Y394^{Lck}

The original data set, using pY418^{Src}-specific antibody, which showed unchanged levels of pY394^{Lck} after TCR stimulation seems to be inconclusive.[7] The caveat of this set of experiments is that anti-CD3ε signaling preferentially targets Fyn over Lck[12–14] thus the increase in pY394^{Lck} levels is usually only marginal. Moreover, the inability to detect these minute changes in pY394^{Lck} content in antigen activated T cells on a global level often relates to the limited sensitivity of western blot analysis.[7] Accordingly, our experiments showed that antibody-mediated anti-CD3ε or TCRβ activations failed to detect any significant elevation of Lck kinase activity (Figure 2a). In contrast, their combined effect with anti-CD4 antibody exhibited a dramatic increase in pY394^{Lck} levels (Figure 2a). Similarly, the immunoprecipitation (IP) of Lck from *n*-dodecyl-β-d-maltoside-containing lysates supplemented with PP2 confirmed a significant increase in the level of pY394^{Lck} after TCR/CD4 coaggregation, whereas after CD3ε stimulation this level remained unchanged (Figure 2b). Thus, it seems that to use the pool of pY394^{Lck} as a readout for gauging changes in Lck activation status upon anti-CD3 signaling is questionable and would require further experimental justification. It is of note that while the ligation of CD4 alone significantly increased the level of pY394^{Lck}, it was not able to trigger tyrosine phosphorylation of downstream proteins and required co-ligation with TCRβ to achieve this phenotype (Figure 2a).

To test whether a measurable increase in Lck kinase activity accompanies the process of early T-cell activation under more physiological conditions, we tracked the kinetics of global changes in the pY394^{Lck} pattern upon incubation of ovalbumin-specific TCR transgenic CD4$^+$ T cells with ovalbumin-loaded bone marrow-derived dendritic cells (BMDCs). As illustrated in Figure 2c, subtle but significant, on an average 1.5- to 2-fold increases in pY394 levels of Lck during the first 12 min of cell conjugate formation were reproducibly observed. It is important to emphasize that likely due to non-synchronous formation of immunological synapses among T cell-BMDC conjugates some fluctuations in the level of pY394^{Lck} were observed, these values always exceeded those determined in nonactivated T cells.

The unresponsiveness of CD4$^+$ T cells treated with geldanamycin correlates with its adverse pleiotropic effect on components of TCR signaling machinery

The original report suggested that the persistence of high levels of pY394^{Lck} is maintained in resting T cells by a geldanamycin-sensitive HSP90-CDC37 complex. In support of this notion, the authors showed that a 3 h treatment of T cells with geldanamycin, which destabilized these complexes, resulted in a severe reduction of pY394^{Lck} levels which, in turn, correlated with T-cell unresponsiveness to anti-CD3 stimulation measured by the induction of pY142CD3ζ.[7] In contrast, our data showed that not 3- but 24-h geldanamycin treatment was necessary to deplete pY394^{Lck} from both resting primary CD4$^+$ T cells and Jurkat cells (Figure 3a). This prolonged time course of geldanamycin-mediated degradation of Lck is in full agreement with two previous independent studies.[15,16] Moreover, the unresponsiveness of pY394^{Lck}-depleted primary T cells to TCR/CD4 coaggregation (Figure 3b) correlated with the displacement of Lck from the membrane (Figure 3c, upper panel) and its failure to associate with the CD4 co-receptor (Figure 3d). The presence of the cytosolic fraction of Lck as well as GAPDH were also grossly impacted (Figure 3c). Geldanamycin treatment had also a dramatic effect on cell viability (Figure 3e) and down-regulation of surface CD4 and TCR on live cells (Figure 3f). It is of note that the incubation of primary CD4$^+$ T cells with a mixture of geldanamycin and 2 ng ml^{-1} of interleukin-7, the anti-apoptotic cytokine that promotes the survival of T cells, also failed to restore T-cell responsiveness (data not shown). These data suggest that geldanamycin-mediated defective TCR triggering could be accounted for by its adverse pleiotropic effects on the cellular environment supporting TCR signaling rather than targeted depletion of the preactivated pool of Lck from the system.

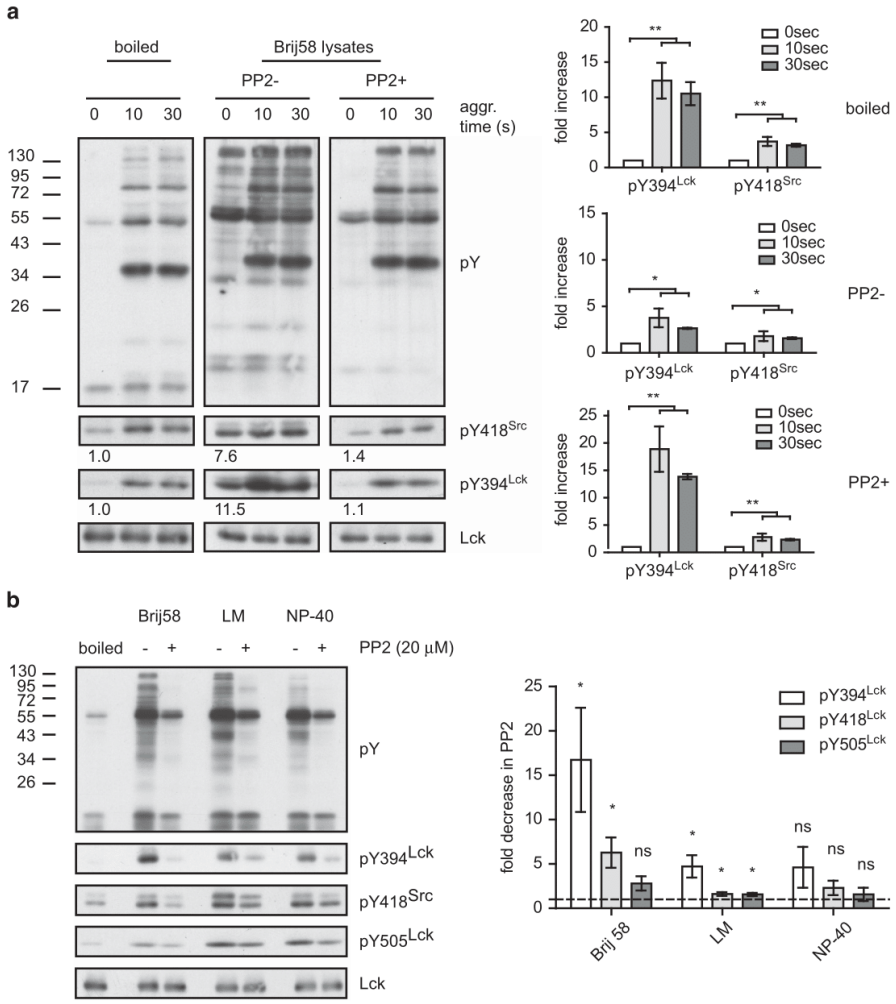


Figure 1 Experimental conditions and the type of reagents affected the quantification of phosphorylation status of Lck in T-cells. (**a**) The solubilization of T-cells in detergent buffers leads to spontaneous tyrosine phosphorylation of Lck that is preventable by the addition of PP2. Freshly isolated primary $CD4^+$ lymph node T-cells were precoated with biotinylated anti-TCR and biotinylated anti-CD4 mAbs and activated, or not (0), with the addition of streptavidin for the time indicated. Cells were then either immediately boiled in Laemmli sample buffer (boiled, left panel) or solubilized for 30 min in TKM+0.5% Brij58 lysis buffer supplemented with or without 20 μM PP2 (PP2+, right panel and PP2-, middle panel, respectively) and subsequently boiled in the Laemmli buffer. The phosphotyrosine content of cell lysates was assessed by western blotting using pY-specific antibodies 4G10, $pY418^{Src}$, $pY394^{Lck}$ and anti-Lck. One representative blot from three independent experiments is shown. Bar graphs on the right side represent the quantification of $pY394^{Lck}$ and $pY418^{Src}$ levels expressed as their fold increment after stimulation in different lysis conditions using indicated antibody reagents. All signals were normalized to total Lck levels where the nonaggregated samples in each condition were given a reference value of '1'. The statistical analysis presented as mean ± s.d. was performed using one sample *t*-test, $*P<0.05$; $**P<0.01$. (**b**) Differences in the phosphorylation status of Lck were dependent on the presence of PP2, the type of buffer detergent and the phospho-specific antibody used. The primary $CD4^+$ T cells were lysed in TKM buffer containing the indicated detergent and supplemented with (+) or without (−) PP2. The bar graph on the right shows the quantification of $pY394^{Lck}$, $pY418^{Src}$ and $pY505^{Lck}$ signals normalized to total Lck and expressed as their fold decrease after PP2 treatment in different lysis conditions using the indicated antibodies. The statistical significance of this decrease, calculated from three independent experiments using a one-tailed Student's *t*-test, is presented as mean ± s.d., $*P<0.05$; ns, not significant. The dashed line represents relative values of indicated site-specific phosphorylation in boiled untreated control samples which were given a reference value of '1'.

The pervanadate treatment of $CD4^+$ T cells dramatically elevates the amount of $pY394^{Lck}$

The standby model of TCR depicts a scenario of ~40% of cellular Lck is found in a preactivated pY394 state[7] and that this Lck pool is required to drive TCR triggering. In contrast, our previous estimate showed that this percentage could be dramatically lower, ⩽2%. Because the density of kinase active Lck on the membrane is a predictive parameter for the regulation of TCRζ phosphorylation,[17] its

accurate estimate is critically important for the modeling of quantitative aspects of TCR proximal signaling.[18] The treatment of cells with pervanadate (pV), a potent phosphatase inhibitor,[19,20] provides an alternative way to assess the proportion of preactivated Lck in peripheral resting CD4$^+$ T cells. Specifically, the ratio between the signal intensity of pY394^{Lck} in pV-activated and resting T cells

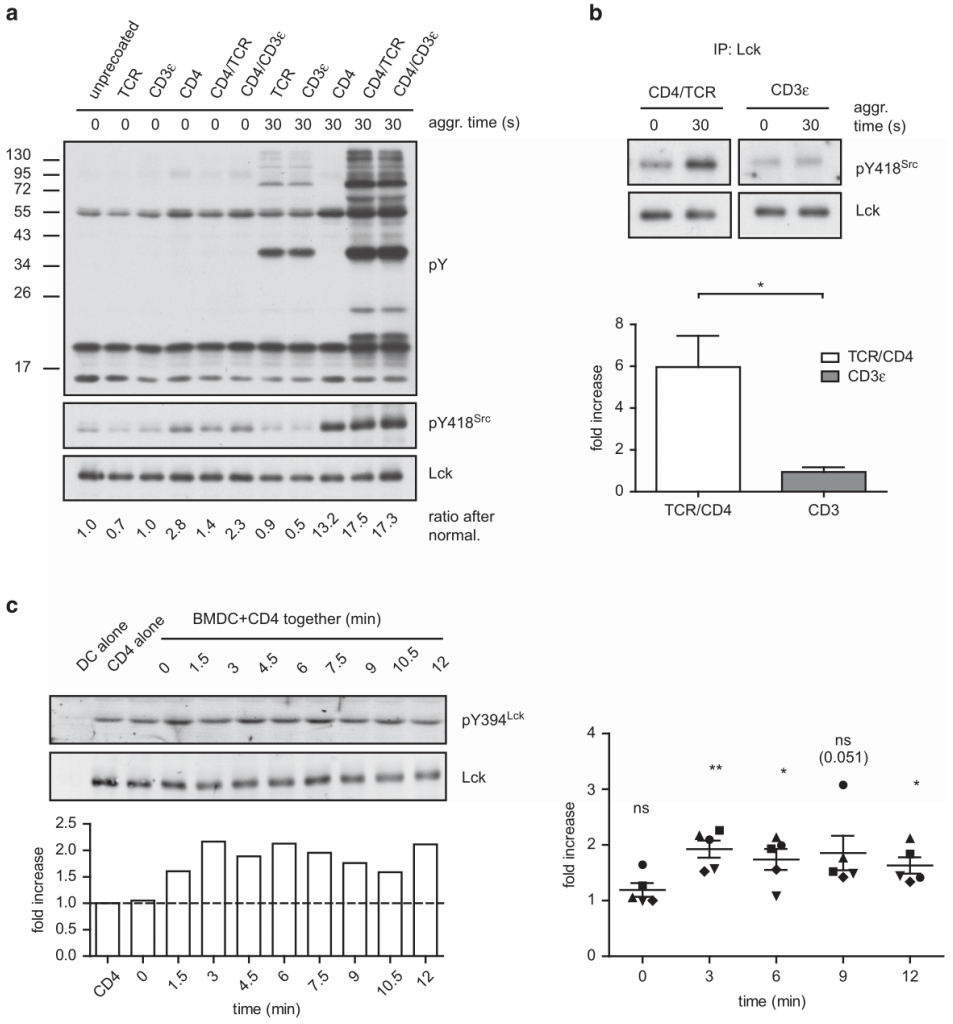


Figure 2 Antigen and TCR/CD4- but not CD3ε-mediated activation of the primary CD4$^+$ T cells induces the phosphorylation of Y394^{Lck}. (**a**) Freshly isolated primary CD4$^+$ lymph node T cells were precoated with the indicated biotinylated antibodies, or left untreated (non-precoated), and co-aggregated with streptavidin, or not (0), for 30 s. Cells were then instantly lysed in Laemmli sample buffer, resolved on a gel and their content probed with the indicated antibodies. The relative level of pY394^{Lck} signals, revealed using anti-pY418^{Src} antibody and normalized to total Lck, is indicated. One representative blot from three independent experiments is shown. (**b**) CD4$^+$ T cells were activated for 30 s, or not (0), by the co-aggregation of biotinylated anti-TCR and CD4 or anti-CD3ε antibodies. Cells were immediately lysed in ice-cold TKM+1% LM lysis buffer supplemented with 20 μM PP2. Lck was immunoprecipitated with anti-Lck antibody (clone 3A5) and probed with anti-pY418^{Src} and anti-Lck. The bar graphs (bottom panel) show the statistical analysis of this experiment as mean ± s.d., $n=3$, performed using a two-tailed Student's *t*-test, $^*P<0.05$. (**c**) Incubation of ovalbumin-pulsed bone marrow-derived dendritic cells (BMDCs) with ovalbumin-specific CD4$^+$ primary T cells resulted in the significant increase of pY394 Lck levels. Cell lysates of the mixture of BMDCs and T cells co-incubated for indicated time points were probed with anti-pY394^{Lck} and Lck (two panels on the left). Levels of pY394^{Lck} and total Lck in individual populations of BMDCs (DC alone) and CD4$^+$ T cells (CD4 alone) are also shown. One representative blot from five independent experiments is shown. The bar graph represents the quantification of pY394^{Lck} levels normalized to total Lck as fold increase over the unmanipulated control T cells (CD4 alone) which was given a reference value of '1'. The scatter plot on the right shows the statistical analysis of all five independent experiments ($n=5$) where data is shown as mean ± s.d., performed using a two-tailed Student's *t*-test between CD4$^+$ controls and samples activated for indicated period of time. $^*P<0.05$, $^{**}P<0.01$, ns, not significant. Values from each independent experiments are labeled with an identical symbol.

provides an estimate of fold increase in pY394 content. If 40% of Lck is indeed preactivated, then the maximum possible achievable increase in the $pY394^{Lck}$ level would be ~2.5-fold.

As illustrated in Fig. 4, left western blot panel, the kinetics of resting $CD4^+$ T cells treated with 500 μM of pV showed a rapid increase in the content of $pY394^{Lck}$, reaching maximal levels between 8 and16 min (Figure 4, left part of each marked line graph). However, due to the fact that total Lck levels dropped after 2 min of treatment, which is likely due to the cellular degradation of Lck, we chose this time point for further assessments of pV dose responses. The accumulation of $Y394^{Lck}$ reached its plateau at 100–1000 μM of pV (Figure 4, right western blot panel), at which its fold increment, compared with untreated cells, was in the range between 60–80 and 20–25, as measured with $pY394^{Lck}$ and anti-$pY418^{Src}$ antibody, respectively (Figure 4, right part of each marked line graph). This suggests that the size of $pY394^{Lck}$ pool in primary resting $CD4^+$ T cells is around 1–2 and 3–5% of total Lck, respectively. Our data also demonstrated a 7- to 14-fold activation-dependent increase in the $pY505^{Lck}$ signal. In addition, a similar increase in $pY394^{Lck}$ accompanied by a moderate increase in $pY505^{Lck}$ was observed in primary $CD8^+$ T cells and thymocytes (Supplementary Figure 2). Unstimulated Jurkat T cells showed much higher levels of phosphorylation on both Y394 and Y505 Lck in comparison with primary T cells and their levels upon treatment with pV increased only slightly (Supplementary Figure 2). Together, these data are consistent with our previous results and support the notion that the basal amounts of $pY394^{Lck}$ in mouse resting T cells, but not Jurkat cells, represent only a very limited proportion of total Lck.

CD45 phosphatase affects the amplitude and rapidity of TCR signaling

The critical question is whether this small pool of preactivated $pY394^{Lck}$ in unstimulated T cells is sufficient for productive TCR triggering. An experimental approach aimed at the diminution of this pool before TCR engagement could provide some clues. As an alternative to geldanamycin treatment, we isolated peripheral lymph node $CD4^+$ T cells from CD45 deficient mice ($Ptprc^{-/-}$) where this population, even though severely decreased in numbers, is still present. The absence of CD45 phosphatase should nudge the Lck phosphorylation balance toward the enrichment of pY505Lck and diminishment of the $pY394^{Lck}$ pool. This, in turn, would provide a good testing ground for the assessment of the consequences of a reduced $pY394^{Lck}$ pool for TCR triggering.

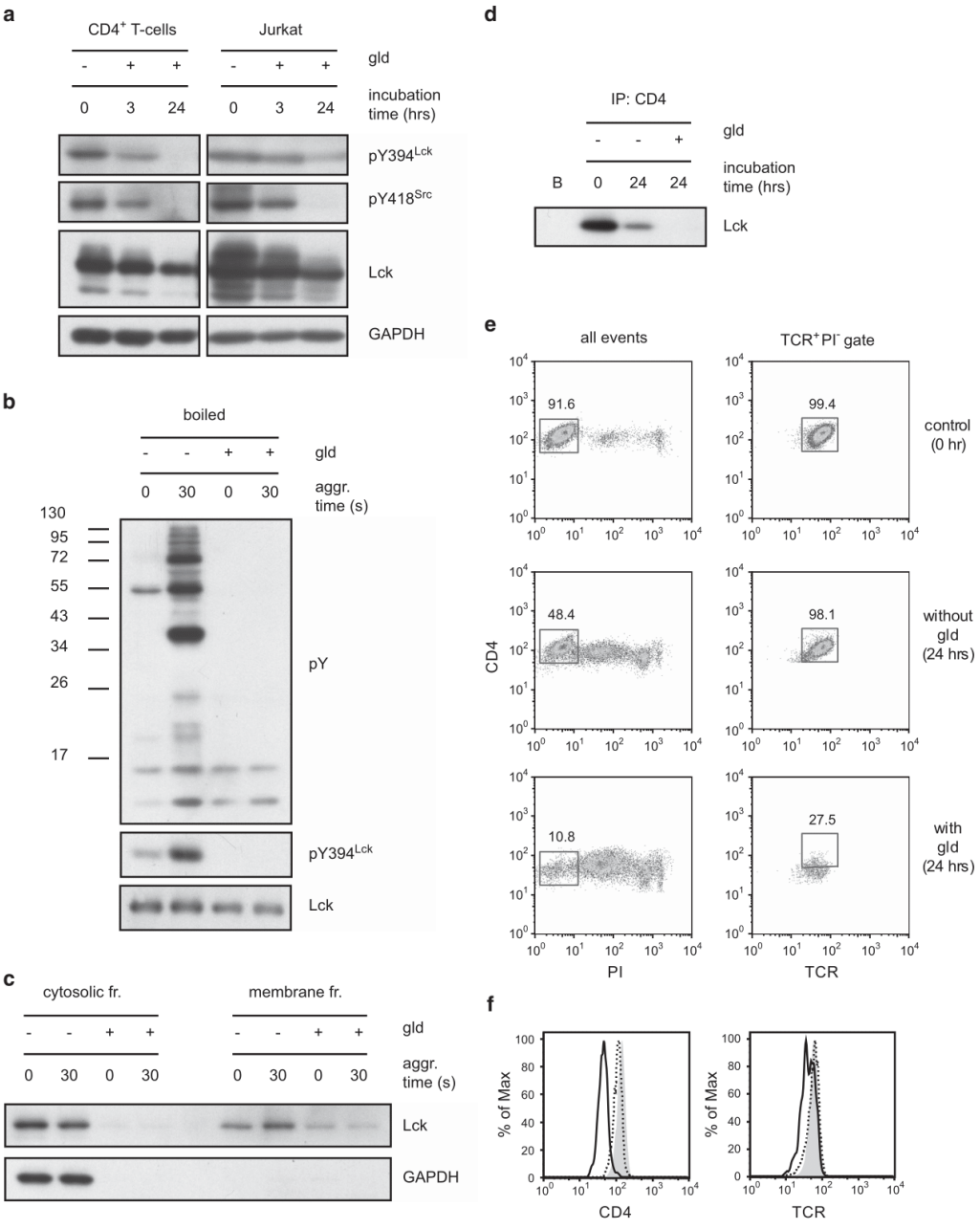
As illustrated in Figure 5a, left panels, the sequential precipitation initiated by anti-$pY505^{Lck}$ followed by anti-Lck, confirmed that in $Ptprc^{-/-}$ mice nearly approximately 90-92% of total Lck in resting $CD4^+$ T cells is phosphorylated on Y505 compared with only approximately 20–25% in the wild type. The control western blot confirmed that the vast majority of Lck was removed from $Ptprc^{-/-}$ T cells by anti-pY505 IP (Figure 5a, right panels). In contrast, as shown in Figure 5b, in both wild-type and $Ptprc^{-/-}$ T cells, the level of $pY394^{Lck}$ in unstimulated cells was barely detectable by both pY394-detecting antibodies ($pY394^{Lck}$ and $pY418^{Src}$), thus confirming their very low levels in resting T-cells. The activation of these cells by pV treatment exhibited a significant and comparable activatory effect on Y394 of Lck in both wild-type and $Ptprc^{-/-}$ T cells, with minor quantitative differences between the two different antibodies used for the detection of $pY394^{Lck}$ levels (Figure 5b). This suggests that there is no principal difference in the capacity of Lck from both wild-type and $Ptprc^{-/-}$ T cells to undergo phosphorylation on Y394.

Simultaneously, and as predicted, a marked difference between wild-type and $Ptprc^{-/-}$ T cells was observed in changes occurring on Y505. Its phosphorylation in wild-type cells increased upon treatment with pV up to eightfold, at which point it approximated the signal intensity in $Ptprc^{-/-}$ T cells. The $pY505^{Lck}$ signal in resting and activated $Ptprc^{-/-}$ T cells remained largely unchanged and saturated, thus corroborating the result presented in Figure 5a. This demonstrates that pharmacologically-mediated or the genetically-engineered lack of CD45 phosphatase activity results in nearly a complete phosphorylation of Lck pool on Y505. Interestingly, notwithstanding this initial difference in the phosphorylation status of Y505 between wild-type and $Ptprc^{-/-}$ T cells, the kinetics and amplitude of pV-induced phosphorylation on Y394 were similar (Figure 5b).

In contrast to pV stimulation, the comparative analysis of signaling through TCR showed a principally different result. The coaggregation of TCR and CD4 on wild-type and $Ptprc^{-/-}$ T cells showed a delayed kinetics and reduced amplitude of TCR triggering in the latter (Figure 6). Notably, the activation-induced appearance of $pY394^{Lck}$ was delayed by at least 20 s and was reduced to about 30% of its

Figure 3 The unresponsiveness of $CD4^+$ T cells treated with geldanamycin correlates with adverse pleiotropic effect on components of TCR signaling machinery. (**a**) The depletion of $pY394^{Lck}$ from T cells required a prolonged, 24 h incubation with geldanamycin. The primary $CD4^+$ T cells were incubated for the indicated period of time with or without 5 μM geldanamycin (gld). Samples were then boiled in Laemmli sample buffer and probed with anti-$pY394^{Lck}$-specific antibody and anti-Lck. GAPDH served as a loading control. One representative blot from four independent experiments is shown. (**b**) The treatment of $CD4^+$ T cells with geldanamycin leads to their unresponsiveness. The cells were incubated for 24 h in the presence of either 5 μM geldanamycin (+) or DMSO as a control (–) and then activated, or not (0), by the antibody-mediated co-aggregation of TCR/CD4 for 30 s. The aliquots of whole cell lysates were probed with phosphotyrosine-specific antibody 4G10 (pY), anti-$pY394^{Lck}$ and anti-Lck. One representative blot from three independent experiments is shown. (**c**) Geldanamycin induces the displacement of Lck and GAPDH from a cytosolic fraction. T cells were pretreated with geldanamycin and subsequently activated as described in **b**. Cytosolic and membrane fractions were collected and probed with anti-Lck antibody. The distribution of the cytosolic marker GAPDH was used to assess the efficiency of fractionation. One representative blot from three independent experiments is shown. (**d**) Geldanamycin induced the dissociation of Lck from CD4. T cells were incubated for the indicated time with 5 μM geldanamycin (+) or with DMSO as a control (–) and lysed in TKM buffer supplemented with 1% NP-40. CD4 was immunoprecipitated and coimmunoprecpitated Lck levels were assessed by western blotting. B, the control sample with precoated beads only. One representative blot from two independent experiments is shown. (**e** and **f**) Geldanamycin treatment resulted in a dramatic decrease in cell viability and downregulation of CD4 and TCR on live cells. (**e**) The cells were incubated, or not (0 h), for 24 h in the presence or absence of 5 μM geldanamycin, stained with directly conjugated antibodies against CD4 and TCR and analyzed by fluorescence-activated cell sorting. Dead cells were visualized by the addition of propidium iodide (PI). The percentage of gated live cells is indicated in left panels. A comparison of TCR and CD4 expression levels (gated on live cells shown in left panels) is presented in right panels. Numbers indicate the proportion of cells expressing physiological surface levels of TCR and CD4 in comparison with those found in freshly isolated T cells (top right panel). (**f**) Histogram overlays show the downregulation of CD4 (left panel) and TCR (right panel) expression in cells treated for 24 h with geldanamycin (solid line) as compared with untreated (dotted line) and freshly isolated T cells (gray filled histogram). Data are based on the fluorescence-activated cell sorting analysis shown in **e**. One representative blot from three independent experiments is shown.

maximal level observed in wild-type T cells 10 s after the coaggregation (Figure 6, pY394^{Lck} blot and bar graph). In agreement with these data, the phosphorylation of LAT, the downstream target of ZAP70, was also delayed with a similar kinetics and reduced to ~60% of its phosphorylation levels in wild-type T cells. Together, these data showed that the cellular pool of Lck that is phosphorylated on Y505 to saturated levels does not prevent its TCR-mediated activation. However, the modestly reduced amplitude of Lck activation and related TCR proximal signals correlated with the retarded onset of these events.



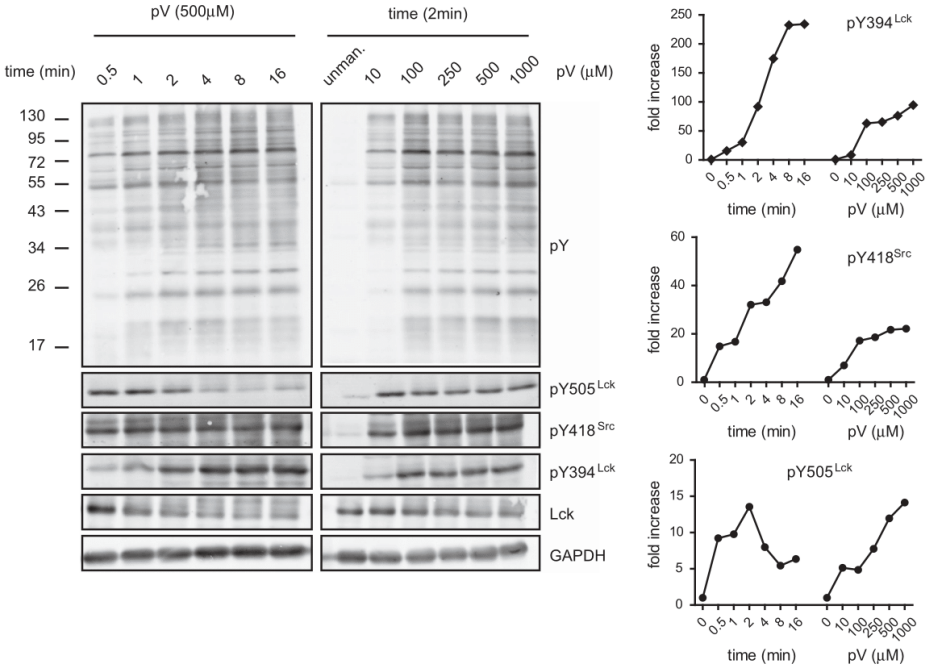


Figure 4 The pervanadate (pV) treatment of CD4+ T cells dramatically elevates amounts of pY394^{Lck}. The phosphotyrosine content of naive CD4+ T cells activated by pV in time- and dose-dependent fashion was probed with pY-specific antibody 4G10, pY505^{Lck}, pY418^{Src}, pY394^{Lck}, anti-Lck and anti-GAPDH. One representative blot from three independent experiments is shown. Line marked graphs on the right side represent the quantification of these experiments. pY394^{Lck}, pY418^{Src} and pY505^{Lck} content is presented as their fold increment after stimulation. All signals were normalized to total Lck and GAPDH levels. Unmanipulated samples were given a reference value of '1'.

DISCUSSION

Whereas several reports have documented the existence of a kinase active pool of Lck persisting in T cells prior to the onset of TCR signaling, Nika *et al.*[7] were first to formalize its existence by proposing the 'stand-by' model of Lck. Although this model has drawn significant attention, the independent verification of its quantitative parameters by other labs has been thus far lacking.

Here, consistent with our previous report, we showed that basal amounts of pY394^{Lck} in resting CD4+ T cells represented only a limited proportion of total Lck. Notably, our data point to the fact that differences in the level of pY394^{Lck} between freshly isolated, but otherwise unmanipulated CD4+ T cells and the same cells activated by antibody-mediated aggregation of anti-TCR/CD4 receptors[9] or by pV treatment (Figure 5b) is approximately 50- and 40- to 60-fold, respectively, thus representing ~2% of total Lck. As we aimed at maximizing the cellular level of pY394^{Lck}, we consider the TCR/CD4 coaggregation as well as pV treatment as a legitimate way to quantify the difference in levels of pY394^{Lck} between resting and globally activated T cells. In this context, we point out that the T cell precoating procedure itself increases background levels of pY394^{Lck} phosphorylation in comparison with non-precoated cells by two to five times (Figure 2a and Ballek *et al.*[9]). Thus, the 40- to 60-fold increase in pY394^{Lck} levels after treatment with pV, which requires no precoating, is equivalent to its 8- to 20-fold increase observed after TCR/CD4 coaggregation. It is also of note that for the quantification of phosphotyrosine-specific signals, we used comparable methods to those employed by Nika *et al.*,[7] the combination of near-infrared fluorescent and chemiluminescent western blot imaging. However, we did not repeat *in situ* fluorescent imaging of intact cells. The reason was that both anti-pY418^{Src} and anti-pY394^{Lck} antibodies, as assayed by western blotting and immunofluorescence on fibroblasts as well as T cells, recognize several intracellular substrates (Supplementary Figure 3), making this approach of limited value.

We would like to emphasize that pY394^{Lck} levels in our relevant experiments were measured in samples that were immediately solubilized by boiling in Laemmli buffer that eliminates the potentially undesirable effect of spontaneous tyrosine phosporylation that is observed in buffer-solubilized samples. In this context, we showed here the necessity to add an Src-family tyrosine kinases inhibitor, such as PP2, to prevent this effect. Our data strongly suggest that the relatively high estimate of pY394^{Lck} levels in T cells in the original report[7] is a direct consequence of T-cell solubilization in the absence of the kinase inhibitor. Nevertheless, we would like to point out that even the addition of PP2 to the lysis buffer might not completely prevent the spontaneous activation of Lck in all experimental conditions. For example, the relatively lengthy IP of Lck by bead-bound antibody can deliberately crosslink the enzyme and thus affect the quantitative aspects of its phosphorylation state. In addition, we showed that the condition of solubilization, in terms of the type of detergent used, as well as the subtle and elusive differences in quality and specificity of antibodies, can affect the

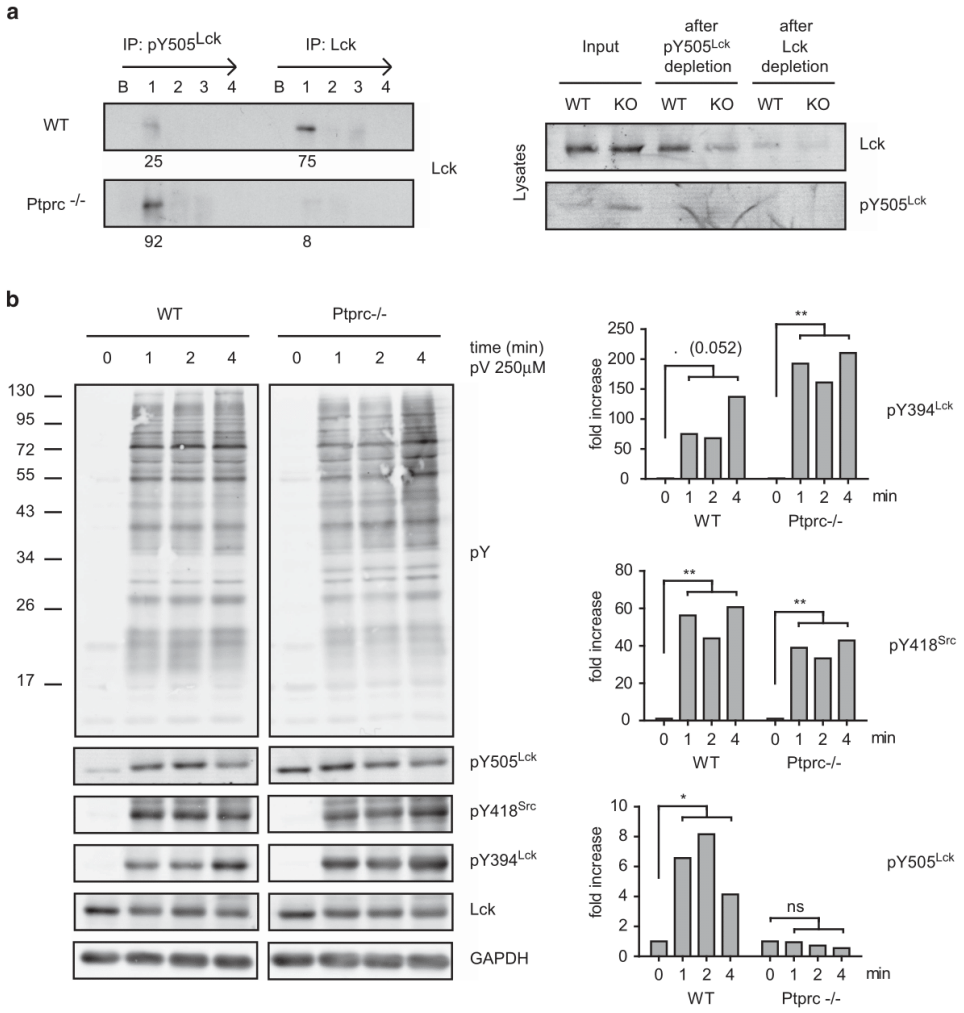


Figure 5 Different levels of pY505^{Lck} have no apparent effect on the kinetics and amplitude of pervanadate-mediated Y394^{Lck} phosphorylation. (**a**) Left panel: naive CD4$^+$ T cells from wild-type (WT) and CD45KO (Ptprc$^{-/-}$) mice were isolated and lysed in 0.4% LM-lysis buffer for 30 min on ice. Lysates were then precleared, denatured by the addition of SDS to a final of 1% concentration and boiled for 5 min at 100 °C. SDS was quenched by the addition of 1% LM-lysis buffer. Samples were then subjected for four rounds of immunoprecipitation (IP) with anti-pY505^{Lck} antibody and subsequent four rounds of IP with anti-Lck antibody. B, the control sample with precoated beads only. Right panel: control of IP efficiency. Part of the lysates was analyzed before and after each IP depletion step and probed with anti-Lck and anti pY505^{Lck} antibody. One representative blot from two independent experiments is shown. (**b**) Naïve CD4$^+$ T cells from wild-type and Ptprc$^{-/-}$ mice were activated by 250 µM pervanadate for 2 min. The activation was stopped by the addition of hot Laemmli buffer. Samples were immediately boiled and probed with the same panel of antibodies as in Figure 4. One representative blot from two independent experiments is shown. Bar graphs on the right side represent the quantification of pY394^{Lck}, pY418^{Src} and pY505^{Lck} levels expressed as their fold increment after pV stimulation. All signals were normalized to total Lck and GAPDH levels. Unmanipulated samples were given a reference value of '1'. The statistical analysis presented as mean ± s.d. was performed using one sample *t*-test, $*P<0.05$; $**P<0.01$; • (0.052), marginal significance with indicated *P*-value; ns, not significant.

quantitative readout of these experiments. However, the fluctuation value of these readouts in the presence of PP2 was diminished to nearly basal levels observed in immediately boiled samples (Figure 1b). Thus, whenever possible and irrespective of the experimental protocol, that is, type of cells, method of activation, technique of detection and type of antibodies used, the background levels of a given protein tyrosine phosphorylation, and not only Lck as presented in this work, should be determined in cellular lysates established by the immediate boiling of samples of interest and compared with those levels obtained by detergent solubilization in the presence or absence of the kinase inhibitor. This will ensure that the levels of tyrosine phosphorylation observed during cellular processes are quantitatively compared with their basal values that approximate those persisting in cells before their solubilization.

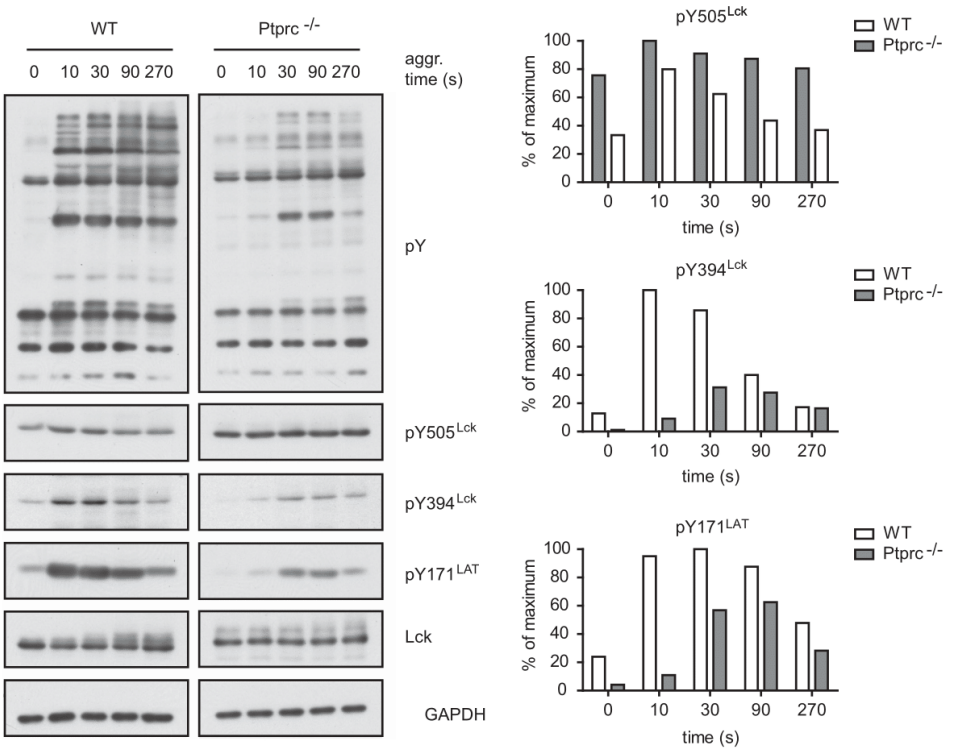


Figure 6 High levels of pY505^{Lck} delay the kinetics and lower the amplitude of TCR triggering. The naive CD4$^+$ T cells from wild-type (WT) and Ptprc$^{-/-}$ mice were precoated with biotinylated anti-TCR and anti-CD4 mAbs and activated, or not (0), with the addition of streptavidin for the time indicated. Cells were immediately boiled in Laemmli sample buffer and probed with pY-specific antibody 4G10, pY505^{Lck}, pY394^{Lck}, pY171^{LAT}, anti-Lck and anti-GAPDH. Bar graphs on the right side represent the quantification of pY505^{Lck}, pY394^{Lck} and pY171^{LAT} levels expressed as their relative levels of maximum measured signal. One representative blot from two independent experiments is shown.

Whether the Lck activity upon productive TCR engagement is increased or not is the subject of intense debate. The standby model of Lck posits that an abundant persistence of preactivated Lck in resting T cells makes the additional activation of Lck dispensable for TCR signaling. Indeed, the authors showed that anti-CD3ε stimulation resulted in only negligible, if any, changes in Lck activity on a global cellular level.[7] Consistent with this report, using a Förster resonance energy transfer-based biosensor that correlates positive changes in an open conformation of Lck with its activation status, Paster *et al.*[21] found no changes upon TCR triggering in Jurkat T cells. In contrast, a later analysis using the same cells and Lck biosensor construct in conjunction with a more sophisticated analytical approach, demonstrated that TCR engagement leads to a significant 20% enrichment of Lck with an active conformation accompanied by a corresponding enhancement of its kinase activity on a global cellular level.[22,23] Others also reported elevated levels of pY394^{Lck} after anti-CD3ε and anti-TCRβ/CD4 antigen stimulation,[24–28] even though an accurate quantification of these data was not put forward. The data that we have presented here is consistent with these observations and suggest that low levels of pY394 Lck persisting in resting T cells is increased upon contact with antigen-presenting cells, suggesting that additional activation of Lck accompanies an antigen-driven T-cell activation process.

Another aspect concerning the modulation of Lck activity upon TCR ligation is the compatibility of data derived from distinct cellular models. This especially relates to essential differences in the expression and initial stoichiometry of TCR signaling elements between the primary resting and Jurkat T cells. The most striking observation is that nonactivated Jurkat cells exhibit unusually high levels of pY394^{Lck} (Supplementary Figure 2) while their surface CD4 levels are very low (data not shown). This implies that the initiation of TCR signaling in Jurkat cells might employ a mechanism distinct from that operating in primary T cells where the majority of membrane-bound Lck is associated with CD4 and hence is not available for TCR signaling prior to the relocation of CD4/Lck complex into the vicinity of TCR/CD3 via CD4-MHCII interaction.[29] This could be important as several reports have suggested the critical role of the CD4/Lck complex for TCR signaling in physiological conditions.[30,31] For this reason, the extrapolation of results obtained on Jurkat cells to primary T cells, and *vice versa*, as well as their generalization, should be done with caution. In addition, CD3ε signaling preferentially targets Fyn over Lck, which makes the use of pY394^{Lck} levels as the main readout for this type of triggering disputable.[12–14,32] Together, although our data and previous reports[24–28] support the notion that the enlargement of the kinase active pool of Lck is required for T-cell activation, the spatio-temporary characteristics of this process have not been

resolved and thus additional experimentations are needed to address these issues.

Integral to the question concerning the modulatory effect of TCR engagement on Lck activity is whether a relatively small pool of kinase active Lck is sufficient to drive TCR signaling without its prior enlargement. Functionally, TCR signaling is designed to discriminate between high-affinity agonist pMHC and self-peptide-MHC interactions. This is achieved through the integration of high specificity and sensitivity of TCR toward the former that translates into a prolonged duration of their interaction and the generation of proximal signals downstream of TCR.[2] This phenomenon also includes LAT phosphorylation, diacylglycerol production and calcium flux which occurs within 4–7 s upon TCR engagement.[30] The results presented here showed that the diminishment of kinase active pool of Lck resulted in a markedly delayed generation of TCR proximal signals (Figure 6) suggesting that the stoichiometry between the active and inactive form of Lck is critical for rapid onset of TCR proximal signaling. Previous studies (reviewed in Chakraborty and Weiss[1] and more recent ones,[17,33] have directly implicated CD45, C-terminal Src kinase-PAG complex and autocatalytic capacity of Lck itself in these regulatory processes. However, modeling the interplay between these enzymatically active components of TCR proximal signaling, their substrates and downstream elements, notably ITAMs of TCR/CD3, ZAP70 and LAT, has proven to be quite challenging due to the variety of methods and distinct cellular models used. The kinetic-segregation model proposes that Lck-mediated tyrosine phosphorylation driven-TCR activation is in resting T cells efficiently inhibited by CD45, a highly expressed membrane phosphatase, which targets both pY394 and pY505 of Lck and pY of ITAM motifs in CD3 chains.[17] Upon TCR engagement, the activation is achieved via physical segregation of CD45 from the preactivated pool of Lck due to the close membrane apposition of interacting T-cell-antigen-presenting cell conjugates and a consequent exclusion of molecules with large extracellular domains, such as CD45, thus releasing a potent activation brake. In general, the Lck standby model simplifies the kinetic-segregation model of TCR signaling by stripping it from the need for its integration with the mechanism of Lck activation.[9] However, although the functional complementarity of these two models is obvious and their integration provides the explanation for the main mechanism underpinning TCR triggering, some aspects still need further clarification. This especially concerns the question regarding the diminished amount of $pY394^{Lck}$, that is, $\geqslant 40\%$ versus $\sim 2\%$, if it would still fit into this integrated model of TCR signaling. Notably, Nika *et al.*[7] estimated that 40% of total Lck in an active $pY394^{Lck}$ state would just fit the requirement for a brisk TCR triggering upon recognition of the cognate ligand. However, if this amount represents only 2% of total Lck, then underlying parameters of the integrated model would require a substantial re-evaluation and modification. Related questions, such as how the $pY394^{Lck}$ pool is generated and what are the structural, spatial and temporal attributes required for its maintenance and integration with processes of TCR triggering also await further elucidation. Recently published comparative studies mapping the interactome of TCR signaling components in resting and activated primary T cells are harbingers of such experimental activities that are expected to also provide critical insight into the physiology of Lck.[34,35]

Our previous and current data suggest that even a small but persistent amount of $pY394^{Lck}$ is sufficient to initiate TCR signaling in timely fashion. We have previously shown that this limited amount of CD4-associated $pY394^{Lck}$ is not freely distributed in the plasma membrane. Rather, it is concentrated and maintained in specific membrane microdomains where it colocalizes with TCR/CD3 and CD45, the latter keeping Lck in an primed conformation and the TCR signaling complex quiescent.[9,10] Upon TCR engagement, CD45 exclusion results in a rapid switch-like response leading to ITAM phosphorylation and downstream signaling.[33,36] Thus, although our data support the kinetic-segregation model, we believe that quantitative parameters of this model must be somehow linked to the locally concentrated pool of $pY394^{Lck}$ that is maintained in resting T cells in specialized membrane microdomains. A detail understanding of the mechanism regarding how these membrane heterogeneities, their resident molecules as well as other non-membrane-associated structures contribute to the regulation of TCR signaling[10,37–39] and especially to the delivery of Lck function is necessary for further advancement in this field.

METHODS

Mice

Three mice strains of C57BL/6 genetic background, 6–8 weeks old were used. Wild-type and homozygote knockout mice of *CD45* gene ($Ptprc^{-/-}$) were purchased from Jackson Laboratories (Bar Harbor, ME, USA)[40] along with OTII transgenic mice with a D011.10 T-cell receptor transgene-specific for ovalbumin peptide.[41] All strains were housed in a specific pathogen-free animal facility at Institute of Molecular Genetics (Prague, Czech Republic).

Antibodies and reagents

Rabbit anti-mouse antibody $pY394^{Lck}$ (SantaCruz Biotechnology), $pY418^{Src}$ (Invitrogen, Paisley, UK), $pY505^{Lck}$, $pY171^{LAT}$ (Cell Signaling Technology, Danvers, MA, USA), GAPDH (Sigma-Aldrich, St Louis, MO, USA), mouse anti-Lck (3A5) and phosphotyrosine-specific platinum 4G10 mAb (Merck Millipore, Darmstadt, Germany) were used for western blotting. An anti-CD4 antibody (clone H129.19) was obtained from BD Biosciences (Heidelberg, Germany). The secondary antibody anti-mouse IrRDye 680RD and anti-rabbit IRDye800CW were obtained from LI-COR Biosciences (Lincoln, NE, USA). Mouse anti-rabbit and goat anti-mouse HRP light chain-specific antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used for protein detection after IP. For IP purposes, antibodies were coupled to Protein A or G magnetic beads (Merck Millipore). Biotinylated anti-mouse CD4 (GK1.5), biotinylated anti-mouse TCRβ (H57) and biotinylated anti-mouse CD3ε (145-2C11) monoclonal antibodies were purchased from eBiosciences (San Diego, CA, USA).

Cell lines, cell preparation and culture

SYF cell line and its Lck infectants were maintained in Dulbecco's Modified Eagle Medium, wild-type and JCAM1.6 Jurkat T-cell line was maintained in RPMI 1640 (Life Technologies, Paisley, UK). Both media were supplemented with 10% inactivated fetal calf serum, 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (Sigma-Aldrich). BMDC culture from OTII transgenic mice was established as described elsewhere.[42] After three days of culture, one half of the media was replenished and on day 6, the cells were harvested and used for further experiments.

Isolation and activation of primary T cells

The procedure was performed as previously described.[25] Briefly, the primary lymph node $CD4^+$ or $CD8^+$ T cells (~95% purity) were isolated from wild-type or $Ptprc^{-/-}$ mice using MACS $CD4^+$ or $CD8^+$ T-cell isolation kit II (AutoMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells were precoated with a specified combination of biotinylated anti-TCR (1 μg ml^{-1}, clone H57), anti-CD4 (0.3 μg ml^{-1}, clone GK1.5) or anti-CD3ε (10 μg ml^{-1}, clone 2C11) antibodies in 500 μl of phosphate-buffered saline+3% fetal calf serum for 30 min at 4 °C, washed and resuspended in 20 μl of phosphate-buffered saline+1% fetal calf serum per tube. Cells were prewarmed in 37 °C and coaggregation-mediated activation was achieved with the addition of streptavidin to the final concentration of 50 μg ml^{-1}. Cells were vortexed and incubated at 37 °C for the indicated period of time. The activation was

stopped by the addition of either ice-cold TKM lysis buffer (50mM Tris-HCl pH 8.0, 25mM KCl, 5mM $MgCl_2$, 1mM EDTA pH 8.0) with the indicated detergent and inhibitors (see below) or immediately boiled in Laemmli sample buffer. For activation by pV, cells were prewarmed for 2 min at 37 °C and a freshly prepared pV (Na_3VO_4 mixed with 1% H_2O_2) was added at the indicated concentration and for the indicated period of time. The activation was stopped by the addition of hot Laemmli sample buffer.

For T-cell activation assay, BMDC's, prepared as described above, were pulsed with 1 μM of ovalbumin$_{323–339}$ peptide for 2 h. For each time point, 10^6 TCR-transgenic $CD4^+$ primary T cells isolated from OTII lymph nodes were mixed with 2.5×10^5 pulsed BMDCs in 10 μl volumes. Cells were then gently spun down for 10–20 s to allow the formation of cell conjugates. The resulting BMDC–T-cell coculture was incubated in serum-free RPMI medium at 37 °C for the indicated period of time and then immediately lysed in hot Laemmli sample buffer.

Cell lysis and IP

Cell lysis of $CD4^+$ T cells was performed in TKM lysis buffer (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA) containing either 0.5% Brij58, or 1% NP-40, or 1% *n*-dodecyl-β-D-maltoside detergent. Buffers also contained a protease and phosphatase inhibitor cocktail (Complete and PhosStop from Roche, Basel, Switzerland) supplemented with 20 μM (unless stated otherwise) of the Src-family tyrosine kinase specific inhibitor, PP2 (Merck Millipore). Lysates were incubated on ice for 30 min, centrifuged at 800 g for 2 min (removal of nuclei) followed by immediately boiling the supernatant in Laemmli sample buffer or used for IP. For Lck and CD4 IPs, the lysates were incubated with antibody-precoated magnetic beads overnight at 4 °C on a rotational wheel. Beads were then intensively washed (6–8 times), boiled in Laemmli sample buffer for 10 min and resolved by SDS-PAGE. For sequential depletion of pY505Lck and then Lck, cell lysates were prepared in denaturation conditions as described previously.[7]

Geldanamycin treatment, cell fractionation and fluorescence-activated cell sorting analysis

Freshly isolated $CD4^+$ T cells were cultured for the indicated time in RPMI media (Sigma-Aldrich) with 10% fetal calf serum and PenStrep (Sigma-Aldrich) supplemented with or without 5 μM of geldanamycin (Merck Millipore) solubilized in dimethyl sulfoxide (DMSO). To obtain membrane and cytosolic fractions, the cells were fractionated according to a previously described protocol.[43] Fluorescence-activated cell sorting analysis was performed using a LSRII flow cytometer (BD Biosciences). Cells were stained with directly conjugated antibodies against CD4 (clone GK1.5) and TCRβ (clone H57), washed and analyzed. Dead cells were identified by the addition of propidium iodide. Data were processed using FlowJo statistical software (FlowJo, Ashland, OR, USA).

Western blotting and quantification

Proteins resolved on polyacrylamide gels were transferred onto PVDF membrane (Merck Millipore) and blocked for 1 h in 5% non-fat milk (for IP) or in 1% bovine serum albumin in TBS-T at room temperature. Blots were then incubated for 1 h with primary antibodies diluted in blocking buffer, washed and incubated for 1 h with secondary antibodies conjugated to horseradish peroxidase or infrared dyes IRDye (LI-COR Biosciences). Signals were visualized by the Odyssey imaging system CLx (LI-COR Biosciences; Figures 2c,4,5b and 6) or developed by incubation with SuperSignal ECL substrate (Thermo Scientific, Waltham, MA, USA) and visualized by exposure to X-ray films (Figures 1,2,3 and 5c, except Figure 2c). Where indicated, densitometry quantification and analysis was performed by AIDA image analyzer software (Straubenhardt, Germany) from the raw image data.

ACKNOWLEDGEMENTS

We thank Z. Cimburek from Flow Cytometry Facility at the Institute of Molecular Genetics for expert technical assistance. This work was supported by Grant 310/09/2084 from Grant Agency of Czech Republic (GA CR). The study was also supported by the Charles University in Prague, project GAUK No. 627112 and by Charles University grant SVV 260083.

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The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)

TCR TRIGGERING INDUCES THE FORMATION OF LCK-RACK1-ACTININ-1 MULTIPROTEIN NETWORK AFFECTING LCK REDISTRIBUTION

The membrane redistribution of Lck kinase during initiation of T-cell activation is critical for delivery of its function (Filipp et al., 2003). In unstimulated T-cells, redistributing Lck was shown to be associated with "heavy" DRMs and after TCR engagement repartitions into "light" DRMs (Ballek et al., 2012). Nevertheless the mechanism underpinning this critically important process for T-cell signalling is not known.

Here we investigated the redistribution process and considered several candidates from previously described molecules which could take a part. RACK1 was shown as a viable option. As an adaptor protein it has a largely promiscuous binding capacity to plethora of molecular partners and was also previously implicated in the redistribution of PKCβII kinase (Ron et al., 1999). For the first time we describe the formation of transient RACK1-Lck complexes very early after TCR triggering. In our microscopy studies we observed timely coordinated movement of both proteins during the formation of IS. In addition, we found out that formation of this complex was dependent on both functional SH2 and SH3 domains of Lck and microtubular network. Nevertheless, the interaction seems to not be direct, suggesting the formation of a larger multiprotein complex involving RACK1 and Lck. The association of RACK1 with cytoskeleton linker α-actinine-1 provided then the evidence of the direct involvement of cytoskeleton in this redistribution. Taken together, we showed that RACK1 orchestrates the formation of multiprotein complex after TCR triggering important for connecting of Lck to cytoskeleton and thus affecting redistribution of Lck within plasma membrane.

frontiers in Immunology

ORIGINAL RESEARCH
published: 27 October 2016
doi: 10.3389/fimmu.2016.00449

TCR Triggering Induces the Formation of Lck–RACK1–Actinin-1 Multiprotein Network Affecting Lck Redistribution

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The initiation of T-cell signaling is critically dependent on the function of the member of Src family tyrosine kinases, Lck. Upon T-cell antigen receptor (TCR) triggering, Lck kinase activity induces the nucleation of signal-transducing hubs that regulate the formation of complex signaling network and cytoskeletal rearrangement. In addition, the delivery of Lck function requires rapid and targeted membrane redistribution, but the mechanism underpinning this process is largely unknown. To gain insight into this process, we considered previously described proteins that could assist in this process *via* their capacity to interact with kinases and regulate their intracellular translocations. An adaptor protein, receptor for activated C kinase 1 (RACK1), was chosen as a viable option, and its capacity to bind Lck and aid the process of activation-induced redistribution of Lck was assessed. Our microscopic observation showed that T-cell activation induces a rapid, concomitant, and transient co-redistribution of Lck and RACK1 into the forming immunological synapse. Consistent with this observation, the formation of transient RACK1–Lck complexes were detectable in primary $CD4^+$ T-cells with their maximum levels peaking 10 s after TCR–CD4 co-aggregation. Moreover, RACK1 preferentially binds to a pool of kinase active $pY394^{Lck}$, which co-purifies with high molecular weight cellular fractions. The formation of RACK1–Lck complexes depends on functional SH2 and SH3 domains of Lck and includes several other signaling and cytoskeletal elements that transiently bind the complex. Notably, the F-actin-crosslinking protein, α-actinin-1, binds to RACK1 only in the presence of kinase active Lck suggesting that the formation of RACK1–$pY394^{Lck}$–α-actinin-1 complex serves as a signal module coupling actin cytoskeleton bundling with productive TCR/CD4 triggering. In addition, the treatment of $CD4^+$ T-cells with nocodazole, which disrupts the microtubular network, also blocked the formation of RACK1–Lck complexes. Importantly, activation-induced Lck redistribution was diminished in primary $CD4^+$ T-cells by an adenoviral-mediated knockdown of RACK1. These results demonstrate that in T cells, RACK1, as an essential component of the multiprotein complex which upon TCR engagement, links the binding of kinase active Lck to elements of the cytoskeletal network and affects the subcellular redistribution of Lck.

Keywords: TCR triggering, RACK1, Lck, membrane redistribution, lipid rafts, α-actinin, cytoskeleton

OPEN ACCESS

Edited by:
Bernard Malissen, Centre d'Immunologie de Marseille-Luminy, France

Reviewed by:
Salvatore Valitutti, French Institute of Health and Medical Research, France
Christoph Wülfing, University of Bristol, UK

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Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *05 August 2016*
Accepted: *10 October 2016*
Published: *27 October 2016*

Citation:
Ballek O, Valečka J, Dobešová M, Broučková A, Manning J, Řehulka P, Stulík J and Filipp D (2016) TCR Triggering Induces the Formation of Lck–RACK1–Actinin-1 Multiprotein Network Affecting Lck Redistribution. Front. Immunol. 7:449. doi: 10.3389/fimmu.2016.00449

INTRODUCTION

Signaling through the T-cell antigen receptor (TCR) has the potential to trigger a broad range of cellular responses (1). During TCR triggering, two Src family tyrosine kinases (SFKs) – Lck and Fyn – provide critical enzymatic and structural functions that predicate the generation of the most proximal signals emanating from the TCR (2). In $CD4^+$ resting T-cells, Lck is targeted to the inner leaflet of the plasma membrane *via* its NH_2-terminal myristate/palmitate motif. A considerable portion of this membrane-associated Lck has been shown to be non-covalently attached to the TCR co-receptor, CD4 (3). Lck kinase activity is positively and negatively regulated by the phosphorylation of two tyrosine residues, Y394 and Y505, respectively, the former being associated with fully active Lck (4). Upon TCR binding to a cognate peptide which is recognized in the context of MHCII, CD4 interacts with the non-variable region of the same MHCII and juxtaposes its bound kinase active Lck within the vicinity of immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 chains of TCR. Lck then phosphorylates ITAMs that serve as docking sites for activated tyrosine kinase ZAP-70, which in turn proceeds to phosphorylate the adaptor protein LAT at multiple sites. This leads to the recruitment of downstream signaling elements such as phospholipase C-γ1 and adaptor proteins Grb2 and GADS which trigger complex signaling cascades, Ca^{2+} flux, cytoskeletal reorganization, and integrin activation (5, 6).

There is a general consensus that a T-cell membrane structural network provides the necessary milieu for coordination and integration of processes that regulate the onset of T-cell signaling. Several types of membrane heterogeneities that concentrate specific and distinct sets of signaling molecules have been proposed. These account for, but are not limited to, lipid rafts (LRs), nanoclusters, protein islands, pickets and fences, transient confinement zones, microclusters, immunological synapse (IS), and supramolecular activation cluster (SMAC) (7). LRs, which represent a sizable fraction of the plasma membrane, are in terms of their composition, structure, and function among the most studied (8, 9). Due to their enrichment in cholesterol and sphingolipids, LRs exist in a liquid-ordered phase, hence are largely resistant to solubilization by mild non-ionic detergents, and can be isolated as detergent-resistant membrane (DRM) fractions. While DRMs are not equated with native LRs, their content and properties allow the examination of changes in membrane raft content induced by TCR signaling (10–13).

The compartmentalization of membrane-residing signaling proteins into LRs provides the basis for their physical segregation and transient clustering (14). Two distinct types of DRM fractions have been documented in resting T-cells: light and heavy DRMs, which are enriched for non-overlapping subsets of signaling molecules (15). Importantly, TCR activation-induced LR redistribution of Lck and several other signaling molecules which are involved in the initiation of signaling cascades, such as CD3ζ, LAT, and CD45, have been documented (14).

While TCR triggering is enzymatically initiated by Lck-mediated tyrosine phosphorylation of CD3 ITAMs, Lck does not remain in a stationary position. There are several lines of evidence that demonstrate that the delivery of Lck function is accompanied by its rapid and targeted membrane redistribution. Notably, we previously reported that LR plays an essential role in temporal and spatial coordination and activation-dependent redistribution of Lck and Fyn kinases (16, 17). A proposed Lck-dependent Fyn activation model posits that antibody-mediated TCR–CD4 co-aggregation-induced Lck activation outside LR results in Lck translocation to light LR where the activation of LR-resident Fyn ensues. Similarly, the "Lck standby model" which does not specifically account for the existence of LR, proposes that upon TCR triggering, the constitutively kinase active fraction of membrane-bound Lck is targeted to areas where it colocalizes with ITAMs of TCR/CD3 complex (18). Studies by Rossy et al. also demonstrated the impact of TCR stimulation on Lck distribution, which was dependent on active conformation of the kinase (19). Furthermore, it has been reported that the early redistribution of Lck to the forming IS with its maximum kinase activity occurs between 2 and 5 min after initiation of T-cell–APC conjugation (20). While these data collectively established Lck as a mobile signaling element that is indispensable for proximal T-cell signaling (21), the underlying process regulating its redistribution is currently unknown. The main aim of the study was to gain insight into the molecular mechanism and its functional elements that regulate the early recruitment of Lck to LR and the formation of the IS.

To consider proteins that could act in aiding the process of activation-induced redistribution of Lck, we searched for previously described molecules with the following attributes: (i) regulation of intracellular translocation of kinases, (ii) ability to interact with SFKs and modulate their kinase activity, (iii) capacity to associate with elements of the cytoskeletal network, and (iv) capacity to interact with multiple partners. Among several candidates, receptor for activated C kinase 1 (RACK1) turned out to be a viable option. RACK1 is a 36-kDa scaffolding protein, which is evolutionary highly conserved in a wide range of eukaryotes including members of the plant, fungi, and yeast kingdoms (22). It is expressed in all mammalian cells, and its deficiency is embryonically lethal (23). It contains seven WD40 repeats (24) with a propeller structure, which provides RACK1 with multiple binding sites allowing interaction with a large number of functionally and structurally distinct proteins such as G proteins, kinases, phosphatases, and IP3 receptors (25). This interactivity predisposes RACK1 to be involved in a broad range of cellular processes, from signal transduction, transcription, translation, viral infection, cell migration, development, and epigenetics to cancer (25). Its interactions with protein kinase C (PKC) (26), membrane-bound receptors such as integrin β (27, 28), NMDA receptor (29), FAK, PDE, and SFKs such as Src and Fyn (30–32) have been studied in detail. Importantly, it has been shown that RACK1 can positively or negatively modulate the kinase activity of SFK Src, Fyn, and Lck, with the resulting effect being cell context specific (27–33). However, the specific role of RACK1 in proximal T-cell signaling has not been investigated so far.

An important and yet enigmatic function of RACK1 is the coordinated translocation and redistribution of its activated

binding partners to a distinct subcellular compartment (25). While mechanisms regulating these dynamic processes are still obscure, the multiple protein-binding capacity of RACK1's seven WD40 domains and its association with beta-spectrin and/or plectin which mediate interactions with major cytoskeletal elements, actin, intermediate filaments, and possibly microtubules most likely contribute to these processes (34, 35). Interestingly, RACK1 is also a constituent of the ribosomal assembly where it recruits signaling components that enable the regulation of translation (36, 37).

Here, we report microscopic, biochemical, and genetic data characterizing RACK1 as an integral part of signaling transduction network capable of transiently co-binding the activated Lck and elements of cytoskeleton, thus revealing its potential in aiding Lck redistribution by integrating TCR/CD4–Lck signals with filament networks.

MATERIALS AND METHODS

Mice

Three mice strains, 6–8 weeks old, were used: wild-type (WT) C57BL/6, OTII transgenic mice with transgenic (tg) TCRα/TCRβ receptor specific for OVA peptide (38), and double tg DO11.10 TCR/tg CARΔ (TgCAR) (Taconic), which express human coxsackie/adenovirus receptor lacking a cytosolic domain (39, 40). All the three strains were housed in a specific pathogen-free animal facility at the Institute of Molecular Genetics (Prague, Czech Republic).

Antibodies and Reagents

For western blotting, mouse anti-RACK1, rabbit anti-pY394^{Lck} (Santa Cruz), anti-pY505Lck (Cell signaling), mouse anti-Lck (3A5), and phosphotyrosine-specific platinum 4G10 mAb (Millipore) were used. Mouse anti-α-actinin-1 (BM-75.2), rabbit anti-RACK1 C-end, anti-GAPDH, and cholera toxin–horseradish peroxidase (HRP) conjugate were purchased from Sigma-Aldrich, anti-α-tubulin from GeneTex, and anti-GADS and anti-LASP from Millipore. Mouse anti-rabbit and goat anti-mouse HRP light chain-specific antibodies (Jackson IRLab) were used for protein detection after immunoprecipitation (IP). For IP purposes, antibodies were coupled to Protein A or G magnetic beads (Millipore) or the bead-conjugated anti-RACK1 from Santa Cruz were used. Biotinylated anti-mouse CD4 (GK1.5) and biotinylated anti-mouse TCRβ (H57) mAbs were purchased from eBiosciences (USA). For immunofluorescence, CellTrace™ Far Red DDAO-SE and Alexa Fluor 488, 555, or 647 goat anti-mouse IgG or goat anti-rabbit IgG (H + L) were used. Actin filaments were visualized by Alexa Fluor 488 Phalloidin purchased from Life Technologies. Streptavidin, Brij58 (polyoxyethylene-20-cetyl-ether), and nocodazole were purchased from Sigma-Aldrich. Latrunculin B, lauryl maltoside (*n*-dodecyl-β-D-maltoside, LM), and Src-family kinase inhibitor PP2 were obtained from Calbiochem (Merck). Ultrapure grade paraformaldehyde (PFA) was obtained from Polysciences. $OVA_{323–339}$ peptide from GenScript Corporation, and staphylococcal enterotoxin E (SEE) was purchased from Toxin Technology.

Cell Lines, Cell Preparation, and Culture

The NIH3T3 cell line and Lck infectants cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM), WT, and JCAM1.6 The Jurkat T-cell line and RAJI B-cell line were cultured in RPMI 1640 (Life Technologies). Both media were supplemented with 10% inactivated fetal calf serum (FCS) and 100 U of penicillin/10 μg streptomycin antibiotics (Sigma) per 1 ml of media. Bone marrow-derived dendritic cells (BMDCs) from OTII transgenic mice were isolated from mice femur and tibia cavities. The cells were cultured for 6 days in RPMI medium supplemented with GM-CSF-containing supernatant, which was produced by the LUTZ cell line (final concentration was adjusted to 30 ng/ml). After 3 days of cultivation, one half of the media was replenished, and on day 6, the cells were harvested and used for further experiments.

cDNA Vectors

Lck constructs, described previously (21), were inserted into the murine stem cell virus (MSCV)-based internal ribosome entry site (IRES)-enhanced green fluorescent protein virus MigR1 (41), permitting the concurrent expression of a given gene and EGFP. EGFP–RACK1 and mCitrine–RACK1 was a gift from Dr. Vomastek, and CFP–Lck (clone W25) was a gift from Dr. Stockinger (42). Generation of retrovirus packaging cell lines and retrovirus stock as well as the infection of NIH3T3 or Jurkat T-cells was performed as outlined elsewhere (43, 44). Successfully infected cells were sorted to the comparable protein expression level of the desired gene.

Isolation and Activation of Primary T-Cells

The procedure was performed as previously described (14). Briefly, primary lymph node $CD4^+$ T-cells (~95% purity) were isolated from mice using MACS $CD4^+$ T-cell isolation kit (AutoMACS, Miltenyi Biotec). $CD4^+$ T-cells were precoated with biotinylated anti-TCR (1 μg/ml, clone H57) and anti-CD4 (0.3 μg/ml, clone GK1.5) antibodies in 500 μl of PBS + 3% FCS for 30 min at 4°C, washed, and resuspended in 20 μl of PBS + 1% FCS per tube or were indicated in 20 μl of PBS + 1% FCS + 20 μM PP2. Cells were pre-warmed in 37°C, and co-aggregation-mediated activation was achieved with the addition of streptavidin to a final concentration of 50 μg/ml. Cells were vortexed and incubated at 37°C for the indicated period of time. The activation was stopped with the addition of either ice-cold lysis buffer with indicated detergent and inhibitors (see below) or immediately boiled in Laemmli sample buffer.

Cell Lysis and Immunoprecipitation

Cell lysis of $CD4^+$ T-cells was performed in TKM (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Brij58) or TNE (50 mM Tris, pH 8, 12.5 mM EDTA, 1% NP-40) lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Roche). Lysates were incubated on ice for 30 min, spun down at 800 × *g* for 2 min (removal of nuclei), and used for further applications. For RACK1 IPs, the lysates were incubated with antibody-precoated RACK1 beads overnight at 4°C on a rotational wheel. Beads were then intensively

washed (six to eight times), boiled in Laemmli sample buffer for 10 min, and immunoprecipitated proteins resolved by SDS-PAGE.

Western Blotting and Quantification

Proteins resolved on polyacrylamide gels were transferred onto a PVDF membrane (Millipore) and blocked for 1 h in 5% non-fat milk (for IP) or in 1% BSA in TBS-T at room temperature. Blots were then incubated for 1 h with primary antibodies diluted in blocking buffer, washed followed by a 1-h incubation with secondary antibodies conjugated to HRP, and developed by incubation with ECL substrate (Thermo Scientific). Where indicated, densitometry quantification was performed by AIDA image analyzer software from raw image data obtained from a GS-800 Biorad densitometer scanner.

Immunofluorescence Microscopy

For immunofluorescence, the staining protocol was adopted from the website www.cellsignal.com with some modifications. Briefly, $CD4^+$ T-cells were seeded on poly-L-lysine-coated coverslips and allowed to adhere for 15 min at 37°C, 5% CO_2 followed by cell fixation in 4% PFA for 15 min. The following procedure was applied to all cell types. After PFA fixation, the cells were permeabilized with ice-cold methanol for 10 min in −20°C (methanol step had to be omitted when phalloidin was used to stain actin cytoskeleton). The cells were blocked for 1 h with PBS containing 0.3% Triton X-100 (PBT) with the addition of 2.5% FCS and 2.5% BSA and incubated with primary and secondary antibodies for 1 h consecutively. Where indicated, cells were stained with DAPI for 10 min. Coverslips were mounted using 4% *n*-propyl gallate in glycerol. Samples were analyzed by sp5 confocal microscopy (Leica) or N-SIM super-resolution microscopy (Nikon). Image reconstruction was performed by Huygens Professional (SVI) or NIS elements (Nikon) software. Image post-editing and image analysis including computing of Pearson's colocalization coefficient (PCC) were done with Fiji imaging software and its plug-in JACoB.

Preparation of APC–$CD4^+$ T-Cell Conjugates

The preparation of conjugates for microscopic analysis of IS formation *in vitro* was performed as previously described (45). Briefly, BMDCs were prepared in parallel as described above. Cells were then pulsed with $OVA_{323–339}$ peptide for 2 h and TCR-transgenic $CD4^+$ primary T-cells isolated from OTII mice were admixed with APCs at 3:1 ratio. The formation of APC–T-cell conjugates was achieved by short centrifugation. Conjugates were incubated in serum-free RPMI medium at 37°C. The cells were fixed with 4% PFA for 15 min, stained with anti-RACK1 and anti-Lck, and used for the microscopy analysis.

Formation of RAJI–Jurkat-Cell Conjugates, Live Cell Imaging

The WT or Lck-deficient Jurkat T-cell line JCAM1.6 was retrovirally transfected with either an EGFP–RACK1 or CFP–Lck construct, respectively. In addition, the mCitrine–RACK1 construct was electroporated (BTX ECM 830, 300 V, 10 ms, 4 mm cuvette) into CFP–Lck stable infectants. Jurkat T-cells that were positive for both CFP and mCitrine were sorted (BD, Influx cell sorter) and rested for 1 day in 37°C/CO_2. The formation of RAJI–Jurkat conjugates using live cell imaging was performed as previously described (46). Briefly, RAJI cells, serving as APCs, were labeled with DDAO-SE (Life Technologies), then loaded with 1 μg/ml SEE, and transferred into cover glass chamber (Ibidi). CFP–Lck/mCitrine–RACK1 Jurkat T-cells were subsequently added at a 1:1 ratio. Cells were observed in a 37°C/CO_2 climate chamber using a DeltaVision Core/Olympus IX71 microscope under CFP/YFP/mCherry filter cubes. Images were acquired every 15 s. Image post-editing and time-lapse movies were done with Fiji imaging software.

Quantification of Fluorescence in Microscopic Images

To analyze the concentricity and apposition of RACK1 and Lck (or GADS), the distance from the centroid of the cell to the cell edge was measured using a fluorescent intensity profile (see details in Figure S1 in Data Sheet 1 in the Supplementary Material). All microscopic quantitative analyses were performed by ImageJ program. Statistical analyses were performed with GraphPad Prism 5 using a paired two-tailed *t*-test.

Quantitation of the recruitment of RACK1 to the IS was calculated as described previously (47). Briefly, using the selection brush tool in ImageJ program, the T cell area adjacent to the synapse region, outside synapse region and the background area outside of the cell, was demarcated. The relative recruitment index (RRI) was calculated as the [mean fluorescence intensity (MFI) at synapse region minus the background]/[MFI of the outside synapse region minus background]. Quantitative measurements of MFI were performed with the program ImageJ. Statistical analysis was performed with GraphPad Prism 5 using a paired two-tailed *t*-test.

Gel Filtration

This size-exclusion chromatography procedure is based on a previously described protocol (48) with slight modifications (14). Briefly, a 5-ml pipette tip plugged with a small piece of glass wool and filled with Sepharose 4B beads (Sigma-Aldrich) was used as the column. Cells were lysed in TKM-Brij58 lysis buffer for 30 min and spun down for 2 min at 800 × *g*. Supernatant at a total volume of 1/10 of the stationary bead volume was loaded onto the top of the column and eluted with cell lysis buffer. All steps were performed at 4°C. In this setting, fraction #4 contains complexes of $>10^7$ Da; most of the pentameric IgM and IgG standards eluted in fractions #7 and #9, respectively (48).

Isolation of Detergent-Resistant Membranes

The isolation of DRMs was performed as described previously (14, 16). For the assessment of protein distribution in fractions obtained by gel filtration, pooled fractions were mixed 1:1 with 80% sucrose and subjected to the same protocol. Light DRMs, corresponding to classical LRs, are enriched in top fractions (#1–3), while the bottom fractions (#8–10) concentrate heavy DRMs together with soluble proteins.

Cytosketal Inhibitors

For assessing the involvement of cytoskeletal components in the redistribution of Lck after TCR/CD4 aggregation, $CD4^+$ T-cells were resuspended in PBS + 3% FCS and treated with either 2 μg/ml of latrunculin B or 10 μM of nocodazole for 30 min in 37°C and 5% CO_2. Subsequent precoating of the cells and TCR/CD4 co-aggregation were also performed in the presence of these inhibitors on ice. Before activation, small aliquots of latrunculin B- and nocodazole-treated samples, as well as untreated controls sample, were pre-warmed and fixed at 37°C with PFA for 15 min, stained as described above, and analyzed by microscopy.

Adenoviral Vectors and Transduction of TgCAR T-Cells

Adenoviral vectors and virus particles containing shRNA hairpins, as well as control empty vector/viruses, were prepared using the Knockout RNAi system and the Adeno-X Expression system 1 (Clontech) and used according to the manufacturer's protocol. Target sequences of shRNA for RACK1 are as follows: RAO#2-gcaagatcattgtagatgaat, RAO#4-ctcccacttcgttagtgat, and RAO#5-ggatgagagtcattcagaatg. Transduction of T-cells was performed as described elsewhere (49). Briefly, isolated naïve $CD4^+$ T-cells were resuspended in DMEM medium containing 2% FCS and desired MOI of adenoviral particles and incubated at 37°C/CO_2 for 1 h. Then, the cell/virus mixture was transferred to a culture dish and incubated for the indicated time at 37°C/CO_2 in complete RPMI media supplemented with 2 ng/ml of rmIL-7 (PeproTech). Then, live T-cells were sorted, rested for 4 h, and used for further experiments.

Mass Spectrometry

The stained protein bands were processed according to the standard protocol generally used for mass spectrometry (MS) protein identification (50) with minor modifications. The gel slices containing the proteins of interest were washed, proteins reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. The extracted peptides were separated using a home-made microgradient device (51) with C18 reversed phase capillary column (i.d. 200 μm, length 70 mm) for LC MALDI-TOF/TOF MS and MS/MS analysis using 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) with α-cyano-4-hydroxycinnamic acid as MALDI matrix. Protein database identification was carried out with Protein Pilot 2.0 software using the SwissProt part of the UniProt database server.

RESULTS

Localization of Lck and RACK1 in $CD4^+$ Primary T-Cells

First, we determined the expression of RACK1 in mouse primary lymph node $CD4^+$ T-cells. RT-PCR analysis performed on total mRNA isolated from FACS-sorted cells (99.3% purity) demonstrated detectable levels in non-activated $CD4^+$ T-cells (data not shown). Confocal microscopy confirmed the presence of RACK1 on the protein level and showed that Lck is localized almost exclusively to the plasma membrane. We observed that RACK1 is positioned just beneath Lck, in a constrained cytoplasmic niche between the plasma membrane and nucleus, the latter fulfilling the vast majority of intracellular space (**Figure 1A**). To assess RACK1 subcellular distribution more accurately, we performed super-resolution microscopy. It confirmed that in resting $CD4^+$ T-cells, Lck and RACK1 are juxtaposed concentrically (**Figures 1B–D**) and exhibit a mild overlap (**Figure 1E**) in accordance with the PCC >0.6 (**Figure 1F**).

RACK1 and Lck Co-Redistribute to Forming Immunological Synapse

Next, we assessed the cellular co-distribution of RACK1 and Lck in activated primary lymph node $CD4^+$ T-cells during early phases of IS formation. Microscopic examination of cell conjugates of OVA peptide-pulsed BMDCs with transgenic T-cells specific for OVA peptide showed that Lck and RACK1 concomitantly translocated to and enriched in IS at early phases of its formation (2–5 min) (**Figures 2A,B**). Lck, the accumulation of which in IS has been previously demonstrated (20, 45), was used as an internal control. Interestingly, we observed that RRI for RACK1 is even higher than that for Lck, strongly suggesting the physiological importance of RACK1 enrichment in the forming IS (**Figure 2B**).

To analyze the co-redistribution of RACK1 and Lck during early phases of IS formation in more detail, we performed experiments that would visualize the kinetics of this process. Toward this end, Jurkat T-cells, which are able to form cell conjugates with RAJI cells were infected with a RACK1–EGFP retroviral construct, and the kinetics of RACK1–EGFP translocation into IS was examined in a time-dependent manner using live fluorescent microscopy (**Figure 3A**; Video S1 in Supplementary Material). RACK1–EGFP protein was found moderately enriched in the forming IS (**Figure 3B**) with the total time of its transient residency from 6 min to more than 18 min (**Figure 3C**).

To visualize the Lck and RACK1 co-redistribution event, we co-transfected Jurkat T-cells with two constructs: RACK1–mCitrine and Lck–CFP. mCitrine and CFP double-positive cells were FACS-sorted, and time-lapse images were recorded (Video S2 in Supplementary Material). As presented in **Figure 3D**, shortly after conjugate formation (1–2 min, see the arrow in the 2 min time frame), RACK1 and Lck co-redistributed to the forming IS where they both persisted for several minutes. Between 6 and 8 min, RACK1–mCitrine slowly moved distally from IS. At the 9-min mark until 11 min (**Figure 3D**, bottom two rows of images), suddenly and rapidly RACK1–mCitrine re-translocated back to the bulk volume of cytoplasmic space of the T-cell, leaving Lck behind in the IS where it remained for at least 7–8 min (**Figure 3E**). It is of note that the Jurkat T-cell which appears in the upper right hand corner of the 2-min image of the time-lapsed video and image and in which the amount of RACK1–mCitrine markedly exceeded that of Lck–CFP (red color is below the visible range) displays similar kinetics of RACK1 translocation to the IS and reverse re-translocation back to the cytoplasm as described above (**Figure 3D**; Video S2 in Supplementary Material).

Taken together, these data suggest that T-cell activation induces a rapid, cooperative, and IS-directed movement of Lck and RACK1. This co-redistribution pattern provided the

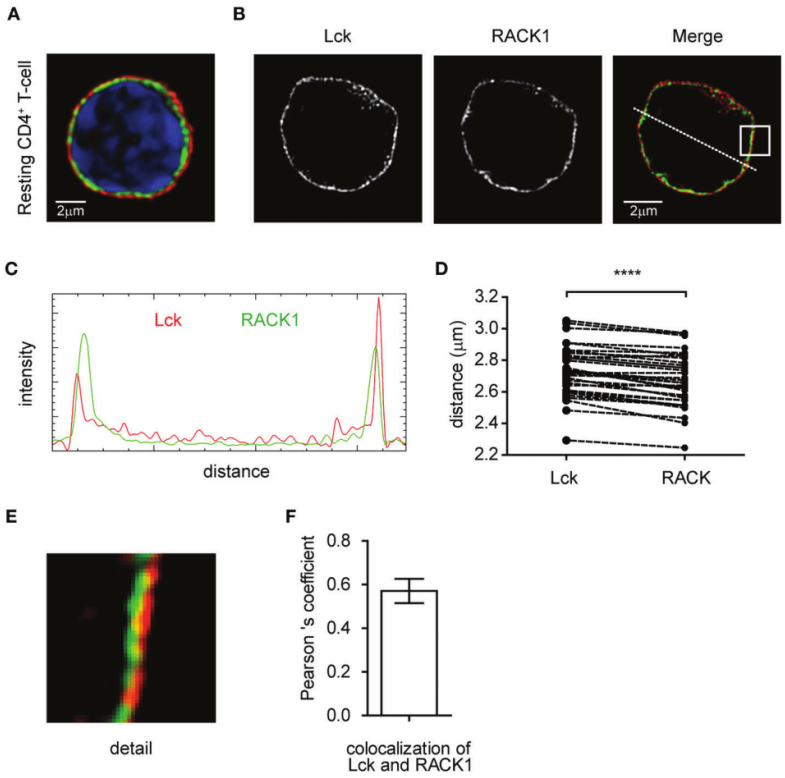

FIGURE 1 | Subcellular distribution of Lck and RACK1 in primary CD4⁺ T-cell. Fixed CD4⁺ T-cells were stained for RACK1 (green), Lck (red), and nuclei (blue) and visualized by confocal microscopy **(A)** or super-resolution N-SIM microscopy **(B)**. **(C)** Fluorescence intensity profile plot of Lck (red) and RACK1 (green) along the dotted line shown in the merged image of figure **(B)**. **(D)** Statistical analysis of the concentric juxtaposition of Lck and RACK1, which shows a larger distance of Lck from the cell centroid to its periphery ($n = 30$), $p \leq 0.0001$. **(E)** Magnification of the rectangle inset from the Merge image presented in **(B)** showing a subconcentrical juxtaposition of RACK1 to membrane-bound Lck. **(F)** The bar graph represents the statistical analysis of Lck and RACK1 colocalization using Pearson's colocalization coefficient ($n = 20$ cells). Error bars denote SD.

first evidence that RACK1 could participate in the mechanism guiding the redistribution of membrane Lck upon TCR engagement.

RACK1–Lck Complex Formation in the Primary CD4⁺ T-Cells

To determine if RACK1 is involved in proximal T-cell signaling *via* its interaction with Lck and if it is constitutive or activation inducible, we utilized the model of antibody-mediated T-cell activation enabling to study the kinetics of interaction between two interacting proteins during the first seconds after TCR/CD4 co-aggregation (14, 16) (**Figure 4A**). On average, a threefold to sixfold enrichment of Lck in complexes with RACK1 reached its maximum level 10 s after TCR–CD4 engagement (**Figure 4B**). Then, in the following 30–90 s, these levels diminished to those observed in non-activated cells (**Figures 4A** and **5A,C**). Consistent with the colocalization analysis (**Figures 1C–F**) only low, background levels of RACK1–Lck complexes were detected in the non-precoated control sample (**Figures 4A** and **5A**, "non-precoated" lane).

Subcellular Distribution of RACK1–Lck Complexes

We recently demonstrated that a small preactivated pool of pY394^{Lck}, which plays an important role in TCR triggering in resting primary CD4⁺ T-cells, associates with an LM-sensitive type of membrane microdomains called heavy DRMs (14). This high molecular weight (HMW) fraction can be obtained from Brij58 detergent-solubilized cells by gel filtration (53, 54). Interestingly, it is the heavy DRM-associated pool of Lck which, upon TCR/CD4 engagement, not only significantly increases its kinase activity but also exclusively translocates to distinct types of microdomains called light DRMs (14, 55). These considerations led us to assess the subcellular distribution of RACK1 in CD4⁺ T-cells. As illustrated in **Figure 4C**, RACK1, similar to the pool of pY394^{Lck}, is enriched in the complexes that are associated

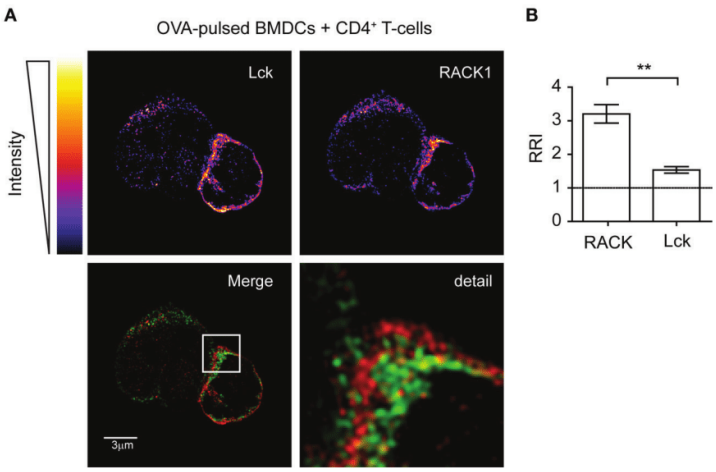

FIGURE 2 | Lck and RACK1 in primary CD4⁺ T-cell co-redistribute into forming immunological synapse. (A) OVA-pulsed bone marrow-derived dendritic cells (BMDCs) were mixed with CD4⁺ T-cells from OTII transgenic mice. After 2–3 min, T-cell–APC conjugates were seeded on cover slips, fixed and probed with anti-Lck (red) and anti-RACK1 (green), and visualized by super-resolution N-SIM microscopy. The upper two panels show the fluorescence intensities of individual Lck or RACK1 signals using pseudocolor digital scaling. The panel titled "Detail" shows the magnification of the rectangle inset seen in the Merge image. An image of one representative cell conjugate is presented. Consistent with a previous report (52), the low expression of Lck was also detected in BMDCs. **(B)** Statistical analysis of the relative recruitment index (RRI) showed that while both RACK1 and Lck are enriched at the site of the forming IS, RRI for the former is significantly higher than that for Lck ($n = 20$), $p < 0.01$.

with high HMW fractions #4–6. Next, we assessed the physical association of Lck and RACK1 in the fractions that were prepared from resting and activated T-cells. As shown in **Figure 4D**, the immunopreciptation of RACK1 from pooled HMW fractions #4 + 5 and #6 + 7 confirmed the activation-induced formation of RACK1–Lck complexes that were detected only in fractions #4 + 5 that were prepared from activated T-cells. These results suggest that in resting T-cells, the pool of preactivated $pY394^{Lck}$ and RACK1 are in physical proximity by co-distributing to heavy DRMs, which spatially restricts their transient interaction upon TCR activation. These data thus support the prediction that upon TCR/CD4 triggering, RACK1 can bind a spatially restricted pool of kinase active Lck and functions as a transportation vehicle that assists the redistribution of Lck to light DRMs, as documented previously (14, 16).

Structure–Function Analysis of RACK1–Lck Interaction

Next, we sought to determine which domain or tyrosine residue, which regulates Lck kinase activity, mediates its interaction with RACK1. Toward this end, we prepared a NIH3T3 fibroblast cell line expressing wild type Lck (WT), constitutively active Lck (Y505F) and Y505F Lck backbone with additionally inactivated either SH3 (W97K) or SH2 (R154K) domain (Figures S2A–C in Data Sheet 1 in the Supplementary Material). Endogenous RACK1 was immunoprecipitated with anti-RACK1 antibody, and its aliquots were blotted for Lck and RACK1. We found that in NIH3T3 cells, the WT, Y505F, and even kinase compromised Y505F/Y394F Lck interacted comparably with endogenous RACK1. However, this complex formation was nearly abolished in the variant expressing non-functional SH3 domain and severely compromised in the SH2 mutant of Y505F Lck (**Figures 4E,F**). This suggests that the presence of both functional modular SH3 and SH2 domains of Lck is a prerequisite for RACK1–Lck complex formation.

RACK1 Complexes with Lck Regardless of Lck Activation Status

The above results revealed that RACK1–Lck complex formation, at least in fibroblast cells, proceeds irrespective of the activation status of Lck (**Figure 4E**, Y505F versus Y394F/Y505F Lck). We next assessed if such a mode of interaction would also occur in primary CD4⁺ T-cells. As shown in **Figure 5A**, the kinetics of TCR/CD4 co-aggregation allowed the IP of RACK1–Lck complexes, which contain detectable levels of $pY394^{Lck}$. Their highest levels were observed at 5–10 s after activation (**Figure 5A**, $pY394^{Lck}$ panel, and **Figure 5B**), whereby the latter time point correlated with the peak of enrichment of Lck in these complexes (**Figure 5A**, Lck panel, and **Figure 5C**). This would suggest that a TCR/CD4-induced increase in $pY394^{Lck}$ precedes and thus predicates the formation of RACK1–Lck complexes. However, the activation-induced RACK1–Lck complex formation was not ablated in T-cells that were pretreated with the SFK kinase inhibitor, PP2, which effectively inhibited the activation-induced global tyrosine phosphorylation of TCR downstream substrates (**Figure 5D**, 4G10 bottom panel, right lane, PP2+) and blocked the activation-induced enhancement of $pY394^{Lck}$ levels (**Figure 6D**, $pY394^{Lck}$ panel, PP2+). Together, these biochemical results strongly suggest that the physical interaction between

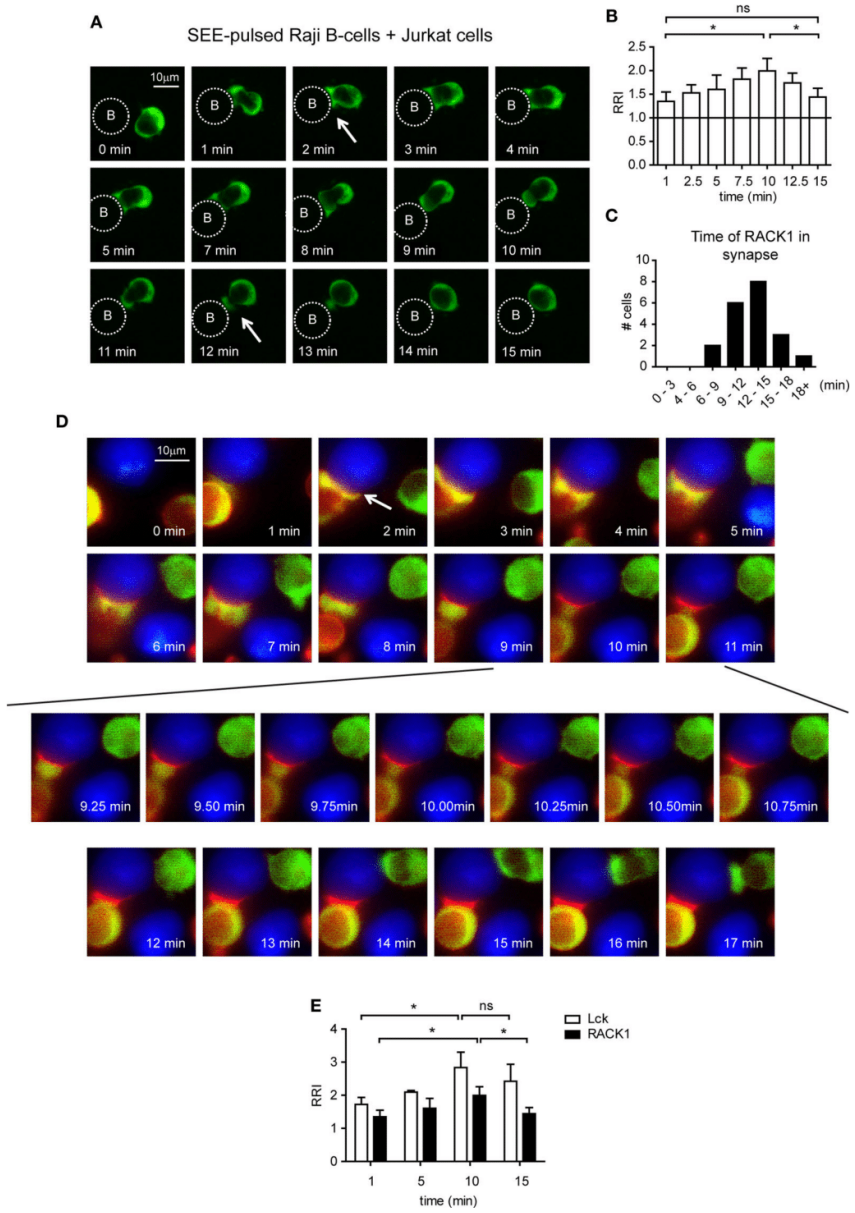

FIGURE 3 | **The kinetics of Lck and RACK1 co-redistribution into forming immunological synapses (IS) in Jurkat T-cells**. **(A)** Jurkat T-cells expressing RACK1–EGFP (green) were mixed with SEE-pulsed RAJI B-cells (denoted by the dotted circle), and the redistribution of RACK1 was observed by live cell imaging microscopy. Sequential time-lapse fluorescence microphotographs from one representative movie (Video S1 in Supplementary Material) are shown. **(B)** Statistical analysis of the kinetics of the relative recruitment index (RRI) of RACK1 to the forming IS measured after cell contact initiation ($n = 20$). **(C)** The bar graph shows the time distribution of RACK1 residency in IS; the start and end points of RACK1 residency are marked by two white arrows shown in **(A)**; $n = 20$ cells. **(D)** Lck-deficient JCAM1.6 Jurkat T-cells co-expressing Lck–CFP (red) and RACK1–mCitrine (green) constructs were mixed with SEE-pulsed RAJI B-cells (blue). Their redistribution during the formation of IS was observed by live cell imaging microscopy. Sequential time-lapse fluorescence microphotographs from one representative movie (Video S2 in Supplementary Material) are shown. An arrow points to the forming IS. **(E)** Statistical analysis of the kinetics of the relative recruitment index (RRI) of RACK1 and Lck to the forming IS measured after cell contact initiation ($n = 20$).

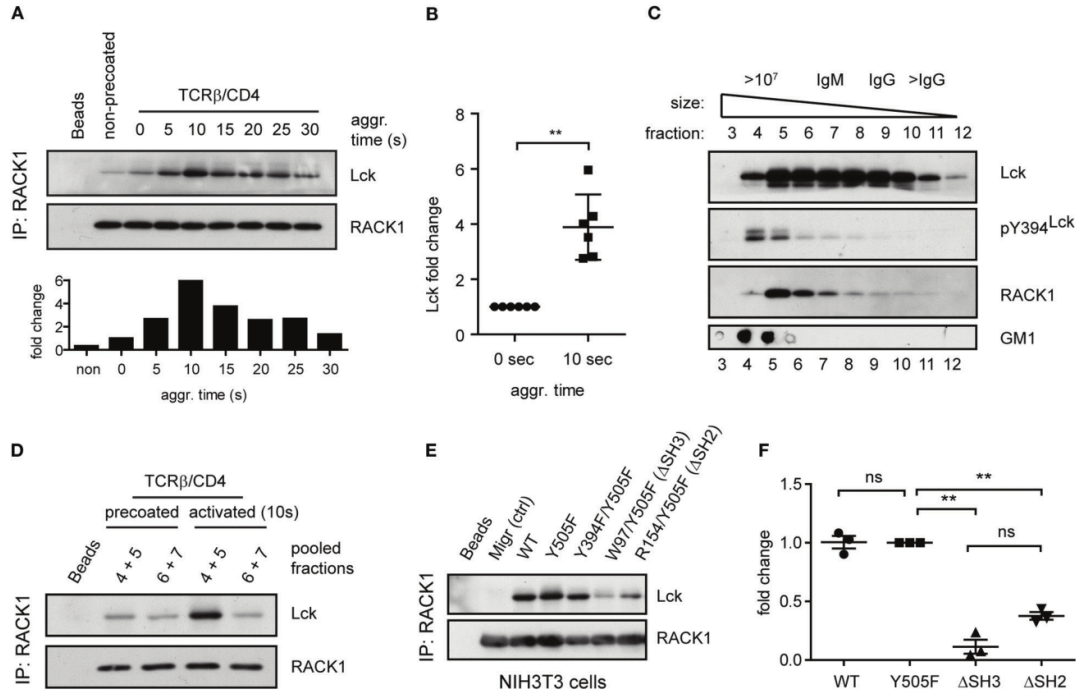

FIGURE 4 | Antibody-mediated engagement of TCR/CD4 receptors induces RACK1–Lck complex formation in primary CD4⁺ T-cells. **(A)** CD4⁺ T-cells precoated or non-precoated with biotinylated anti-TCR and anti-CD4 mAbs (TCRβ/CD4) were co-aggregated, or not (0 s), with streptavidin for the indicated time. RACK1 immunoprecipitates were blotted against Lck and RACK1. The bar graph at bottom shows the relative amount of co-immunoprecipitated Lck after normalization to RACK1. **(B)** The graph plot presents the relative fold increase of co-immunoprecipitated Lck (10 s after activation) from six independent experiments. The statistical analysis presented as mean ± SD was performed using the Student's two-tailed *t*-test, $^{**}p < 0.01$. **(C)** RACK1–Lck interaction is localized into HMW fractions. CD4⁺ T-cells fractions (#3–12) obtained from a Sephadex column were probed with anti-Lck, anti-pY394^{Lck}, anti-RACK1, and cholera toxin B subunit–HRP, which detects the surrogate marker of light DRMs, GM1. The fraction elution profile of the molecular weight size marker is shown on the top. **(D)** Fractions #4–5 and #6–7 from non-activated (precoated) or activated (TCRβ/CD4, 10 s) CD4⁺ T-cells were pooled, immunoprecipitated with anti-RACK1, and blotted with anti-Lck and RACK1 antibody. **(E)** RACK1–Lck interaction depends on functional SH2 and SH3 Lck domains. Endogenously expressed RACK1 was immunoprecipitated from NIH3T3 Lck infectants and blotted with anti-Lck and anti-RACK1 antibodies. **(F)** Statistical analysis of **(E)** represents the relative fold-change of RACK1 co-immunoprecipitated Lck variants from three independent experiments. The statistical analysis presented as mean ± SD was performed using the Student's two-tailed *t*-test, $^{**}p < 0.01$, n.s., not significant. Blots shown in **(C–E)** are representatives of at least three independent experiments.

Lck and RACK1 is independent of the activation status of Lck. However, under normal circumstances during TCR/CD4 engagement, upon which the preactivated pool of Lck is significantly increased (56), these complexes contain a sizeable fraction of pY394^{Lck} (**Figure 5A**).

Identification of Additional Components of RACK1 Complexes in CD4⁺ T-Cells

Our observation that the formation of RACK1–Lck complexes was dependent on both the SH2 and SH3 domain of Lck (**Figure 4E**) suggested that this interaction could be indirect and involved additional structural components. Consistent with this notion are results from our pull-down experiments using *in vitro* translation that failed to show direct RACK1–Lck binding (data not shown). To gain added insight into the complexity of RACK1–Lck interaction, we first assessed the presence of additional components in RACK1 immunoprecipitates from activated primary CD4⁺ T-cells. To recognize their presence in immunoprecipitated RACK1 complexes, we took advantage of the fact that TCR signaling is a tyrosine phosphorylation (pY)-driven event, and thus, at least those components of RACK1 complex which change their pY status could be readily detectable.

As illustrated in **Figure 6A**, several tyrosine phosphorylated proteins co-immunoprecipitated with RACK1. Specifically, we found four major phosphoproteins in activated T-cells, three of which possessed the molecular weights (MWs) of ~100, ~70, and ~38 kDa, and their pY status was associated with RACK1 in an activation-inducible manner. The fourth phosphoprotein which possessed an MW of ~56 kDa matched the MW of Lck. To reveal the identity of the former three phosphoproteins, the

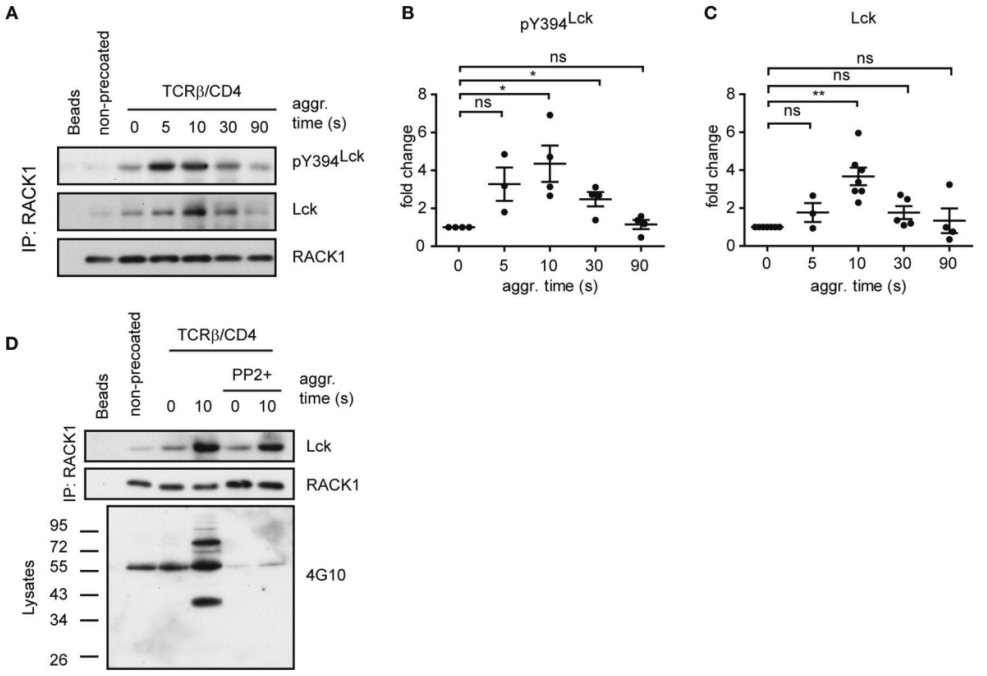

FIGURE 5 | RACK1–Lck complexes can form irrespective of Lck kinase activity, but those from activated T-cells contain a sizeable fraction of pY394^{Lck}. (A) CD4$^+$ T-cells precoated or non-precoated with biotinylated anti-TCR and anti-CD4 mAbs (TCRβ/CD4) were co-aggregated, or not (0 s), with streptavidin for the indicated period of time. RACK1 immunoprecipitates were blotted against pY394^{Lck}, total Lck, and RACK1. **(B,C)** Statistical analysis of pY394Lck and total Lck blots from **(A)**, respectively, represent the normalized to total RACK1 from at least three independent experiments. The statistical analysis presented as mean ± SD was performed using the Student's two-tailed *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, n.s., not significant. **(D)** CD4$^+$ T-cells were pretreated, or untreated, with SFK inhibitor (PP2+), TCRβ/CD4 co-aggregated, and lysed in TNE lysis buffer. RACK1 was immunoprecipitated and aliquots blotted against total Lck and RACK1; total cell lysates were blotted with anti-pY 4G10 antibody. Blots shown in **(A,D)** are representative of at least three independent experiments.

corresponding areas from a coomassie-stained gel were excised and their protein content subjected to MS analysis (Table S1 in the Supplementary Material). Due to the fact that RACK1 is a scaffold protein that plays an essential role in transcription, epigenetics, and translation as well as binds diverse signaling and structural proteins, we zoomed in on those proteins that potentially act outside these activities. Among them, we focused on proteins that were known to be involved in proximal TCR signaling and cytoskeleton regulation of the forming IS (**Figure 6B**). The capacity of these candidate proteins to bind RACK1 before and after T-cell activation was assessed. GADS, LASP1, and α-actinin-1 exhibited sizeable changes in the kinetics of interaction with RACK1 upon TCR/CD4 co-aggregation. Notably, while α-actinin-1 binds to RACK1 with increasing intensity (from 0 to 90 s, **Figure 6C**, upper panel), the level of RACK1 interaction with GADS and LASP1 was diminished with similar kinetics over the time tested (**Figure 6C**, GADS and LASP1 panels). RACK1–GADS complex formation in resting primary T-cells was microscopically corroborated by their colocalization in resting T-cells (Figure S3 in Data Sheet 1 in the Supplementary Material).

Only Kinase Active Lck–RACK1 Complexes Bind α-Actinin-1

While RACK1 interactome involves unusually high number of partners (BioGRID database; http://thebiogrid.org), the binding of α-actinin-1 to RACK1 complexes is an original finding. Actinins are primarily considered to be actin-cross-linking proteins but can also link transmembrane proteins to the cytoskeleton and membrane trafficking events (57). Our data showed that in resting CD4$^+$ T-cells, α-actinin-1 inducibly associates with RACK1 upon TCR/CD4 co-aggregation (**Figure 6D**, TCRβ/CD4, 0 versus 10 s, α-actinin-1 panel, and **Figure 6E**). However, as described above, while RACK1–Lck complex formation proceeds even in the presence of SFK inhibitor PP2 when Lck kinase activity is severely compromised (**Figure 6D**, TCRβ/CD4, 10 s/PP2+, pY394^{Lck} panel), binding of this complex to α-actinin-1 is blocked (**Figure 6D**, last lane, α-actinin-1 panel, and **Figure 6E**). This advocates for a scenario in which TCR/CD4 co-ligation induces the formation of complexes which contain RACK1 and kinase active Lck (**Figure 5A**), the latter required for linking these complexes to α-actinin-1 component within the cytoskeleton. If Lck

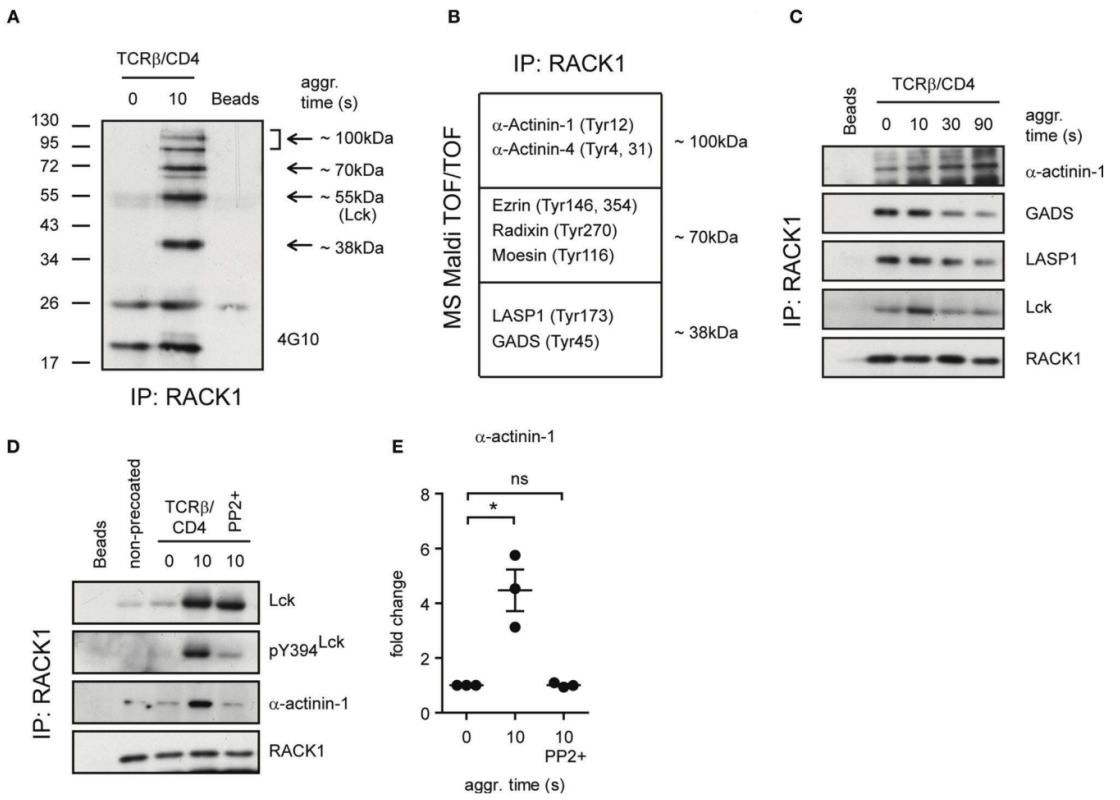

FIGURE 6 | **Identification of additional components of RACK1–Lck complexes**. **(A)** RACK1 immunoprecipitates from non-activated (0 s) and activated (10 s) CD4+ T-cell samples, and beads alone, were probed with anti-pY antibody (4G10). The arrows point to areas that show readily detectable phosphoproteins co-immunoprecipitating with RACK1. In the coomassie blue-stained gel, the areas depicted by arrows from all three samples were extracted (except for the 55-kDa area) and subjected to MALDI TOF/TOF MS analysis. A selected list of size-related and identified proteins with their potential pY sites are shown in **(B)**. The bead sample was negative for these proteins. **(C)** The kinetics of complex formation between RACK1 and indicated proteins before (0) and at the indicated time points after TCRβ/CD4 co-aggregation. RACK1 immunoprecipitates were probed with anti-α-actinin-1, anti-GADS, anti-LASP, anti-Lck, and anti-RACK1. **(D)** CD4+ T-cells were treated (PP2+) or not with PP2 inhibitor, activated, and lysed in TNE lysis buffer. RACK1 was immunoprecipitated, and samples were blotted against Lck, pY394Lck, α-actinin-1, and RACK1. **(E)** Statistical analysis of **(D)**, actinin-1 panel, represents the relative fold change of RACK1 co-immunoprecipitated actinin-1 normalized to total RACK1. The statistical analysis presented as mean ± SD was performed using the Student's two-tailed *t*-test, $^*p < 0.05$, n.s., not significant. Blots shown in **(C,D)** are representative of three independent experiments.

activity is blocked, the formation of RACK1–Lck complexes still proceeds, but α-actinin-1 is not engaged.

Destabilization of Microtubules Prevents RACK1–Lck Complex Formation

The timely and spatially coordinated complex formation between Lck and RACK1 in heavy DRM, their co-redistribution to the forming IS and linkage to α-actinin-1 suggest that the cytoskeletal network is actively involved. We have also reported that the activation-induced translocation of Lck to light DRMs is blocked by nocodazole, an inhibitor of microtubular assembly (14). To further investigate the potential involvement of RACK1 in the microtubular network-assisted translocation of Lck, we assessed the effect of nocodazole treatment on RACK1–Lck complex formation. The results showed that nocodazole, but not latrunculin-mediated inhibition of the actin cytoskeleton, effectively blocked the formation of RACK1–Lck complexes (**Figure 7A**, Lck panel, and **Figure 7B**). Of note, while lantrunculin and nocodazole treatment effectively disrupted the actin and microtubular network, respectively (**Figure 7C**), the activation-induced global tyrosine phosphorylation in the presence of these inhibitors was comparable to the untreated control sample (**Figure 7A**, bottom panel, 4G10). These data potentially provide a mechanistic explanation for the activation-dependent redistribution of Lck to light DRMs by virtue of linking the TCR/CD4-Lck complex to the microtubular cytoskeletal network *via* RACK1. This also suggests that at the very early stages of T-cell engagement, actin cytoskeleton does not affect Lck mobility even though α-actinin,

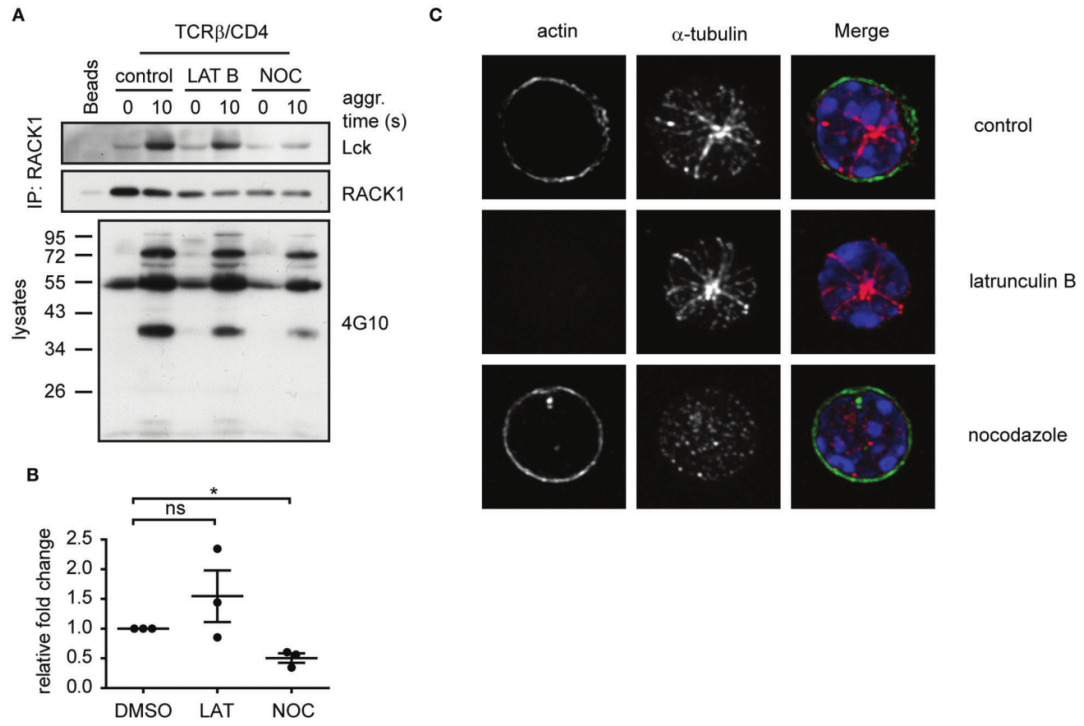

FIGURE 7 | Functional microtubular network is important for RACK1–Lck complex formation upon T-cell activation. **(A)** CD4+ T-cells were pretreated with latrunculin B (LAT B), nocodazole (NOC), or DMSO (control) and then activated for the indicated period of time followed by lysis in TNE lysis buffer. RACK1 was immunoprecipitated and blotted against Lck and RACK1. Samples from lysates before IP were boiled and subjected to pY western blot analysis with 4G10 antibody. Blots are representative of three independent experiments. **(B)** Statistical analysis of **(A)**, Lck panel, represents the relative fold-change of RACK1 co-immunoprecipitated Lck normalized to total RACK1. The untreated control samples were given reference value "1." The statistical analysis presented as mean ± SD was performed using the Student's two-tailed *t*-test, $*p < 0.05$, n.s., not significant. **(C)** CD4+ T-cells treated with DMSO (control); nocodazole or latrunculin B were pre-warmed at 37°C and fixed by PFA on polylysine coverslips. Tubulin (red) and actin (green) were visualized by confocal microscopy. Nuclei were stained with DAPI (blue). Representative images show the maximal intensity projection (MIP) of *z*-stacks.

which can potentially crosslink actin microfilaments, is already part of the Lck redistribution machinery.

Knockdown of RACK1 Hinders Activation-Induced Lck Translocation to LR

We next attempted to evaluate the potential functional relevance of RACK1–Lck interaction for the redistribution of Lck to light DRMs (16). CD4+ T-cells from TgCAR transgenic mice expressing the receptor for adenovirus on T-cells were infected with adenovirus containing shRNAs to downregulate RACK1 (**Figure 8A**). After 96 h of adenovirus infection, the cells were harvested and activated by TCR/CD4 co-aggregation. The distribution of Lck to light DRMs was then compared to cells that were infected with a mock construct. Regardless of the technical caveats that are associated with our limited ability to consistently generate viable RACK1 knockdown (KD) immune cells (see commentary in Figure S4 in Data Sheet 1 in the Supplementary Material), our data showed that in activated T-cells, the reduced levels of RACK1 correlated with diminished redistribution rate of Lck to light DRMs (**Figure 8B**). These results points to the potential involvement of RACK1-based multiprotein signaling network in Lck redistribution during proximal T-cell signaling.

DISCUSSION

The overarching goal of our investigation was to provide the initial insight into the molecular mechanism involved in the early recruitment of Lck to light DRMs and the forming IS. As targeting Lck to light DRMs predicates enhanced TCR-mediated IL-2 production (21) and alternations in the association of Lck with accessory molecules within light DRMs supports abnormal T-cell signaling in autoimmune diseases (58), elucidation of the nature of this process on the molecular level represents a topic of academic and clinical interest. Our data provide compelling evidence that RACK1 orchestrates spatial redistribution of Lck *via* tethering to cytoskeletal elements.

Results presented in this study are the first to reveal and characterize the role of RACK1 in early T-cell activation. RACK1–Lck

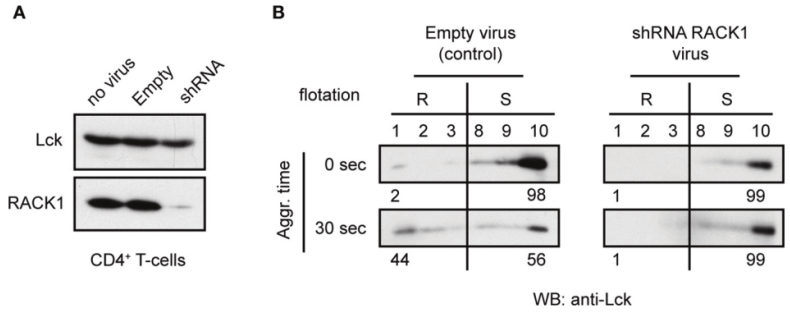

FIGURE 8 | Adenovirus mediated knockdown of RACK1 impedes translocation of Lck to light DRMs. CD4+ T-cells from TgCAR transgenic mice were infected with either empty control virus (Empty) or with a mixture of adenoviral RACK1-targeting shRNA constructs (shRNA) or were not infected (no virus). **(A)** The effectiveness of RACK1 downregulation is shown 96 h after infection. Cells were harvested and probed with anti-Lck and anti-RACK1. **(B)** CD4+ T-cells infected with Empty virus (control) or shRNAs RACK1 virus were activated by TCR/CD4 co-aggregation (30 s), or not (0 s), and the redistribution of Lck to light DRMs was assessed. Raft (#1–3, R) and soluble (#8–10, S) fractions were probed with anti-Lck. Numbers represent relative distribution of Lck to these fractions. Blots are representative of two independent experiments.

complex formation in primary CD4+ lymph node T-cells is activation-inducible, transient, and wanes shortly after activation. We have previously shown that only the pool of Lck that is associated with the HMW fractions translocates to light DRMs (14). Co-purification of RACK1 with these fractions in resting T-cells and the confinement of its activation-induced interactions with Lck to these fractions is consistent with its involvement in the shuffling of Lck to light DRMs. In addition, Lck and RACK1 co-redistribute with the same kinetics to the forming ISs. This argues for the existence of an underlying mechanism by which Lck and RACK1 are physically coupled and mechanistically transferred to these structures, likely *via* an association with common cytoskeletal elements (34, 59). In this context, our biochemical data showed that the formation of RACK1–Lck complexes, as well as the subsequent translocation of Lck to light DRM (14) depends on an intact microtubular network. While the relevant mechanism is obscure, there is precedence for the involvement of microtubular network in the proximal T cell signaling. As demonstrated previously, microtubules are located in close proximity to the cell membrane at the activation site and together with dynein regulate early microcluster transport and TCR signaling events (60). Thus, it is quite possible that microtubules can assist the binding of RACK1 and Lck, directly or indirectly, and *via* an unknown mechanism regulate their TCR/CD4-induced redistribution. Importantly, KD of RACK1 in primary CD4+ T-cells profoundly hampered the translocation of Lck to light DRMs. Thus, our data demonstrate that RACK1 fulfills the role of an adaptor protein that is involved in the regulation of Lck redistribution to light DRMs through the linking of TCR/CD4–Lck to the cytoskeletal network.

It has been previously reported that a Src–RACK1 interaction occurs through the binding of Src–SH2 domain to the tyrosine in position 246 (Y246) in the sixth WD40 domain of RACK1, which is phosphorylated by Src itself (32). As Src and Lck share the same structural components and domain organization, one could assume that RACK1–Lck interaction should also be dependent on the SH2 domain of Lck. However, we were unable to detect tyrosine-phosphorylated residues (pY) on RACK1 10 s after TCR/CD4 co-aggregation (data not shown). Thus, it is unlikely that RACK1–Lck interaction is mediated *via* binding of Lck–SH2 to pY on RACK1. Unexpectedly, RACK1–Lck interaction was also abrogated in the SH3-inactivated mutant of Lck. This indicates an equal importance of these two domains in RACK1–Lck complex formation. However, the structural basis for RACK1 binding to SH3–Lck is uncertain. Alternatively, if these interactions are mediated through some intermediary, it would predict the formation of multiprotein complexes involving numerous protein–protein interactions. Our data support this scenario. Notably, in addition to RACK1, Lck redistribution machinery might include the adaptor protein GADS (61) and components of the cytoskeleton such as α-actinin (62) and LASP1 protein (63). Other potential components such as serine/threonine protein phosphatases PP1 and 2A, protein SEC13 homolog, F-actin capping protein, Annexin A2, and ERM proteins were detected (Table S1 in the Supplementary Material), but validation of their presence and potential function in RACK1 complexes in T-cell proximal signaling needs further experimentation.

Interestingly, our data showed that Lck formed complexes with RACK1, irrespective of its kinase activity status, suggesting that conformational changes of TCR and/or CD4 may play a role in their induction (64). While the underlying mechanism awaits some resolution, we also demonstrated that it is only when these complexes contain kinase active Lck, they recruit α-actinin-1. The binding of α-actinin-1 to RACK1–Lck complexes adds another layer of complexity to the schematic of TCR signaling. Notably, it has been previously reported that α-actinin-1 associates with the membrane fraction of mouse lymphocytes (65) and resides in heavy DRMs of in immune cells (66). It has been also shown that α-actinin binds directly to both phosphatidylinositol-(4,5) biphosphate ($PtdInsP_2$) and PI-3 kinase which when activated, converts PIP_2 to phosphatidylinositol-(3,4,5) triphosphate [PtdIns(3,4,5)-P_3] at the inner leaflet of the plasma membrane (67). α-actinin-1 is also a target of activated tyrosine kinase (68), the nature of which, in T-cells, has not

been elucidated. Interestingly, while α-actinin primarily acts to bundle actin filaments, this function upon the initiation of TCR signaling would be likely subjected to negative regulation by all the three mechanisms mentioned in this study. Notably, binding to α-actinin of PtdIns(3,4,5)-P_3, which is generated by activated PI-3 kinase (69) and tyrosine phosphorylation with increased binding of Ca^{2+} to EF domains of α-actinin (70), would additively or synergistically reduce actinin's affinity for actin. Thus, while TCR/CD4 engagement recruits α-actinin-1 to RACK1–Lck complexes, during proximal T-cell signaling, α-actinin-mediated actin bundling would be compromised. However, whether such a mechanism in T-cells is indeed operational is currently under investigation.

Our data from primary $CD4^+$ T-cells also confirmed a previous finding that RACK1 physically associates with GADS in resting Jurkat T-cells (71). GADS and SLP76 are critical components of the signaling pathway which, upon TCR activation, inducibly bind to the phosphorylated scaffold protein LAT (61), which is responsible for subsequent actin cytoskeletal rearrangement (5). While speculative, a more plausible scenario would be that after TCR/CD4 triggering, tyrosine kinases Lck and ZAP70 are activated with the latter phosphorylating LAT at multiple sites (pY-LAT). The RACK1–GADS–SLP76 complex is then recruited to pY-LAT *via* SH2 of GADS, brought into the proximity of the TCR/CD4 complex and gains access to activated Lck. RACK1 then dissociates from GADS and forms multiprotein complexes which include several signaling components. Inclusion of kinase active Lck in this complex is predicated by the presence of an unperturbed microtubular cytoskeleton, which then mediates Lck redistribution to light DRMs, where Lck phosphorylates Fyn. This model is consistent with the kinetics of Lck translocation to light DRMs: TCR/CD4 co-aggregation-induced Lck enrichment in light DRMs reaches its maximum at 30 s, which correlates with the already decreasing amount of activation-induced RACK1–Lck complexes (16). Binding of α-actinin-1 to the RACK1 complex exclusively in the presence of kinase active Lck suggests that formation of RACK1–$pY394^{Lck}$–α-actinin-1 module acts as a regulatory switch for the engagement of actin cytoskeleton upon productive TCR/CD4 triggering. A precise molecular mapping and structure–function analysis will be needed to dissect the parameters of this transient multiprotein complex formation and its interactome.

Our data also raise a fundamental question concerning the spatiotemporal regulation of interaction between microtubular and actin cytoskeleton during T-cell proximal signaling. We need to take into account evidence that not only the two filament systems interact with each other *in vivo* (72) but also that α-actinin specifically plays an integral role in the cooperative regulation of microtubular and actin cytoskeleton dynamics (73–75). In addition, as both RACK1 and α-actinin have been implicated in costimulatory and/or adhesion signaling which closely follows the TCR triggering event (71, 76, 77), it would be not entirely surprising that their TCR-induced complex formation would integrate signals from multiple receptors, including TCR/CD4, CD28, and integrins, and orchestrate the complex cooperative microtubular and F-actin cytoskeleton rearrangement. Importantly, as during early phases of TCR signaling, due to the adverse effect of tyrosine phosphorylation of α-actinin and increased levels of Ca^{2+} and PtdIns(3,4,5)-P_3, actin cytoskeleton would not be able to effectively engage, allowing T-cell membrane relaxation, rapid redistribution of signaling membrane protein to the forming IS, and coalescence of various types of membrane LRs, including redistribution of Lck to light DRMs. Later, once the initial wave of secondary messengers wanes, actinin engages and bundles filamentous actin so that the formation and maturation of actin architecture surrounding IS can be accomplished.

Taken together, data presented in this study advocate for the existence of a novel mechanism that integrates the engagement of TCR/CD4 receptors with cytoskeletal network *via* forming RACK1-based multiprotein network. While there is no doubt that more experimentation is necessary to fully elucidate its composition, structure, dynamics, kinetics, and the type of activation-dependent behavior, to the best of our knowledge, these are the first data that revealed its involvement in proximal T cell signaling with potential impact on the activation-induced repartitioning of Lck.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Institute of Molecular Genetics under the number #175/2011. The use of animals was also approved by the Academy of Sciences of the Czech Republic.

AUTHOR CONTRIBUTIONS

DF initiated and designed the experiments, and with OB analyzed the data. OB performed most of the experiments. JV and AB were involved in confocal microscopy and IP experiments. MD, OB, and JM prepared NIH3T3 transfectants, assisted with FACS analysis, and performed all animal work. PŘ and JS prepared samples and preformed MS TOF/TOF analysis and data mining. OB, JM, and DF wrote the initial draft of the manuscript, and DF finalized the manuscript.

ACKNOWLEDGMENTS

We thank Zdeněk Cimburek and Martin Čapek from flow cytometry and microscopy facility at the Institute of Molecular Genetics for expert technical assistance. This work was supported by Grant P302/12/G101 from Grant Agency of Czech Republic (GA CR).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00449/full#supplementary-material

VIDEO S1 | Time-lapse imaging RACK1 redistribution into immunological synapse upon T-cell activation. WT Jurkat T-cells expressing RACK1-EGFP (green) constructs were mixed with SEE pulsed RAJI B-cells and the formation of IS was observed by live cell imaging microscopy.

VIDEO S2 | Time-lapse imaging of Lck and RACK1 co-redistribution into immunological synapse upon T-cell activation. Lck-deficient JCAM1.6 Jurkat T-cells co-expressing Lck-CFP (red) and RACK1-mCitrine (green) constructs were mixed with SEE pulsed RAJI B-cells (blue) and the formation of IS was observed by live cell imaging microscopy.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

LCK, MEMBRANE MICRODOMAINS, AND TCR TRIGGERING MACHINERY: DEFINING THE NEW RULES OF ENGAGEMENT

The emerging of new models of TCR triggering resulted in intensive discussions among the scholars which model is the most appropriate one. Almost all of them assume the requirement for some kind of membrane spatial-temporal organization as a TCR triggering regulation tool. Also, the question how Lck activity is regulated, what initiates its function and many additional questions still remain unanswered.

Here, in this review article, we gave an overview of TCR triggering in the context of new findings. We focused mainly on the role of membrane microdomains and Lck activation during TCR engagement. We discussed how redistribution mechanisms within plasma membrane could provide additional regulatory step essential for T-cell activation. As a result, we propose our working model of TCR triggering.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 12 June 2012
doi: 10.3389/fimmu.2012.00155

Lck, membrane microdomains, and TCR triggering machinery: defining the new rules of engagement

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In spite of a comprehensive understanding of the schematics of T cell receptor (TCR) signaling, the mechanisms regulating compartmentalization of signaling molecules, their transient interactions, and rearrangement of membrane structures initiated upon TCR engagement remain an outstanding problem. These gaps in our knowledge are exemplified by recent data demonstrating that TCR triggering is largely dependent on a preactivated pool of Lck concentrated in T cells in a specific type of membrane microdomains. Our current model posits that in resting T cells all critical components of TCR triggering machinery including TCR/CD3, Lck, Fyn, CD45, PAG, and LAT are associated with distinct types of lipid-based microdomains which represent the smallest structural and functional units of membrane confinement able to negatively control enzymatic activities and substrate availability that is required for the initiation of TCR signaling. In addition, the microdomains based segregation spatially limits the interaction of components of TCR triggering machinery prior to the onset of TCR signaling and allows their rapid communication and signal amplification after TCR engagement, via the process of their coalescence. Microdomains mediated compartmentalization thus represents an essential membrane organizing principle in resting T cells. The integration of these structural and functional aspects of signaling into a unified model of TCR triggering will require a deeper understanding of membrane biology, novel interdisciplinary approaches and the generation of specific reagents. We believe that the fully integrated model of TCR signaling must be based on membrane structural network which provides a proper environment for regulatory processes controlling TCR triggering.

Keywords: Lck, Fyn, membrane microdomains, heavy and light DRMs, TCR triggering, compartmentalization, spatio-temporal regulation

INTRODUCTION

T cells are prototypical examples of highly sophisticated and meticulously regulated signaling cells. Signaling through the T cell receptor (TCR) has the potential to trigger a broad range of cellular responses. The utility of this potential, on the molecular level, is controlled by the existence of multiple signaling pathways that can be triggered individually or in parallel, in additive or synergistic fashions (Friedl et al., 2005; Acuto et al., 2008; Davis, 2009). However, despite having a comprehensive list of TCR signaling elements and their pathways, there is still a paucity of information concerning very proximal events that initiate T cell activation. This especially relates to the following questions: (i) how is the engagement of TCR by a peptide ligand presented in the context of MHC (pMHC) transduced across the plasma membrane (PM) to the interior of T cell and (ii) how is this signal spatially and temporarily coupled to the initiation of receptor's immunoreceptor tyrosine-based activation motif (ITAM) tyrosine phosphorylation itself. In this review, we will give a short account of the past and current literature concerning the mechanism controlling the initiation of enzymatic reactions by two Src-family tyrosine kinases (SFKs), Lck and Fyn, that drive the process of activation in mature peripheral $CD4^+$ T cells. We will argue that the tight control of TCR signaling is achieved via a highly regulated process of membrane microdomain-based segregation of various signaling molecules and that this compartmentalization represents the basic organizational principal underpinning the integrity, coordination, and spatio-temporal correlates of TCR proximal signaling.

TCR SIGNALING MODULES

The functional roadmap of TCR activation is usually presented as a two-dimensional cartoon densely populated by numerous proteins positioned sequentially along the multitude of signaling cascades spanning from the PM to the nucleus. While this simplification is of practical use, recent data suggest the existence of spatially restricted multiprotein modules designed to regulate and perform specific T cell signaling-related functions. The spatial integration of these modules is usually achieved either by a direct interaction of these proteins or by a common spatial confinement imparting their proximity. Based on their functional and spatio-temporal relationships, Acuto et al. (2008) conveniently grouped the signaling elements in T cells into three main signaling modules: (i) TCR triggering module (TCR module); (ii) Lck/Fyn regulation module (SFK module); and (iii) signal diversification and regulation module (D/R module).

TCR module is one of the most complex biological signal-transducing systems that contains a highly diversified receptor and

ligand components (Wucherpfennig et al., 2010). TCR consists of a ligand recognizing variable TCRαβ heterodimer electrostatically assembled with its signal-transducing chains CD3γ, CD3δ, CD3ε, and CD3ζ that in their cytoplasmic portion contain 10 ITAMs (Call et al., 2002). This structural complexity of TCR is dictated by the functional requirement for the generation of a biologically relevant signal which is able to translate the affinity of TCR-pMHC interaction, gauged by the duration of their engagement, to quantitatively distinct ITAM phosphorylation patterns (Kersh et al., 1998; Acuto et al., 2008). A critical step in this process is the ordered phosphorylation of ITAMs (Kersh et al., 1998), controlled by SFK module and executed mainly by Lck (van Oers et al., 1996; Hegedus et al., 1999). Phosphorylated ITAMs serve as docking sites for tyrosine kinase ZAP-70 which after being activated by Lck proceeds to phosphorylate the adaptor protein LAT on multiple sites. This in turn, leads to the engagement and assembly of the D/R module initiating downstream signaling. Notably, the recruitment of phospholipase $C\gamma_1$, as well as the adaptor proteins Grb2 and GADS to these sites allows the activation of MAPK signaling pathways, Ca_2^+ flux, and formation of multimeric signaling complexes which lead to cytoskeletal reorganization and integrin activation (Nel, 2002; Smith-Garvin et al., 2009). These pathways then converge on both the activation and translocation to the nucleus of critical transcription factors NF-AT, NF-κB, and AP1, which control the expression of the cytokine IL-2, required for activation and proliferation of T cells in addition to the expression of other activation-inducible genes. Due to the complexity of TCR mediated signaling, a tight regulation of this process is, at least partly, provided by a membrane-based compartmentalization.

LIPID RAFTS, CYTOSKELETON, AND TRANSLOCATIONS

To achieve unencumbered coordination, specificity, and efficiency in signaling, much of the biochemistry in cell signaling is spatially restricted and timely regulated by the physical segregation of signaling elements into an appropriate confinement. This process of compartmentalization is highly complex and dynamic. It involves not only the engagement of immune receptors, translocation of critical signaling elements to cell–cell contact zones and nucleation of active signaling complexes, but also a cellular architecture that supports it. This includes the lateral segregation of PM into distinct microdomains, called lipid rafts (LR; Horejsi, 2003), and a cytoskeletal network that regulates its structural and dynamic changes (Billadeau et al., 2007; Viola and Gupta, 2007; Burkhardt et al., 2008).

Lipid rafts, also called membrane rafts, are defined as small (5–50 nm in diameter), heterogeneous, highly dynamic, cholesterol, and sphingolipid-enriched microdomains that compartmentalize cellular processes (Simons and Ikonen, 1997; Simons and Toomre, 2000; Marmor and Julius, 2001; Sharma et al., 2004; Rodgers et al., 2005; Manes and Viola, 2006; Goswami et al., 2008; Lindner and Knorr, 2009; Lingwood and Simons, 2010; Simons and Sampaio, 2011). Due to their stable and highly ordered structure, LR tend to be resistant to solubilization by mild non-ionic detergents and can be isolated as a detergent-resistant membrane (DRM) fraction by discontinued sucrose density gradient centrifugation (SDGC; Edidin, 2003; Lingwood and Simons, 2007). However, this operational definition of LR, originally based solely on their resistance to solubilization and coupled with inconclusive early microscopic investigations into LR organization in living cells, cast serious doubts not only about the content-related fidelity between DRMs and bona-fide native LR, but also challenged the existence of LR *in vivo* (Munro, 2003; Shaw, 2006; Leslie, 2011). Nowadays, however, using the state-of-art imaging techniques on live cells, the evidence demonstrating lipid and protein-dependent functional subcompartmentalization of cell membrane is being increasingly accepted (reviewed in Lingwood and Simons, 2010; Simons and Sampaio, 2011).

Nevertheless, the question if and to what extent can DRMs be equated to native LR is the subject of ongoing debates. As this review often cites work related to the utility of cell solubilization methods used to obtain various DRMs and the credibility of which is frequently discounted, we feel obliged to provide several remarks justifying the implementation of this experimental approach. While there is a general consensus that DRMs are indeed of an altered composition, it has been demonstrated that a critical factor affecting the degree of their content-related difference is in the type of detergent used (Schuck et al., 2003; Garner et al., 2008). In this context, the widely used Triton X-100 detergent (TX) seems to be a suboptimal choice as it has been shown to induce considerable rearrangement of the original membrane (Koumanov et al., 2005), facilitates the merging of membrane patches of distinct cellular origin (Madore et al., 1999) and scrambles membrane symmetry (Radeva and Sharom, 2004). It has been also documented that TX-derived DRMs represent extraction intermediates in the solubilization of which is a function of time (Lindner and Naim, 2009). In contrast, these adverse effects were not observed for Brij-type detergents such as Brij-96 and 98 (Schuck et al., 2003; Chen et al., 2007). Moreover, the latter type of detergent generates stable (up to several months), content-wise reproducible, small diameter DRMs that selectively retain proteins (Drevot et al., 2002; Chen et al., 2009; Knorr et al., 2009; Otahal et al., 2010; Ballek et al., 2012). As discussed below, another important fact is that Brij solubilization extracts distinct types of DRMs that can be further separated by immunoprecipitation (Drevot et al., 2002; Brugger et al., 2004; Chen et al., 2009; Ballek et al., 2012). While not equating DRMs with native LR, these data suggest that Brij-type DRMs display properties that are compatible with those of native LR, at least to the extent that allows us to use them as a biochemical means to probe the heterogeneity of membrane raft environments (Chen et al., 2007, 2009; Lindner and Naim, 2009; Horejsi et al., 2010). It should be noted that DRM partitioning is not only the most frequently used method to assess raft-related transmembrane signaling, but often is instrumental in providing insight into the nature of its components and the underlying mechanism(s) (Lingwood and Simons, 2007).

Given that the solubilization-dependent cofractionation method is the only available biochemical approach to evaluate the protein distribution to DRM, and thus its raft potential, the choice of an appropriate detergent is of critical importance (Lichtenberg et al., 2005; Brown, 2006, 2007; Lingwood and Simons, 2007). In this respect, Brij-type detergents are becoming the tools of choice in characterizing the potential for raftophilicity of signaling molecules before and after initiation of signaling (Horejsi et al., 2010; Quinn, 2010). Thus, the primary importance of the

solubilization approach is that it can provide the initial insight into the activation-induced changes in DRM association and lay the conceptual network for assessment of the role of lipid-based nanodomains in membrane signaling. However, due to the unavoidable degree of artificiality inherent to this approach, these findings require verification by means of other types of methods. As an example, the correlation between Brij-type DRM cofractionation of CD4 and CD45 molecules and their membrane confinement assessed by using advanced microscopic technologies lent the credibility to the Brij-based solubilization approach (James et al., 2011; Ballek et al., 2012).

Another important aspect of Brij-based membrane solubilization is that at least two distinct fractions of DRMs can be isolated. The first type represents the light DRMs that are easily obtained by SDGC as low density fractions that float to the top of the gradient (Edidin, 2003; Lingwood and Simons, 2007). Recently, a new type of microdomains called high density or heavy DRMs has been documented in immune cell signaling (Rollet-Labelle et al., 2004; Otahal et al., 2010, 2011; Ballek et al., 2012). The light and heavy DRMs share both their resistance and sensitivity to solubilization by a polyoxyethylene type of detergent (such as Brij58 and Brij98) and lauryl maltoside (LM), respectively. On the contrary, due to their different protein-to-lipid ratios, light, and heavy DRMs can be separated from each other by SDGC (Otahal et al., 2010; Ballek et al., 2012). It is of note that Brij58 and Brij98 display a relatively high degree of specificity for a distinct type of LR, thus preserving distinct subpopulations of DRMs (Drevot et al., 2002; Knorr et al., 2009; Lindner and Knorr, 2009). As critical signaling molecules such as pY394Lck, CD3ζ, H-ras, CD5, CD28, CD45, LAX associate with heavy DRMs, this non-conventional and so far largely overlooked type of microdomains could represent important membrane structures that are able to support T cell signaling (Otahal et al., 2010, 2011; Ballek et al., 2012).

Several complementary approaches lend credibility to the notion that LR indeed have a role in the initiation of T cell signaling. These include but are not limited to (i) functional assays targeting critical signaling molecules to distinct membrane subcompartments (Kabouridis et al., 1997; Zhang et al., 1999; Otahal et al., 2010, 2011); (ii) state of the art electron, fluorescent microscopy, and nanoscopy (reviewed in Lindner and Naim, 2009); (iii) biochemical data suggesting the existence of several distinct types of DRMs extracted from the same cellular membranes (Drevot et al., 2002; Knorr et al., 2009; Ballek et al., 2012); and (iv) requirement for a distinct ganglioside composition of functional LR in $CD4^+$ versus $CD8^+$ T cells (Nagafuku et al., 2012). The latter study is of significant importance as it shows that activation of $CD4^+$ T cells, but not their $CD8^+$ counterparts, isolated from knock-out animals deficient in GM3-derived ganglioside, is severely compromised and animals fail to develop experimental asthma. As GM3 is the main component of LR, these data provide strong *in vivo* evidence for the functional involvement of LR in T cell signaling (Nagafuku et al., 2012).

TCR/CD3 proximal signaling appears to depend on the integrity of LR (Dykstra et al., 2003). A demonstration that TCR ligation by pMHC complex on antigen-presenting cells (APC) results in both (i) the clustering of LR at the interphase between T cell and APC, referred to as the immunological synapse (Davis and Dustin, 2004; Chichili et al., 2010), and (ii) the recruitment of CD3ζ, ZAP-70 kinase, adaptor SLP76, PKCθ, $PLC\gamma_1$, Itk, and Carma1 kinase to LR, provide strong evidence for the function of LR in T cell signaling (Horejsi, 2003; Filipp and Julius, 2004; Palacios and Weiss, 2004; Kabouridis, 2006; Ballek et al., 2012).

Available data strongly suggest that raft dynamics depends on F-actin rearrangements. According to this model, interactions between actin and actin-binding transmembrane proteins and lipids in the inner leaflet of the PM form a membrane skeletal mesh (MSM) functioning as a structural regulator of LR dynamics (Kusumi et al., 2005). In this respect, several signaling molecules, such as CD3ζ, CD2, PAG, and CD45, have been shown to be directly or indirectly linked to actin cytoskeleton (Zeyda and Stulnig, 2006). The MSM structure is further supported by its interaction with filamin A (FLNA) which crosslinks actin proteins to generate and sustain the sub-cortical actin cytoskeleton (Tavano et al., 2006). As FLNA also interacts with the co-stimulatory molecule CD28, it has been suggested that TCR-CD28 coaggregation is the principal regulatory mechanism for LR-based compartmentalization of signaling elements at an immunological synapse (Lee et al., 2002; Tavano et al., 2006). Very recent key studies also suggest that actin cytoskeleton plays an indispensible role in the nanoscale organization at the PM of critical T and B cell signaling molecules (Harwood and Batista, 2011; Sherman et al., 2011).

Another important consideration is the time frame of T cell activation. It has been demonstrated that in resting T cells the formation of a mature immunological synapse with TCR receptors localized in its central zone called c-SMAC is observed 15–30 min after the initiation of T cell-APC interaction (Lee et al., 2002). Importantly, this event is preceded by the early recruitment of kinase active Lck to the forming immunological synapse, with maximum activity between 2 and 5 min after the initiation of T cell-APC conjugation. This pattern was closely followed by the presence of kinase active ZAP-70. Thus, the peak of Lck- and ZAP-70-mediated tyrosine kinase signaling initiated upon the TCR engagement occurs well before a mature synapse is fully formed. These data suggest that the initiation of compartmentalization of signaling elements linked to the translocations of critical kinases occurs rapidly after the T cell-APC contact has been established.

SFK MODULE: NON-REDUNDANT ROLE OF Lck AND Fyn IN PROXIMAL TCR SIGNALING

Whereas all three signaling modules – SFK, TCR, and D/R- contribute to the regulation of threshold, magnitude, and duration of T cell activation signals, the full understanding of how SFK and TCR modules are integrated is central to the understanding of the initiation of T cell activation. Lck and Fyn provide the critical functions predicating the generation of the most proximal signals emanating from the TCR (Latour and Veillette, 2001). While they share common structural features (Boggon and Eck, 2004; Salmond et al., 2009), mode of regulation (Mayer, 1997), membrane localization (Kabouridis et al., 1997; Resh, 2006), the ability to phosphorylate CD3ζ *in vitro* (Hegedus et al., 1999; Mustelin and Tasken, 2003) and display a partial capacity to compensate for each other's deficiency (Groves et al., 1996), biochemical, genetic, and topographical evidence suggest that their roles in the cellular activation process, while interdependent, are functionally distinct

(Filipp et al., 2003; Filipp and Julius, 2004). This is in agreement with the fact that Fyn, but not Lck, has been shown to associate with the CD3 complex via the interaction of its unique domain with ε-chain, although at low stoichiometry (Samelson et al., 1990; Salmond et al., 2011). On the other hand, the delivery of Lck function in TCR proximal signaling is modeled through its stoichiometric interaction with CD4 which, due to its interaction with the non-variable region of peptide presenting MHCII, relocate Lck to ITAMs of the TCR/CD3 complex (Meuer et al., 1982; Abraham et al., 1991; Maroun and Julius, 1994; Bonnard et al., 1997). While the co-binding of CD4 to the MHCII ectodomain is not essential for the stability of TCR-pMHC complex (Artyomov et al., 2010), it significantly augments T cell sensitivity under circumstances when low numbers (<30) of specific peptides are presented (Irvine et al., 2002).

The differential "wiring" mechanism of Lck and Fyn to the TCR/CD3 complex is extended to their capacity to interact with distinct downstream targets and signaling pathways (Zamoyska et al., 2003). Specifically, anti-CD3 stimulation induced tyrosine phosphorylation of the Fyn substrate Cbl but, not the ZAP-70 substrate LAT (Tang et al., 2002). Several studies also demonstrated that Fyn can activate the Ras/Erk pathway in an Lck-independent fashion via the recruitment of Grb2-SOS to hypophosphorylated CD3ζ chains (Chau et al., 1998; Chau and Madrenas, 1999; Denny et al., 2000; Methi et al., 2007). In addition, Fyn, via its stimulation through CD3, has been implicated in the induction of T cell anergy. This mechanism involves the Fyn-mediated phosphorylation of the LR-associated adaptor protein PAG and the sequestration of active Ras to LR (Davidson et al., 2007; Smida et al., 2007). Consistent with these data, Zamoyska and colleagues, in a series of elegant experiments with inducible expression of Lck or Fyn on their relevant genetically deficient background, demonstrated different effects of Lck and Fyn on T cell development and TCR signaling (Zamoyska et al., 2003; Salmond et al., 2009). The argument for the functional distinction of Lck and Fyn is further supported by the topographical studies demonstrating that LR separates these two kinases. In primary resting T cells, 75–95% of Lck resides outside LR while more than 98% of Fyn is associated with LR (Filipp et al., 2003). Collectively, these data suggest that Fyn plays an important role in the generation of Lck- and ZAP-70- independent signals directly through the CD3 complex. This functional uncoupling of Lck and Fyn signaling is important when building the model of proximal T cell signaling (see below).

REGULATORY CIRCUITS OPERATING IN SFK MODULE

Lck displays prototypical structural features of SFKs. It consists of a short N-terminal region post-translationally modified by lipid acylation which is required for its association with the PM. It is followed by unique, SH3 and SH2 domains, a short linker region, a catalytic domain and a C-terminal segment (Boggon and Eck, 2004). Its kinase activity is regulated via the reversible phosphorylation of two critical tyrosine residues: (i) positive regulatory Y394 located in the kinase domain and (ii) negative regulatory Y505 in the C-terminal segment (**Figure 1**). The phosphorylation of Y394, achieved through trans/autophosphorylation, predicates the full kinase activity of Lck and exhibits a dominant effect over the inhibitory Y505 if both tyrosine residues are phosphorylated in tandem (Hardwick and Sefton, 1997; Ashwell and D'Oro, 1999). As Lck provides enzymatic activity which drives the T cell activation process, the full understanding of mechanisms regulating this activity is essential to build the realistic model of T cell proximal signaling. Taking advantage of the fact that T cells are one of the most intensively studied cellular models in respect of the role of membrane subcompartmentalization in signaling, in the context of this review we will highlight those features of SFK regulatory circuits which are connected to raft structures.

The post-translational biochemistry of Y505 is relatively well understood. When phosphorylated (pY505) by C-terminal src kinase (Csk), the C-tail clamps on its own SH2 domain, locking the kinase domain in an inactive, closed conformation (Mayer, 1997). However, since ^{505}pYQPQP is not an optimal sequence for the interaction with the SH2 domain, it is assumed that the Lck tail would dynamically swing on and off its binding pocket in SH2, making this interaction weak and unstable (Mayer, 1997; Nika et al., 2007). This structural instability is counteracted by the ability of a linker region to interact with its own SH3 domain and thus maintain the repressed conformation of Lck (Yamaguchi and Hendrickson, 1996; Sicheri et al., 1997; Xu et al., 1997; Rudd et al., 2006; **Figure 1**). As a consequence, the interaction of SH2 and/or SH3 with their high affinity intracellular ligand can release this inhibitory conformation and positively regulate its kinase activity (**Figure 1**; Moarefi et al., 1997; Marti et al., 2006).

Csk is a cytosolic protein and its proximity to the PM is a prerequisite for SFK's C-tail tyrosine phosphorylation (Palacios and Weiss, 2004). The recent demonstration that an acute inhibition of Csk activates TCR signaling independently of ligand binding strongly supports the idea that Csk is one of the most critical elements of basal signaling machinery negatively regulating Lck and Fyn activities in resting T cells (Schoenborn et al., 2011). In mouse thymocytes, approximately 20% of cytosolic Csk is recruited to the PM via its interaction with PAG (Davidson et al., 2003). PAG is a DRM-associated constitutively tyrosine phosphorylated adaptor which serves as docking sites for various SH2 domain-containing proteins such as Lck, Fyn, Shc, Vav, GAP, PI3K, ZAP-70, and Syk (Brdicka et al., 2000; Kawabuchi et al., 2000). In resting T cells, the SH2 domain of Csk binds to pY317 of PAG. Because the phosphorylation of this site is attributed to DRM-associated Fyn that, in turn, is negatively regulated by Csk, PAG-Csk complex represents the critical component of a negative homeostatic regulatory feedback mechanism keeping Fyn activity restrained. Importantly, upon TCR engagement, pY371 dephosphorylation releases Csk to the cytosol resulting in a membrane environment conducive to Fyn activation (Brdicka et al., 2000). Interestingly, while one study suggested that CD45 phosphatase can act on pY371 of PAG (Davidson et al., 2003) another failed to confirm this finding (Brdicka et al., 2000).

In the context of membrane compartmentalization, it is not clear how Csk is positioned in the vicinity of most membrane Lck which is not DRM-associated (Filipp et al., 2003). The fact that approximately 50% of Lck in resting cells is phosphorylated on Y505 (Luo and Sefton, 1990) suggests an alternative way for Csk recruitment to non-DRM fractions. The same notion relates to the recruitment of Csk to DRMs since PAG deficiency has no tangible effect on thymocyte development and proximal T cell signaling

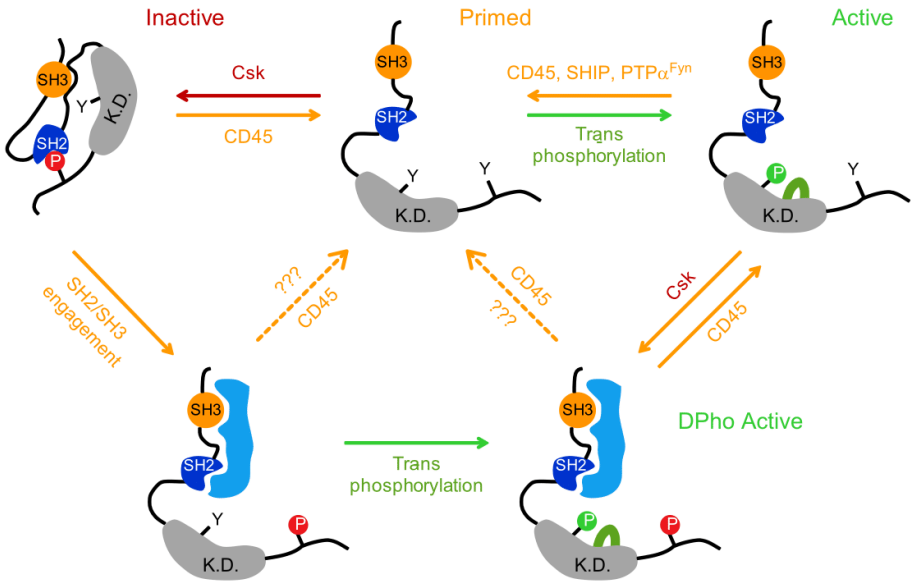

FIGURE 1 | A traditional model postulates that Lck kinase activity is regulated by reversible phosphorylation of two tyrosines residues: positive regulatory tyrosine Y394 in kinase domain (K.D.) and the negative regulatory tyrosine Y505 in the C-terminal end. When Y505 is phosphorylated (depicted here as P in red circle) by C-terminal src kinase (Csk), it forms an intramolecular bond with its own SH2 domain, locking the kinase domain in a substrate inaccessible, closed inactive conformation (top left structure). CD45 can dephosphorylate pY505, leaving the Lck structure in an open, primed conformation displaying a relatively low kinase activity (top middle structure). To achieve full kinase activity, the phosphorylation of Y394 (depicted here as P in green circle) must be achieved through trans/autophosphorylation (top right structure); its dephosphorylation is performed by CD45 or SHIP-1 or alternatively by PTPα in case of Fyn. It has been recently demonstrated that Lck can also persist in a double phosphorylated state (DPho), when both regulatory tyrosines (Y505 and Y394) are phosphorylated concurrently and the kinase retains its activity (bottom structure). DPho state is likely achieved in a two-step process. The first step involves the interaction of high affinity ligand(s) with Lck SH2 and/or SH3 domain which disrupts the closed conformation and is followed by CD4-Lck aggregation-mediated trans/auto phosphorylation of pY394. Alternatively, it is generated by the action of Csk on pY394 Lck. Whether CD45 converts these isoforms to a dephosphorylated, primed conformation or to an active pY394 state *in vivo*, is unclear (dashed arrows). Red arrows denote negative regulation; green arrows denote positive regulation.

(Veillette et al., 2002; Davidson et al., 2003; Dobenecker et al., 2005). The alternative interaction partners could include DRM-resident adapter protein LIME and soluble fraction-associated SIT, paxillin, focal adhesion kinases, and Dok-related adaptors (Veillette et al., 2002; Tedoldi et al., 2006; Yasuda et al., 2007; Schoenborn et al., 2011). Thus, while there is a general consensus that Csk is a critical enzymatic effector of TCR signaling inhibitory mechanism acting on Lck and Fyn, we are still far from a full understanding of the molecular infrastructure supporting its function. From the point of view of TCR triggering, this notion chiefly concerns the process of compartmentalization of those adapter proteins that recruit Csk to the PM as well as the characterization of kinases and phosphatases that regulate this process via acting on tyrosine residues through which Csk is recruited to unidentified adaptors such as Y371 of PAG.

The action of Csk is opposed by a transmembrane phosphatase CD45 which is able to dephosphorylate pY505, leaving the Lck structure in an open, primed conformation with a basal level of kinase activity (D'Oro and Ashwell, 1999; Hermiston et al., 2003; **Figure 1**). Phosphatase activity is critical for Lck physiology, as CD45 deficiency results in the hyperphosphorylation of pY505, leading to a dysfunctional Lck and a severely impacted ability to trigger TCR signaling (Stone et al., 1997). This signaling-incompetent Lck phenotype can be reverted by the expression of a constitutively active mutant Y505F Lck allele (Seavitt et al., 1999). The positive regulatory role of CD45 contrasts with its enzymatic activities negatively affecting TCR responses. Those include the dephosphorylation of a positive regulatory pY394 residue on Lck and pY-ITAMs on CD3 chains (Trowbridge and Thomas, 1994). Mechanistically, it is unclear how this functional dichotomy of CD45 is coordinated on the molecular level. Attempts to explain it by the mutual interaction of CD45 with a specific extracellular ligand failed to obtain supportive experimental data. It has been reported that within the context of animals, positively (dephosphorylation of pY505) and negatively (dephosphorylation of pY394) directed CD45 activities are predicated by a low to medium and high level of its expression, respectively. Thus, an abundant expression of CD45 can be regarded as a physiologically relevant mechanism to suppress the hyperactivation of T cells via dephosphorylating pY394 Lck (Mustelin et al., 1989; McNeill et al., 2007). However, the question remains of how opposing activities of CD45 are delivered on a single cellular level, where the

existence of several pools of Lck with a combinatorial pattern of Y505 and Y394 phosphorylations have been recently demonstrated (**Figure 1**; Nika et al., 2010).

Available data suggest that membrane compartmentalization plays a critical role in controlling CD45 involvement in regulating Lck activities and TCR signaling. The first evidence for non-homogenous membrane distribution of CD45 in T cells comes from the observation that the effect of CD45 deficiency on the phosphorylation status of Lck, especially on its negative regulatory tyrosine, is much more pronounced than that on Fyn (Hurley et al., 1993; Stone et al., 1997). That correlates with the significantly reduced kinase activity of membrane associated Lck, but not Fyn in these cells (Biffen et al., 1994). While a possible explanation could relate to sequence differences in the C-terminal tail of Lck and Fyn (Xu et al., 1997) and their ability to interact with CD45 (Nam et al., 2005), it seems that in *in vitro* conditions CD45 dephosphorylates Lck and Fyn with similar efficiencies (Ostergaard et al., 1989; Mustelin et al., 1992). Thus, the most likely explanation is that the proximity of CD45 with Lck and Fyn is affected by their membrane compartmentalization. Indeed, several groups have reported that only 0–5% of CD45 was found to be associated with DRMs, while the rest of CD45 pool colocalizes with Lck in a soluble fraction (Edmonds and Ostergaard, 2002; Maksumova et al., 2005; Ballek et al., 2012). It is thus possible that another DRM-associated phosphatase, PTPα, which can dephosphorylate both pY528 and pY417 residues of Fyn, substitutes for CD45 activity (Maksumova et al., 2005; Vacaresse et al., 2008). In addition, the capacity of SHIP-1 phosphatase to target pY394 Lck residue may likely account for the deactivation of kinase active Lck associated with DRMs (Chiang and Sefton, 2001; Kosugi et al., 2001).

Thus, an enzymatic control of the formation and breakage of intramolecular interactions that impact the conformation of Lck and Fyn can be adjusted by a variety of regulatory inputs that harness or activate their kinase activity. TCR-independent regulation of Lck and Fyn through constitutively driven action of Csk and CD45 establishes the net phosphorylation balance at critical tyrosine residues dictating the basal level of their kinase activities. Since in primary resting $CD4^+$ T cells LR segregate a majority of Lck and Fyn, their regulatory circuits must be coordinated within the limits of redistribution and recruitment of CD45 and Csk, respectively, to these distinct membrane compartments. While our knowledge of these processes is still incomplete, consideration of these parameters should provide a deeper understanding of the constraints on the mechanism controlling TCR triggering.

MODELING THE INVOLVEMENT OF Lck AND Fyn IN TCR TRIGGERING MECHANISM

Conventionally, Lck in resting non-stimulated T cells is considered to be inactive due to its pY505 status. The binding of the pMHC complex leads to the coaggregation of TCR with CD4, clustering-mediated trans/autophosphorylation of CD4-associated Lck and its juxtaposition to and subsequent phosphorylation of ITAMs of CD3 chains (Mendieta and Gago, 2004; Smith-Garvin et al., 2009). This type of aggregation model of Lck activation relies on regulatory proteins CD45 and Csk that can swiftly and reversibly turn Lck on and off, respectively. A caveat to this model is that during the aggregation period, CD45 would have to dephosphorylate those CD4-associated Lck molecules that accumulated in the vicinity of the TCR-pMHC complex. While the low stoichiometry of interaction between CD45 and CD4-Lck complex has been reported (Bonnard et al., 1997; Irles et al., 2003), the mechanism coupling TCR triggering with CD45-mediated dephosphorylation of pY505 Lck remains elusive.

The main argument discounting this version of the aggregation model is that under some circumstances the CD4 coreceptor is dispensable for TCR signaling (Haughn et al., 1992; Straus and Weiss, 1992; Van Laethem et al., 2007; Acuto et al., 2008). Consistent with this notion, $CD4^-$ but not $CD4^+$ T cells responded to mAb-mediated anti-TCRCβ stimulation, arguing that the sequestration of Lck by CD4 makes the kinase unavailable for TCR signaling (Haughn et al., 1992). The expression of the mutated form of Lck which was unable to bind to CD4 recapitulated the anti-TCRCβ responsive phenotype and correlated with the enrichment of Lck in DRMs, concomitant activation of DRM-colocalized Fyn and the presence of hypophosphorylated ZAP-70-pp21ζ complexes (Haughn et al., 1992, 1998; Filipp et al., 2004). Thus, targeting active Lck locally to the site of TCR engagement via CD4 can provide the much needed sensitivity when signaling a low number of pMHCII (<30), while a less sensitive signaling supported by a "diluted," membrane bound CD4-"free" Lck is driven by a distinct mechanism, likely involving Fyn (Haughn et al., 1992, 1998; Irvine et al., 2002; Filipp et al., 2004).

Lck and Fyn provide critical enzymatic activities that drive TCR triggering (Filipp and Julius, 2004). We, and others, have previously demonstrated that membrane microdomains play an essential role in temporal and spatial coordination of these two kinases (Filipp et al., 2003, 2004; Maksumova et al., 2005). Accordingly, the coaggregation of TCR and CD4 results in the activation of Lck within seconds in soluble fractions followed by its translocation into DRMs and activation of colocalized Fyn. We further showed that only DRM-associated, kinase active Y505F Lck reciprocally co-immunoprecipitates with and activates Fyn. Mutational analyses revealed a profound reduction in the formation of Lck-Fyn complexes and Fyn activation using kinase domain mutants K273R and Y394F of Y505F Lck, both of which have severely compromised kinase activity. Thus, these results suggested a model where Lck-dependent Fyn activation is predicated by proximity-mediated transphosphorylation of the Fyn kinase domain in LR (Filipp et al., 2008). It is of note that while the above described studies are consistent with the notion that function of Lck is proximal to that of Fyn when TCR and CD4 are coaggregated, the reciprocal role of Fyn in Lck activation when stimulated via CD3 has also been reported (Sugie et al., 2004).

An unexpected outcome of our study was the characterization of the C-terminal YQPQP sequence acting as a novel *cis*-acting component essential for the partitioning of Lck to LR (Filipp et al., 2008). Notably, the sequential truncation of YQPQP resulted in an increased reduction of kinase active Lck partitioning to LR, in both fibroblasts and T cells. This, in turn, correlates with ablation of the capacity of these truncates to enhance TCR mediated IL-2 production (Filipp et al., 2008). This is in agreement with our previously published data reporting a requirement for an enrichment of Lck in DRMs for the initiation of proximal TCRαβ signaling (Filipp

et al., 2004). Our preliminary data pointed to the existence of multiprotein translocation molecular machinery that is responsible for the targeting of Lck to DRMs via the tethering of its C-terminal sequence directly to microtubular cytoskeletal elements. Indeed, we have already demonstrated that, nocodazole, but not latrunculin B, pharmacological agents inhibiting the polymerization of tubulin and actin, respectively, blocked the TCR/CD4 activation-induced enrichment of Lck in light DRMs (Ballek et al., 2012). These data suggests that the C-terminal sequence of Lck, on the top of its negative regulatory function, targets Lck to microtubular network resulting in the reorganization of cytoskeleton and redistribution of Lck to LR.

A POOL OF PREACTIVATED Lck IN T CELL PROXIMAL SIGNALING

The exact mechanism regulating the level of kinase activity of Lck prior to stimulation remains obscure. However, precisely quantifying the phosphorylation pattern of Lck in resting T cells may provide a context from which to base correlations with its main regulators such as CD45 and Csk.

In doing so, recently published data has provided new insight into the source of pY394Lck used for the initiation of TCR signaling (Nika et al., 2010). The authors demonstrated the existence, in primary naïve $CD4^+$ T cells, of a substantial pool of preactivated pY394Lck (∼40%) and showed that this amount remained unchanged on the global cellular level after anti-CD3 as well as antigen mediated TCR stimulation. These findings are consistent with a previous study that was unable to provide evidence of any measurable increase of the structurally open conformation of Lck upon anti-CD3 activation (Paster et al., 2009). Results of these studies invite a discussion concerning the origin, maintenance, and function of the preactivated Lck in spatio-temporal coupling of TCR engagement to the generation of the most proximal biochemical signals (Berry, 2011; Davis and van der Merwe, 2011).

Therefore, with good justification, the search for the mechanism triggering T cell activation has centered on the organization of membrane structures that support membrane segregation and redistribution of preactivated Lck to the proximity of its physiological substrates. We have recently shown that in resting $CD4^+$ T cells the pool of pY394 Lck is associated with heavy DRM fractions and that T cell activation led to its redistribution from heavy to light DRMs in a microtubular network-dependent fashion (Ballek et al., 2012). We also found that the kinase active, heavy DRM-associated pool of Lck is membrane confined with CD45 phosphatase. Moreover, most of CD3ζ was also associated with heavy DRMs.

Importantly, TCR/CD4 aggregation resulted in up to a 50-fold increase in the total level of pY394 Lck, whereby the heavy DRM-associated fraction of Lck contributed by ∼70%. If we theoretically assume that TCR/CD4 coaggregation activates the entire pool of cellular Lck (this approximation is obviously incorrect as about 10–30% of Lck is not membrane associated), we estimate that the pool of preactivated pY394 Lck in resting $CD4^+$ T cells represents only about ∼2% of total kinase (Ballek et al., 2012). However, as the cytosolic pool of Lck which is not involved in TCR signaling is found to be constitutively active (Haughn et al., 1998), this estimate can be even lower. The reason for this discrepancy with a previously published report is unclear but, likely stems from using various types of T cells, different mode of activation, solubilization protocols, and detection methods (discussed in Ballek et al., 2012). However, in this context, the key observation here is that the lowest levels of pY394 Lck in resting freshly isolated but otherwise unmanipulated primary $CD4^+$ T cells were observed when their solubilization was performed instantly in boiled Laemmli buffer. A lysate buffer-mediated cell solubilization reproducibly showed elevated basal levels of pY394. This, in turn, caused a significant diminution of differences in pY394 levels between TCR/CD4 aggregated and non-aggregated samples when Lck was immunoprecipitated (manuscript in preparation).

INCORPORATING MEMBRANE COMPARTMENTALIZATION INTO THE MODEL OF TCR TRIGGERING

The above described data support Davis and van der Merwe's (2006) kinetic-segregation (KS) model of T cell activation. The model posits that mutual random interactions between Lck, Fyn, tyrosine phosphatases CD45/CD148, and Csk as well as Lck and Fyn themselves, in a resting state, establishes the net balance of Lck and Fyn phosphorylation predicating their kinase activity. According to this model, spontaneous kinase activation is prevented by CD45 phosphatase which is able to dephosphorylate critical regulatory residues pY505 and pY394 on Lck as well as pY-ITAMs on CD3 chains (Trowbridge and Thomas, 1994; Hermiston et al., 2003). Its abundance (two to three molecules of CD45 per TCR complex), high enzymatic activity (100–1000 times faster rate of dephosphorylation compared to the rate of phosphorylation by SFKs), and broad specificity (Trowbridge and Thomas, 1994; Davis and van der Merwe, 2006), make CD45 by far the most efficient suppressor of tyrosine phosphorylation-driven T cell activation processes (Davis and van der Merwe, 2006; Choudhuri and van der Merwe, 2007).

The presence in T cells of a preactivated pool of Lck has been a welcome addition to the schematics of KS model, as it allows one to ignore the requirement for a specific mechanism which would couple Lck activation to TCR triggering (Chakraborty and Das, 2010; Davis and van der Merwe, 2011). However, our estimation that in resting T cells less than 2% of total Lck is kinase active, raises the question of whether this pool is sufficient to provide support for TCR signaling without its prior enlargement (Ballek et al., 2012). A rather low amount of this pool can also significantly impact quantitative aspects of biochemical reactions initiating T cell activation (Chakraborty and Das, 2010). Moreover, both pY394Lck and a sizeable pool of CD45 were found in heavy DRMs suggesting that kinase activity of pY394Lck is spatially restricted and controlled by its membrane colocalization with this phosphatase (Krotov et al., 2007; Ballek et al., 2012). This observation found its support in a recent demonstration that a fraction of CD45 and CD4 are colocalized by a virtue of membrane confinement accounting for their enormously enhanced association rate (Haugh and Lauffenburger, 1997; James et al., 2011). The significance of this finding is highly relevant to the methodology used for the solubilization of cells as it indicates that Brij58 heavy DRMs selectively concentrate molecules that colocalize to a certain type of membrane environment *in vivo*.

The critical TCR signal-generating molecules distribute preferentially to a distinct type of lipid microdomains (**Figure 2**). Notably, most of CD3ζ (and likely the entire TCR/CD3 complex) fractionate to heavy DRMs and display a kinetically synchronized redistribution with active Lck to light DRMs. Whether DRMs with which CD3ζ and pY394 Lck are preferentially associated represent a common or slightly distinct type of microdomains is unknown at the present time. Either way, heavy DRMs can support two independent mechanisms inhibiting TCR triggering. First, the membrane colocalization of CD45 with pY394 Lck and/or CD3ζ can keep their net tyrosine phosphorylation on a minimal level. Second, as posited by the Safety TCR triggering model, due to its negatively charged lipid composition, these microdomains could provide a suitable environment for the sequestration of intracellular chains of CD3ζ and CD3ε via the interaction of its basic residue-rich sequences with the inner leaflet of the PM, thus preventing them from being targeted by active Lck (Kuhns and Davis, 2008; Xu et al., 2008). Despite the fact that the mechanism liberating these CD3 chains from the PM (Fernandes et al., 2010; Gagnon et al., 2010) as well as the lipid composition of heavy DRMs have not been elucidated, coupling the presence of CD45 with the "Safety TCR Trigger" mechanism endows these domains with a powerful anti-pY mechanism to maintain a non-signaling phenotype in resting T cells. Moreover, it is quite likely that adaptor proteins PAG and LAT reside in a slightly distinct type of membrane microdomains, as their profile of Brij58 solubilization pattern is only partially overlapping (Brdicka et al., 2000). LAT in resting T cells is not tyrosine phosphorylated, suggesting that its resident microdomains are either devoid of tyrosine kinases, or heavily enriched in tyrosine phosphatases. Using two-color photoactivated localization microscopy it has been recently shown that LAT-containing membrane nanoclusters are juxtaposed to and partially overlap with the pool of preassembled complexes of hypophosphorylated CD3ζ and ZAP-70 (Haughn et al., 1998; Sherman et al., 2011). Thus, the membrane pool of LAT resides in nanodomains functionally positioned to the vicinity of and efficiently phosphorylated by activated ZAP-70 (Sherman et al., 2011). Alternatively, the activation-dependent recruitment and phosphorylation of LAT from subsynaptic vesicles delivered to these activation zone nanodomains could establish the LAT signaling networks (Williamson et al., 2011). By the same token, light DRMs, preferentially concentrate Fyn with its inhibitory machinery consisting of PAG-Csk complex, PTPα, and likely a small fraction of CD45 (Filipp and Julius, 2004; Ballek et al., 2012). This suggests that while membrane microdomains serve to segregate functionally distinct signaling molecules in resting cells, their juxtaposition allows brisk interaction of their signaling components leading to amplification of the signal upon activation.

An interesting question that arises is how many functionally distinct lipid microdomains are there in the PM of T cells and how do they relate to the generation of the most proximal activation signals? According to the recent study of Horejsi and colleagues who studied the effect of Csk attachment to various types of raft and non-raft membranes it seems that this number is limited (Otahal et al., 2011). Csk targeting to light DRMs using membrane attachment motifs derived from either Lck, Fyn, PAG, or LAT inhibited TCR signaling with a comparable efficiency. Targeting Csk to heavy DRMs was in this context inefficient. While the mechanism is unclear, what seems to be critical is the enrichment of ITAM-phosphorylated TCR/CD3 complex with kinase active Lck in DRMs. This notion is consistent with the importance of targeting activated Lck to DRMs as it has been demonstrated in previous studies (Filipp et al., 2003, 2004, 2008; Pizzo et al., 2004). This points not only to the structural but possibly also functional distinction between light and heavy DRMs in the context of their roles in the T cell activation process (**Figure 2**). Heavy DRMs accommodating the signal-inducing Lck and TCR/CD3/ZAP-70 complex are equipped with mechanisms preventing spontaneous activation of TCR signaling. Upon TCR-pMHC interaction and the initiation of signaling, activated Lck and phosphorylated TCR/CD3/ZAP-70 complexes coalesce with light DRMs whereby Lck and ZAP-70 engage and activate their prime targets Fyn and LAT, which can further amplify or dampen the signal. Thus, according to this model, light DRMs likely function as a spatially restricted checkpoint regulating the strength and quality of TCR proximal signals. The very recent data with LAT mutants which are unable to target to their natural LR environment provide a strong support for such role of lipid nanodomains in the mechanism of T cell proximal signaling (Otahal et al., 2010; Sherman et al., 2011).

TOWARD THE INTEGRATED MODEL OF TCR SIGNALING: NEW RULES OF ENGAGEMENT

The models of TCR signaling that detail triggering mechanisms have been described in several previously published papers and reviews. These mechanisms revolves around three basic molecular processes explaining how extracellular TCR engagement is transduced across the PM and translated to a relevant biochemical signal: segregation, conformational change, and aggregation (Werlen and Palmer, 2002; Choudhuri and van der Merwe, 2007; Minguet et al., 2007; Smith-Garvin et al., 2009; Sigalov, 2010). There is compelling evidence that all three processes contribute to TCR triggering, but the mode of their integration is still matter of debate (Choudhuri and van der Merwe, 2007). An integrated model of TCR triggering should accommodate the contribution and spatio-temporal relationships between all participating signaling molecules and membrane structures that guide, coordinate, and support the complex process of T cell activation. An incorporation of LR to this model has been hampered by insufficient knowledge and controversies related to the quantitative and qualitative parameters of these membrane structures. A major challenge is to provide a mechanism of how TCR engagement invokes its association with LR (Choudhuri and van der Merwe, 2007). Recent data suggest that the TCR receptor in resting cells resides in a special type of microdomains where it colocalizes with a small fraction of pY394 Lck and where upon pMHC-TCR interaction, TCR signaling is initiated (**Figure 2**; Ballek et al., 2012).

We suggest that the following features of membrane compartmentalization should be considered when building an integrated model of TCR proximal signaling:

– All critical components of TCR signaling triggering machinery including the TCR/CD3 complex, Lck, CD45, Fyn, PAG, and LAT are membrane associated. Sizeable fractions of ZAP-70 and Csk are also recruited to the membrane via their interaction

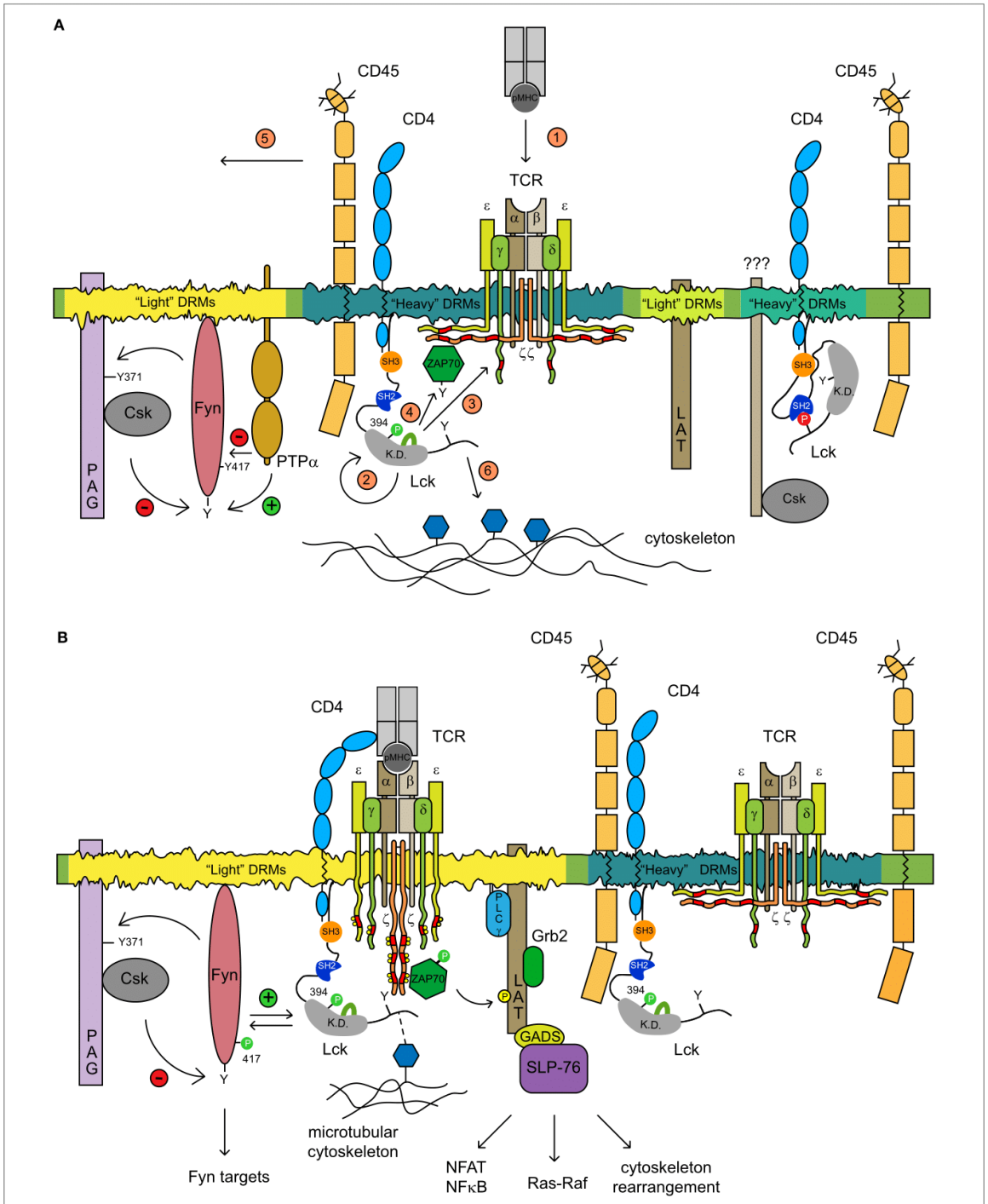

FIGURE 2 | Continued

FIGURE 2 | Continued
The proposed model of TCR triggering mechanism. (A) Several types of membrane microdomains segregate distinct pools of functionally related molecules. Two SFK kinases Lck and Fyn reside in different types of microdomains where their kinase activities in resting T cells are controlled via distinct homeostatic mechanisms. The spatial confinement of Fyn within the light DRMs which are conducive to self-phosphorylation is subjected to a negative feedback loop mechanism controlled by PAG-recruited Csk. Kinase active Fyn phosphorylates PAG on Y371 that allows the membrane recruitment of Csk which in turn phosphorylates the negative regulatory Y528 of Fyn, thus dampening its activity. The dephosphorylation of pY528 as well as pY417 Fyn is mediated by either PTPα or by CD45. The pool of pY394 Lck associated with CD4 resides in heavy DRMs, where it colocalizes with TCR/CD3 and CD45. In resting T cells, CD3ζ is hypophosphorylated and precomplexed with the inactive ZAP-70 kinase. A distinct pool of heavy DRM-associated kinase inactive CD4-Lck complexes that are largely depleted of CD45 has been also detected. The identity of adaptor proteins able to recruit Csk to Lck is unknown. The adaptor protein LAT likely occupies another type of light DRMs. The activation of T cells is accompanied by a cascade of events depicted in the figure by numbers 1–6 in orange circles. pMHC-TCR engagement (1) promotes the clustering of CD4-Lck resulting in the activation of Lck (2). In the situation where only a few pMHC complexes are engaged, the preactivated pool of pY394Lck should be sufficient to initiate the signaling cascade; (3) activated Lck proceeds to phosphorylate ITAMs of CD3 chains which are concomitantly released from sequestration by the inner leaflet of PM upon TCR engagement, by a mechanism that has not been elucidated; ZAP-70 kinase recruited to pY-ITAMs is activated by Lck (4); CD45 and other phosphatases that possess a bulky ectodomain are moved laterally from contact zones by a size-exclusion mechanism and likely by virtue of their association with the cytoskeleton which coordinate their membrane redistribution (not depicted; 5); the free C-terminal end of activated Lck is able to interact with elements of the microtubular cytoskeletal network which aid its enrichment in light DRMs likely via the coalescence of heavy and light DRMs (6). **(B)** The amplification of the TCR signal in light DRMs seems to be critical for the engagement of downstream signaling components. A fraction of TCR/CD3/ZAP-70^{active} and kinase active pY394 Lck translocates to light DRM and amplifies the signals in two independent ways: (i) colocalization with Fyn disrupts its negative regulatory homeostatic mechanism allowing the proximity-mediated transphosphorylation of the Fyn kinase domain, its activation and subsequent phosphorylation of Fyn targets Pyk2 and ADAP; and (ii) activated ZAP-70 phosphorylates its main targets LAT and SLP76 adaptors, thus allowing the engagement of downstream targets such as NF-AT, Ras-Raf signaling, and cytoskeleton rearrangement.

with pY-ITAMs of CD3 and PAG, respectively. Importantly, all of these signaling molecules are preferentially associated with certain types of lipid-based membrane microdomains.

– Membrane microdomains represent the smallest structural and functional units of membrane confinement of signaling elements that are able to control enzymatic activities supporting the initiation of TCR signaling. This control is imposed by microdomain-localized negative regulatory feedback loops and by biophysical constraints imposed by the properties of lipid-forming membrane structures that control the activities of Lck and Fyn kinases and the phosphorylation of CD3 chains, respectively. Thus, to keep the TCR triggering apparatus on a low signaling potential, TCR/CD3, Lck, and Fyn are regulated separately by inhibitory mechanisms tailored to each of these components via their association with microdomains where these components reside.
– Protein-lipid interactions support the functional infrastructure of microdomains in resting T cells. Their importance is accentuated by a recent finding that the TCR triggering apparatus and notably the TCR/CD3 complex, CD4-Lck, CD45, and LAT, are composed of monovalent or monomeric proteins. This finding emphasizes the functional contribution of membrane confinement for enhancement of their association rates (James et al., 2011). Once the signaling is triggered and the protein tyrosine phosphorylation drives the formation of the signalosome, protein–protein interactions become predominant (Zeyda and Stulnig, 2006).
– Critical for the generation of Lck-dependent signals is the effect of CD45 on the maintenance of a small pool of pY394 Lck colocalized in a specific type of microdomains. A soluble antibody mediated TCR/CD4 coaggregation resulted in the rapid exclusion of most CD45 from these microdomains suggesting that the membrane apposition formed at the contact zone between APC and T cell is not the sole mechanism accounting for the removal of phosphatases from the vicinity of juxtaposed TCR/CD3-CD4/Lck complexes (Burroughs et al., 2011). The attachment of most if not all microdomain-associated elements of the TCR triggering apparatus to components of microtubular and F-actin-based cytoskeletal network also contributes to the activation-induced redistribution of CD45 and other signaling molecules (Louie et al., 1988; Rozdzial et al., 1998; Ha-Lee et al., 2000; Brdickova et al., 2001; Kwik et al., 2003; Tavano et al., 2004; Cairo et al., 2010).
– The microdomains based spatial segregation of the components of TCR triggering machinery serves in resting T cells to prevent them from interacting with each other and thus further diminish the probability of spontaneous activation. On the other hand, the juxtaposition of various types of microdomains, as seen for those harboring TCR/CD3 and LAT, allows rapid communication and the signal transfer to downstream components (Sherman et al., 2011). The distribution of Lck and Fyn to distinct types of membrane microdomains provides also the structural resolution for substantiation of Lck-independent Fyn signaling via anti-CD3 stimulation.

CONCLUSION

Thus, rather than freely diffusing in the membrane, the segregation of the components of TCR triggering machinery into several types of microdomains represent an essential membrane organizing principle in resting T cells (**Figure 2A**). These trans-acting structural membrane components spatially limit the availability of signaling molecules prior to the onset of TCR signaling and mediate their communication after engagement, via a process of mutual coalescence. This model is consistent with the suggestion that TCR activation leads to an increased affinity of heavy DRMs to coalesce with light DRMs juxtaposing the pY394Lck/pYCD3ζ/ZAP-70/complex to the vicinity of the LAT adaptor protein (Otahal et al., 2010; Kennedy et al., 2011; Sherman et al., 2011), allowing thus the amplification of the activation signal. A complementary model of membrane compartmentalization called "fences and pickets," that is based on the presence of physical barriers formed by transmembrane proteins attached to the submembrane cytoskeletal network has been

recently described (Kusumi et al., 2005). While this model can explain the significantly retarded coefficient of membrane diffusion of signaling molecules and lipids in resting T cells as well as their rapid immobilization upon initiation of TCR signaling, the microdomain model provides the resolution for the physiological regulation of the "ground state" of the TCR signaling apparatus. The integration of structural and functional aspects of microdomain and "fences and pickets" models with various aspects of distinct mechanisms underpinning TCR triggering will require a deeper understanding of membrane biology, novel interdisciplinary approaches and the generation of specific reagents. We believe that major advances in this exciting and rapidly progressing field of T cell biology can be achieved only when regulatory processes controlling TCR triggering will be considered in the context of the membrane structures that support them.

It has been 20 years since the publication of the seminal paper by Brown and Rose (1992) which opened up a world of studies on the role of lipids rafts in membrane bioactivities and significantly enhanced our knowledge of membrane signaling. Hence, we would like to convey our conviction that the biochemical approach still provides a valuable tool to assess the validity of protein potential for membrane rafts. The careful interpretation of experimental data allows the construction of a hypothetical model that can be further scrutinized by the state-of-art imaging and biophysical techniques. These techniques in combination with the solubilization method would produce many new exciting discoveries in the years to come.

ACKNOWLEDGMENTS

This work was supported by Grant 310/09/2084 and Grant P302/12/G101 from Grant Agency of Czech Republic (GA CR) and Grant AVOZ50520514 from the Academy of Sciences of the Czech Republic. We thank V. Horejší for critical reading of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 February 2012; accepted: 25 May 2012; published online: 12 June 2012.

*Citation: Filipp D, Ballek O and Manning J (2012) Lck, membrane microdomains, and TCR triggering machinery: defining the new rules of engagement. Front. Immun. **3**:155. doi: 10.3389/fimmu.2012.00155*

This article was submitted to Frontiers in T Cell Biology, a specialty of Frontiers in Immunology.

GENERAL DISCUSSION AND CONCLUSIONS

The understanding how T-lymphocytes are activated from cellular to molecular levels is for T-cell biologist like a "Holy Grail" quest. Probably, there is not a more studied cell in the immune system then T-cell. Of course, we already possess a great knowledge about these cells and their activation, but many key questions await their resolution. In our study we focused mainly on molecular mechanisms of very early stages of T-cell activation. There are two fundamental questions which have not yet been fully answered. First, how the activation signal is transmitted to the cell and second what is the mechanism which triggers biochemical reactions leading to the initiation of TCR signalling pathways.

Our contribution to this topic revealed several novel characteristics of proximal TCR signalling components as well as we challenged several well-established dogmas within already existing models of T-cell activation. Considering all publically available data and our own results, we proposed a newly adapted model for TCR triggering.

One very important question concerns the role of lipid microdomains, lipid rafts, or more generally, changes in plasma membrane organization during T-cell activation process. It was suggested that the spatial regulation of signalling molecules which prevent them from their random mutual interactions must play an essential role. Whereas K-S model suggests that segregation of proteins is driven via their size and does not account for lipid microdomains involvement (Anton van der Merwe et al., 2000), the standby model explicitly requires some kind of compartmentalization between constitutively active Lck and its substrates to prevent spontaneous T-cell activation (Nika et al., 2010). Similarly the conformational model could be dependent on the specific membrane composition which enables binding of cytoplasmic tails of CD3s to inner membrane leaflet (Xu et al., 2008). Taken together, the existence of distinct membrane structures, their activation-dependent merging and interaction could be a common denominator of nearly all models of T-cell activation proposed so far.

So, the lipid microdomains theory could explain why proteins are segregated and what drives their clustering, but such interpretation must be performed with caution. We should be able to distinguish if such segregation is due to intrinsic mechanism or extrinsic manipulation, but this could be problematic. In this sense, we have to be aware of potential artefacts caused by detergent-mediated cell lysis and DRMs extraction (Lichtenberg et al., 2005). We should also have to take into account that solubilisation can affect these interactions. These and other questions are hard to answers because the layout of technical procedures always influences the experimental outcomes. For example, from our experiences we know that the centrifugation step which removes insoluble material upon cell lysis seems to be critical. Different centrifugal forces will result in different amounts of extracted proteins in soluble fractions (e.g. Lck, RACK1). This could be detrimental for cytoskeleton-associated proteins that form larger and heavier complexes and could

be lost in the pellet fraction upon centrifugation at higher speeds. For T-cell activation this could be crucial, because during activation many proteins form *de novo* complexes with cytoskeleton. Of course, inefficient centrifugation with lower speed would also have disadvantages as it could result in sample impurities and unspecific binding to bait proteins. So, the question is how to appropriately setup experimental conditions and correctly interpret the data? Definitively, the parallel use of various techniques to prove experimental outcomes should be a standard. A potentially new promising tool for membrane studies could be SMA (styrene-maleic acid) copolymer, which extracts membrane proteins and complexes while preserving their native lipid environment (Dorr et al., 2016; Scheidelaar et al., 2015). Initial data showed that this extraction is dependent on the membrane composition and potentially, it could be used to extract raft-like structures (Dominguez Pardo et al., 2017). Nevertheless more experimentation and proper evaluation of SMA function is needed.

As lipid microdomains can vary in size, they also vary in their composition (Simons and Gerl, 2010). Recently, it was shown, that by biochemical extraction using very mild non-ionic detergent of Brij series we can distinguish at least two types of them: The “light” and the “heavy” DRMs. Both are characterized as high molecular weight (HMWs) complexes according size exclusion chromatography fractionation that are also sensitive to treatment with lauryl maltoside disrupting lipid interactions and preserving only protein-protein interactions. Difference between them is that “light” DRMs after isopycnic ultracentrifugation float to the top of sucrose gradient whereas “heavy” DRMs remain at the bottom (Otahal et al., 2010) due to their distinct density and protein-to-lipid ratio.

In our experiments we follow up this differential DRMs isolation procedure and characterized the spatial distribution of critical TCR signalling components in resting T-cell to find out if there is any conclusive context of their clustering (Ballek et al., 2012). We observed that TCR/CD3 complex in naïve T-cell almost exclusively localizes to “heavy” DRMs and upon TCR engagement is partially transported to “light” DRMs. This is consistent with a previously described data showing recruitment of TCR into DRMs (Montixi et al., 1998; Xavier et al., 1998). Nevertheless, with improving technical resources, the tracking of TCR distribution gives often contradictory results (Hashimoto-Tane et al., 2010). The authors used advanced microscopy techniques to visualize TCR nanoclusters in live cells and target them to liquid ordered or liquid disordered fractions of plasma membrane by various lipid probes (e.g. laurdan). Whereas some conclude that TCR nanoclusters are in naïve T-cell in ld phase (Beck-Garcia et al., 2015), others showed that they are in lo phase (Dinic et al., 2015). However, and importantly, most of them demonstrate cluster aggregation to larger platforms after T-cell activation (Dinic et al., 2015; Lillemeier et al., 2010; Pageon et al., 2016) what is in agreement with principle of original LR theory.

Besides TCR, we found that "heavy" DRMs in naïve T-cell almost exclusively retain active Lck ($pY394^{Lck}$) together with CD45 phosphatase (Ballek et al., 2012). Whereas CD45 upon TCR engagement remains in "heavy" DRMs and non-DRMs fractions, the $pY394^{Lck}$ redistribute to "light" DRMs. That is quite important and it points to several possible conclusions.

CD45 phosphatase is very abundant protein in T-cells with high phosphatase activity. It exerts a dual regulative role on T-cell activation – positive by activating Lck and negative by dephosphorylating ITAMs in TCR/CD3 complex (Furukawa et al., 1994). Then, it is not surprising that all these molecules could localize into one type of cluster or microdomain. It is easy to imagine that CD45, as a master phosphorylation regulator in T-cells, keeps equilibrium which is disrupted by exclusion of CD45 from such clusters or by redistribution of active Lck and TCR into "light" DRMs upon TCR engagement. However, problems could arise when we imagine that all these molecules are in same "heavy" DRM cluster. How to shield TCR from the spontaneous phosphorylation by active Lck? The answer could lie in the conformational change in TCR/CD3 chains which protect phosphorylation of ITAMs as was suggested in the conformational model (Xu et al., 2008) or there are distinct "heavy" DRMs clusters with different composition, or the system is even more complicated as was suggested in reconstituted membrane protein cluster system (Furlan et al., 2014). There, it has been shown that Lck in TCR clusters is autophosphorylated on inhibitory tyrosine and simultaneously dephosphorylated by CD45. This equilibrium is broken by T-cell activation resulting in CD45 exclusion from this cluster, lowering density of CD45 in vicinity of TCR and so confirming positive role of CD45 on TCR triggering. Whether positive or negative, CD45 segregation seem to be one of the most important regulatory mechanisms of TCR triggering.

Phosphorylation of ITAM motif in TCR/CD3 complex by Lck kinase is considered as a first detectable biochemical event of the initiation of TCR signalling pathway. But how Lck is regulated and what triggers Lck activity *per se*, is not known. In our experiments, we identified the pre-existing pool of $pY394^{Lck}$ localizing almost exclusively to "heavy" DRM clusters. Upon T-cell activation, the vast majority of $pY394^{Lck}$ redistributed to "light" DRMs. In addition, the quantitative analysis revealed that $pY394^{Lck}$ levels increased upon TCR engagement using both CD4/TCR cross-linking (up to 50-fold) and antigen stimulation followed by the procedure of immediately boiling cell samples (Ballek et al., 2012; Ballek et al., 2015). This findings are in sharp contrast to previously published results showing that in naïve T-cell almost 50% of total Lck is $pY394^{Lck}$ and T-cell activation does not result in $pY394^{Lck}$ amplification (Nika et al., 2010). Consistent with Nika's report, it was shown that conformational change in Lck structure (from closed to opened – i.e. from inactive to active) during TCR engagement was not detectable (Paster et al., 2009). The large pre-existing pool of constitutively active Lck then conveniently fits into several models including K-S model or stand-by model. In these models one could assume that only disbalance

of phospho/dephospho regulatory mechanism acting via exclusion of CD45 away from TCR is sufficient to trigger T-cell activation. Nevertheless, not only our results reported *de novo* phosphorylation and amplification of pY394^{Lck} upon TCR engagement (Filipp et al., 2003; Holdorf et al., 2002; Philipsen et al., 2017; Stirnweiss et al., 2013; Wang et al., 2011), we also detected much lower amounts of pY394^{Lck} in naïve T-cells than described in Nika's report.

So what causes such a big discrepancy in the amount of pY394^{Lck}? We investigated the possible role of several technical aspects and found out some variances in T-cell activation procedure and pY394^{Lck} detection. The most prominent effect on the quantity of pY394^{Lck} exerted the solubilisation step (Ballek et al., 2015). The standard cell lysis in various detergents do not block efficiently phosphorylation events and results in the global spontaneous phosphorylation of proteins. This severely compromises the quantitative outcomes in such conditions and could explain why there are such discrepancies within different studies. We suggested to add, besides protease and phosphatase inhibitors, also the kinase inhibitors into lysis buffer, to overcome spontaneous activation of SFKs during the cell solubilisation and thus obviate this technical problem. Nevertheless, on the whole, it is alarming that most of the studies failed to consider such important aspect of experimental settings, especially when it concerns the quantification of phosphorylation status which could be heavily compromised by spontaneous phosphorylation during cell lysis a could lead to severe misinterpretation of data.

There are several mechanisms which control proteins redistribution on plasma membrane but the one(s) which regulates the recruitment of crucial T-cell signalling components into "light" DRMs is not clear. Our previous data showed that spatial redistribution of Lck into "light" DRMs upon TCR engagement predicates further signalling and IL-2 production (Filipp et al., 2008; Filipp et al., 2003). Thereafter, we identified RACK1 as a protein which orchestrates the relocation of HMW Lck by tethering to cytoskeletal network (Ballek et al., 2016). Involvement of cytoskeleton in T-cell membrane redistribution processes is expected as was shown previously (Shaffer et al., 2009; Tavano et al., 2006). However, transient biochemical association of Lck and RACK1 as well as their timely coordinated movement into IS was revealed for the very first time.

Our data suggested that RACK1-Lck interaction is not direct and there are likely additional components forming much larger multiprotein complex. Using mass spectrometry analysis we identified several possible interaction partners of RACK1 (Ballek et al., 2016). Similarly to Lck, some of them exhibit transient association with RACK1 upon T-cell activation. The most promising seemed to be GADS (Grb2-related adaptor downstream of Shc) and α-actinin-1 (ACTN1).

GADS was originally identified as an Shc interactor (Liu and McGlade, 1998) but its major role was implicated in TCR signalling in connecting proximal and distal TCR signalling pathways (Figure 2, right part). GADS is expressed mainly in

leukocytes and as an adaptor protein contains one SH2 domain flanked by two SH3 domains which together provide high affinity binding to its partners. It has been shown that it constitutively interacts with SLP-76 and links this protein to LAT upon TCR stimulation (Liu et al., 1999). This results in the redistribution of PLCγ to LAT via Vav1 and ITK kinase (Bogin et al., 2007). The importance of GADS in T-cell development was evaluated *in vivo* by deletion of GADS in mouse or *in vitro* in human Jurkat cells showing defect in thymocyte proliferation – lack of efficient positive selection (Dalheimer et al., 2009; Yoder et al., 2001) and impairment in calcium influx and cytokines production (Bilal et al., 2015). Up today several other interaction partners were identified, including costimulatory molecule CD28 or RACK1 (Ellis et al., 2000).

α-actinins are ancient family of actin filament cross-linking proteins. They are expressed almost in all eukaryotes from yeasts to mammals, excluding plants and some other lineages where they have been lost during evolution. They form dimers containing several spectrin-like repeats flanked by actin-binding domain and calmodulin-like domain (Sjoblom et al., 2008). In mammals, they could be divided into two subgroups – muscle actinins (ACTN2 and ACTN3) and non-muscle actinins (ACTN1 and ACTN4). Non-muscle actinins were implied in cell adhesion, cell migration or cytokinesis, thus providing anchor for microfilaments to cell membrane structures. It was shown that they have capacity to interact with integrins or intracellular adhesion molecules (Celli et al., 2006; Sjoblom et al., 2008). Moreover, in context of T-cells, ACTN1 associates with membrane fraction of mouse lymphocytes (Hoessli et al., 1980). Interaction of ACTN1 with F-actin and other proteins is regulated by several mechanisms including tyrosine phosphorylation (Izaguirre et al., 2001), binding to phosphoinositides (Greenwood et al., 2000) or to calcium (Blanchard et al., 1989). For example, increased levels of Ca^{2+} leads to inhibition in actin binding and bundling. α-actinins thus can act as multipurpose interaction platforms for structural, membrane and signalling proteins as well as cytoskeleton cross-linking proteins.

Both proteins provide new look at the mechanism of Lck redistribution. While RACK1-GADS interaction was detected previously (Ellis et al., 2000), RACK1-ACTN1 association has been described for the first time and seems to be important for connecting RACK-Lck complex to cytoskeleton. How this process could be regulated? While functional actin cytoskeleton is important for RACK1-Lck interaction (Ballek et al., 2016), the microtubular network is essential for Lck redistribution process (Ballek et al., 2012). Since both microtubular and actin cytoskeletal elements play a significant role in TCR signalling (Billadeau et al., 2007; Hashimoto-Tane et al., 2011; Kumari et al., 2014) the interplay and cross-talk between them in this process could be envisioned (Lasserre and Alcover, 2010). Since Lck activity is not necessary for RACK1-Lck association we can speculate that some kind of conformational change in TCR or Lck co-receptor could trigger such association (Kim et al., 2012).

Taking together, the formation of signalling axis consisting of receptor-RACK1-cytoskeleton elements could point to a common mechanism for redistribution processes. While RACK1 is an adaptor protein involved in various cellular events including cell migration, grown, survival, apoptosis and others (Adams et al., 2011), one of its originally described function is the role in PKC redistribution (Ron et al., 1999). Members of PKC family are also express in T-cell (PKCα and PKCθ) and are involved in calcium signalling pathway. In addition, they were shown to redistribute after T-cell activation into IS (Gharbi et al., 2013; Yokosuka et al., 2008). Thus RACK1 association with PKCα and PKCθ and their coordinated movement towards IS could represent another example of activation-induced redistribution mechanisms actively involving RACK1.

With help of our experimental data we decided to revise the view on TCR triggering model. In our working model we started out from the co-receptor aggregation and lipid segregation model (Fig. 10). We assumed that in naïve unstimulated T-cell the signalling proteins are segregated into different clusters. Small pool of basal active Lck is maintained in HMW complexes identified with "heavy" DRMs together with CD45 and TCR. CD45 negatively regulates signalling by dephosphorylating ITAM motifs in TCRs and thus maintaining equilibrium. Upon TCR engagement by cognate peptide, CD45 clusters are excluded and active Lck together with portion of TCR/CD3 redistribute to "light" DRMs. The redistribution of Lck is controlled with help of RACK1 complex which cooperates with cytoskeletal elements.

Co-receptor binding to MHC stabilizes Lck in the vicinity of TCR/CD3 complex providing enough time to phosphorylation and activation of ZAP-70 kinase and other Lck molecules by trans-autophosphorylation. Active ZAP-70 binds Lck and further stabilizes it through preventing its closed conformation. ZAP-70 then activate LAT which is also localized in "light" DRMs. Taking together, T-cell activation results in changes in redistribution of proteins on plasma membrane leading to their aggregation in a common lipid-based signalling platform.

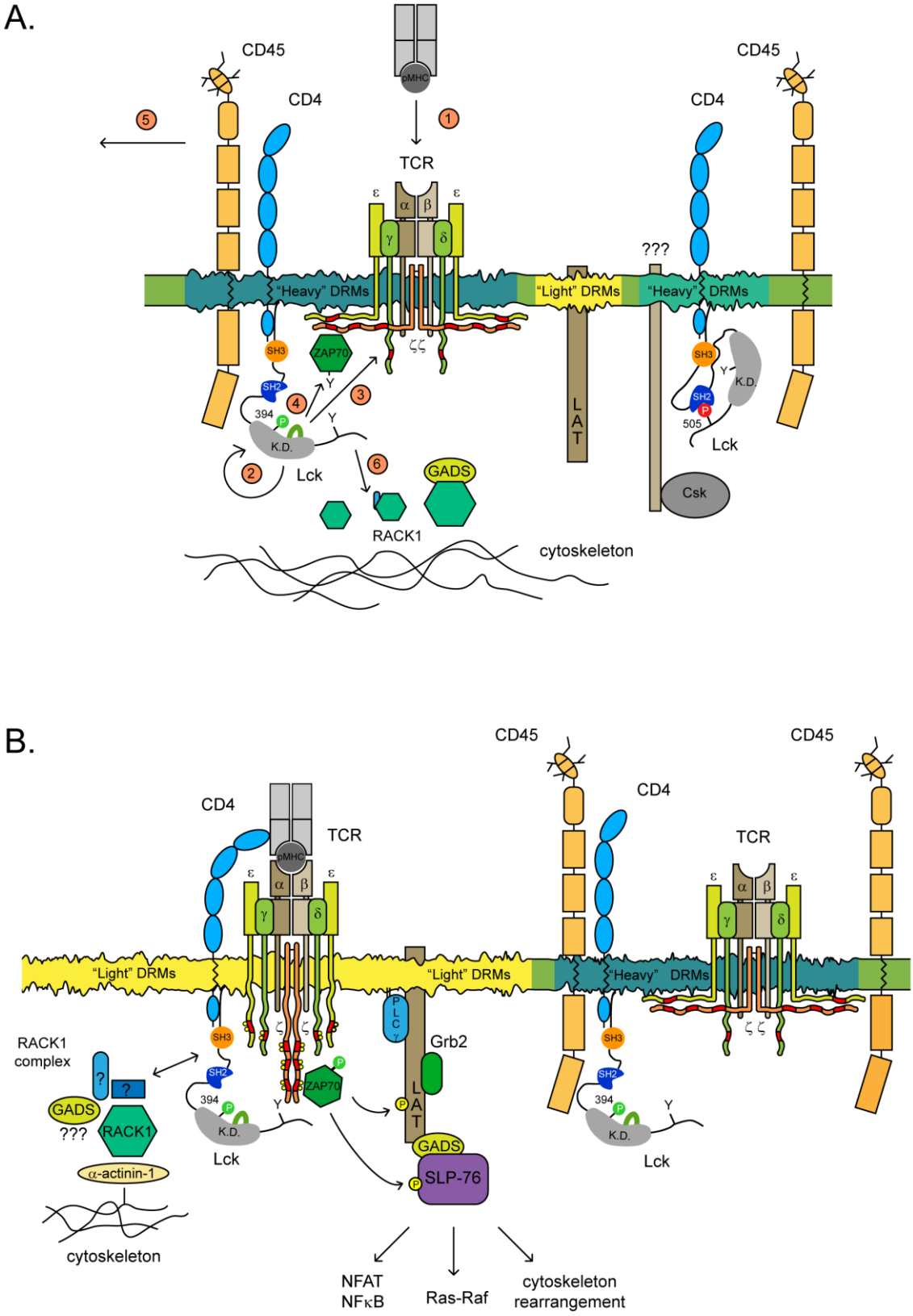
What remains to be elucidated?

What keeps active Lck active in "heavy" DRMs and simultaneously protecting the T-cell activation? Is it the balance of autophosphorylation of its negative tyrosine with dephophorylation by CD45? What is a role of co-receptors? Is their binding to MHC complex indispensable for T-cell activation? Could it be possible that co-receptor aggregation results in conformational changes in their structure, thus providing a signal for the formation of RACK1-Lck complex association with cytoskeleton via ACTN1, as it has been shown in our preliminary data? The question of cytoskeleton involvement in early events of T-cell activation is also an unresolved issue. What is the dynamic of membrane segregation and how is it regulated? Is the cytoskeleton the only driving force of membrane redistribution

processes? Moreover, the process of clustering membrane molecules is not elucidated yet. These and many other questions remain to be answered to better understand the principles of TCR triggering mechanism.

In conclusion, in this study we provided new experimental data pointing at the role of Lck kinase in proximal T-cell signalling. We suggested how it could be regulated together with other critical signalling proteins via membrane segregation processes. Even though, we have not completely solved all questions depicted at the beginning, our results contributed to better understanding of the processes accompanying the proximal T-cell signalling and we are also very confident that they provided a strong stimulus for discussion in the field of T-cell activation.

Figure 10: The proposed model of TCR triggering mechanism. A. *Several types of lipid microdomains segregate distinct pools of functionally related molecules. The pool of pY394^{Lck} associated with CD4 resides in "heavy" DRMs, where it co-localizes with TCR/CD3 and CD45. In resting T-cells, CD3ζ is hypophosphorylated and pre-complexed with the inactive ZAP-70 kinase. A distinct pool of "heavy" DRMs-associated kinase inactive CD4-Lck complexes that are largely depleted of CD45 has been also detected (right side of the figure). The identity of adaptor proteins able to recruit Csk to Lck is unknown. The adaptor protein LAT likely occupies another type of "light" DRMs. The activation of T-cells is accompanied by a cascade of events depicted in the figure by numbers 1-6 in orange circles. pMHC-TCR engagement (1) promotes the clustering of CD4-Lck resulting in the activation of Lck (2). In the situation where only a few pMHC complexes are engaged, the preactivated pool of pY394^{Lck} should be sufficient to initiate the signalling cascade; (3) activated Lck proceeds to phosphorylate ITAMs of CD3 chains which are concomitantly released from the sequestration by the inner leaflet of plasma membrane upon TCR engagement, by a mechanism that has not been fully elucidated; ZAP-70 kinase recruited to pY-ITAMs is activated by Lck (4); CD45 and other phosphatases that possess a bulky ectodomain are pushed away laterally from contact zones by a size-exclusion mechanism and likely also by a virtue of their association with the cytoskeleton which coordinates their membrane redistribution (not depicted) (5); the activated Lck is able to interact with components of RACK1 complex mediating binding to elements of the cytoskeletal network which aid its enrichment in "light" DRMs likely via the coalescence of "heavy" and "light" DRMs (6 and B).* ***B****. The amplification of the TCR signal in "light" DRMs seems to be critical for the engagement of downstream signalling components. A fraction of TCR/CD3/ZAP-70^{active} and kinase active pY394^{Lck} translocates to "light" DRMs and amplifies the signals. Activated ZAP-70 phosphorylates its main targets, LAT and SLP-76 adaptors, thus allowing the engagement of downstream targets such as NF-AT, Ras-Raf signalling and cytoskeleton rearrangement.*


A.
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SH2
394
K.D.
Lck
4
3
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6
GADS
RACK1
LAT
Csk
SH3
SH2
505
K.D.
Lck
cytoskeleton
B.
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TCR
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GADS
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RACK1
α-actinin-1
cytoskeleton
SH3
SH2
394
K.D.
Lck
ZAP70
PLCγ
Grb2
LAT
GADS
SLP-76
SH3
SH2
394
K.D.
Lck
NFAT
NFκB
Ras-Raf
cytoskeleton
rearrangement

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