One-Sentence Summary: Ca^{2+} signaling triggered by adhesion to specific extracellular matrix proteins facilitates T cell activation.

Editor's Summary:
A sticking point for T cells
During the activation of T cells, intracellular Ca^{2+} globally increases and leads to changes in gene expression by promoting the nuclear translocation of the transcription factor NFAT1. Weiβ *et al.* found that T cell activation was facilitated by the formation of Ca^{2+} microdomains that occurred when mouse T cells adhered to extracellular matrix components that might be typically encountered during migration to a site of inflammation. The authors characterized the signaling proteins required and the role of store-operated Ca^{2+} entry in adhesion-dependent Ca^{2+} microdomain formation, which was critical for the global increase in intracellular Ca^{2+} and the nuclear translocation of NFAT1 after T cell receptor stimulation. Thus, the formation of these Ca^{2+} microdomains in response to adhesion sensitizes T cells for activation. –Wei Wong

Title: Adhesion to laminin-1 and collagen IV induces the formation of Ca^{2+} microdomains that sensitize mouse T cells for activation

Authors:

Mariella Weiß[1†], Lola C. Hernandez[1†], Diana C. Gil Montoya[1], Anke Löhndorf[1], Aileen Krüger[1], Miriam Kopdag[1], Liana Uebler[1], Marie Landwehr[1], Mikolaj Nawrocki[2], Samuel Huber[2], Lena-Marie Woelk[3], René Werner[3], Antonio V. Failla[4], Alexander Flügel[5], Geneviève Dupont[6], Andreas H. Guse[1], Björn-Philipp Diercks[1*]

Affiliations:

[1] The Calcium Signalling Group, Department of Biochemistry and Molecular Cell Biology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany.

[2] Section of Molecular Immunology und Gastroenterology, I. Department of Medicine, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany.

[3] Department of Computational Neuroscience, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany.

[4] Microscopy Imaging Facility (UMIF), University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

[5] Institute for Neuroimmunology and Multiple Sclerosis Research, University Medical Centre Göttingen, 37075 Göttingen, Germany.

[6] Unité de Chronobiologie Théorique, Faculté des Sciences, CP231, Université Libre de Bruxelles, B-1050 Brussels, Belgium.

* Corresponding author: Björn-Philipp Diercks (b.diercks@uke.de)

† These authors contributed equally as 1st authors

Abstract:

During an immune response, T cells migrate from blood vessel walls into inflamed tissues by migrating across the endothelium and through extracellular matrix (ECM). Integrins facilitate T cell binding to endothelial cells and ECM proteins. Here, we report that Ca^{2+} microdomains observed in the absence of T cell receptor (TCR)/CD3 stimulation are initial signaling events triggered by adhesion to ECM proteins that increase the sensitivity of primary murine T cells to activation. Adhesion to the ECM proteins collagen IV and laminin-1 increased the number of Ca^{2+} microdomains in a manner dependent on the kinase FAK, phospholipase C (PLC), and all three inositol-1,4,5-trisphosphate receptor (IP_3R) subtypes, and promoted the nuclear translocation of the transcription factor NFAT1. Mathematical modeling predicted that the formation of adhesion-dependent Ca^{2+} microdomains required the concerted activity of two to six IP_3Rs and ORAI1 channels to achieve the increase in the Ca^{2+} concentration in the ER-plasma membrane junction that was observed experimentally and that required SOCE. Further, adhesion-dependent Ca^{2+} microdomains were important for the magnitude of the TCR-induced activation of T cells on collagen IV as assessed by the global Ca^{2+} response and NFAT1 nuclear translocation. Thus, adhesion to collagen IV and laminin-1 sensitizes T cells through a mechanism involving the formation of Ca^{2+} microdomains and blocking this low-level sensitization decreases T cell activation upon TCR engagement.

Main Text:

INTRODUCTION

During an immune response, T cells are activated by T cell receptor (TCR)/CD3-complexes that bind to antigenic peptides presented on major histocompatibility complexes (MHC) at the surface

of antigen-presenting cells (APCs) (*1*, *2*). Adhesion-dependent T cell priming, namely an adhesion-dependent change in TCR sensitivity, may be essential to facilitate full TCR mediated activation (*3*). After T cell activation in lymphatic organs, adhesive interactions between T cells and endothelial cells or extracellular matrix (ECM) proteins occur during T cell migration from blood vessels into the inflamed tissue (*4*). In this process, cell adhesion receptors on T cell surfaces known as integrins are activated. Integrins consist of an α- and a β-subunit, and the α- subunit is essential for integrin ligand specificity (*5*). Hence, binding of integrin receptors to cell adhesion molecules (CAMs) on the vessel wall enable the adhesive interaction, leading to rolling, followed by firm adhesion and finally transmigration between endothelial cells (*4*, *6*). Next, T cells adhere through integrins to ECM proteins of the basement membrane (such as laminin-1 and collagen IV) and of the interstitium (such as collagen VI or fibronectin) during migration to sites of inflammation (*7*).

In Jurkat T cells, integrin-mediated adhesion to ECM proteins triggers an increase in global free cytosolic calcium (Ca^{2+}) concentration ($[Ca^{2+}]_i$) (*8*). Upon binding of integrins to ECM proteins, phosphorylation of focal adhesion kinase (FAK) and subsequent activation of phospholipase C-γ (PLC-γ) (*9*) occurs. Thus, FAK is thought to be a key component of integrin-mediated signaling pathway (*10*). Moreover, Src family tyrosine kinases, the second messengers D-*myo*-inositol-1,4,5-trisphosphate (IP_3) and cyclic ADP-ribose (cADPR), and store-operated calcium entry (SOCE) are components of α6β1-integrin-mediated global Ca^{2+} signaling (*11*, *12*).

We previously described locally restricted Ca^{2+} signals, termed Ca^{2+} microdomains, that are observed within tens of milliseconds and continue for approximately 15 seconds after TCR/CD3 and CD28 stimulation with antibody coated-beads, mimicking an immune synapse, (*13–15*) until they merge into a global Ca^{2+} signal. These Ca^{2+} microdomains are evoked by the second

messenger nicotinic acid adenine dinucleotide phosphate (NAADP), which associate with the protein HN1L (also known as JPT2), thereafter acting on type 1 ryanodine receptors (RYR1). Ca^{2+} released from the endoplasmic reticulum (ER) through RYR1 is sensed by stromal interaction molecule (STIM) proteins which in turn translocate and activate ORAI1 channels (*13–15*). Of note, TCR/CD3/CD28 evoked Ca^{2+} microdomains also depend on purinergic activation of P2X4 and P2X7 (*16*). The underlying mechanisms for global Ca^{2+} signaling in T cells are transient increase of IP_3 that peaks around 2-3 mins (*17*), slow increase of cADPR with elevated concentrations starting 10 min after stimulation (*18*), and SOCE mediated by ORAI channels (*19*, *20*). The formation of adhesion-dependent Ca^{2+} microdomains (ADCMs) in the absence of TCR stimulation depends on the one hand on ORAI1 and STIM1/2 expression but also on P2X4 and pannexin-1 (*13*, *16*). However, it is unknown whether and through which signaling cascade integrins are involved, and whether the formation of TCR-independent Ca^{2+} microdomains represents a sensitization step during T cell activation.

Here, we explored a role for integrins, extracellular matrix proteins and endothelial membrane proteins and the signal transduction molecules FAK, PLC-γ, and IP_3Rs in ADCMs, and adhesion-dependent clustering of ORAI1/STIM, FAK and IP_3R subtypes involved in adhesion-dependent T cell sensitization. Moreover, we investigated the downstream effect of adhesion through nuclear factor of activated T cells (NFAT) 1 translocation to the nucleus after TCR stimulation.

RESULTS

‘Spontaneous’ Ca^{2+} microdomains depend on β1 integrins and on SOCE

In the absence of TCR/CD3/CD28-stimulation but on weakly adhesive coating (in our case poly-L-lysine (PLL)), Ca^{2+} microdomains appeared infrequently in T cells (Fig 1 A, B). We previously termed these local signals ‘spontaneous’ Ca^{2+} microdomains (*13*), but hypothesize here that they

are evoked by weak adhesion and use the term adhesion-dependent Ca^{2+} microdomains (ADCMs) from this point onwards. Because integrins may play an essential role in ADCMs and adhesion-dependent sensitization of T cells, we identified the specific integrin isoforms involved in Ca^{2+} microdomain formation. Monoclonal antibodies recognizing $\alpha_L\beta_2$, $\alpha_6\beta_1$, or $\alpha_2\beta_1$ integrins but not $\alpha_5\beta_1$ integrin resulted in a significant decrease in the number of Ca^{2+} microdomains in mouse T cells on weakly adhesive PLL coating (Fig. 1 C-G). T cells on non-adhesive BSA-coating did not form ADCMs (Fig 1 G, H). ORAI1 is a Ca^{2+} channel involved in Ca^{2+} microdomains in T cells under such weakly adhesive conditions (*13*, *21*), a result we confirmed here using Ca^{2+} free buffer (Fig. 1 H), the SOCE inhibitor STX564 (*22*), and the ORAI inhibitor Synta66 (Fig. 1 I).

Laminin-1 and collagen IV evoke ADCMs

Next, we investigated the effect of different ECM proteins on ADCM formation, and as expected (Fig. 1C-F), mouse T cells adhering to laminin-1 and collagen IV coatings had similar numbers of ADCMs compared to those adhering to PLL (Fig. 2 A-D). In contrast, Ca^{2+} microdomain numbers in T cells placed on ICAM-1 or collagen VI were not different from those on the negative control substrate (Fig. 2 E-G). Thus, integrins binding to ICAM 1 and collagen-VI are not involved in the formation of ADCMs. The mean amplitudes of Ca^{2+} microdomains were in the range of 210 to 250 nM (Fig. 2 H). Together, ADCMs are evoked when T cells bind to laminin-1 and collagen IV, the central ECM proteins of the basement membrane.

Role of ADCMs on downstream signaling after TCR stimulation through NFAT-1 translocation

To determine whether adhesion of T cells to ECM proteins affects processes downstream of T cell activation through the TCR, translocation of NFAT-1 to the nucleus upon TCR stimulation was

examined in mouse T cells on different adhesive surfaces (Fig. 3 A-D). The percentage of nuclear NFAT-1 signal was significantly increased in T cells on PLL and collagen IV compared to those BSA at 7.5 min and 15 min after TCR stimulation using anti-CD3/CD28 coated beads, whereas there was no difference in the absence of TCR stimulation or in the maximum response obtained by thapsigargin (Fig. 3 D). Hence, adhesion to basement membrane proteins and the resulting Ca^{2+} microdomains sensitize the T cells, leading to a faster translocation of NFAT-1 to the nucleus.

Mechanisms underlying ADCM formation

Previously, we reported that *Ryr1* deficiency did not affect ADCM formation in T cells suggesting that the NAADP/RYR1 pathway is not involved (*13*). In contrast, deletion of *Orai1* or preincubation with the ORAI antagonists STX564 or Synta66 almost completely blocked Ca^{2+} microdomain formation (Fig. 1 I). FAK is a major signal transducer of integrins (*10*) and IP_3 signaling (*23*), and the formation of Ca^{2+} microdomains were fully antagonized by the FAK inhibitor PF562,271 and slightly, but significantly blocked by the PLC inhibitor U73122 as compared to the control compound U73343 (Fig. 4 A-E). Moreover, global Ca^{2+} signals evoked by TCR stimulation (Fig. 4 F) and total peak area (Fig. 4 G) were significantly reduced upon FAK inhibition of T cells on both PLL and collagen IV. Accordingly, normalized NFAT-1 translocation to the nucleus evoked by TCR stimulation was significantly decreased in T cells on PLL and collagen IV after FAK inhibition (Fig. 4 H, I).

To further substantiate involvement of IP_3 signaling, the formation of ADCM in primary T cells with deletions of all IP_3R subtypes was assessed (Fig. 5, A to H). Compared to WT T cells on adhesive coatings, the number of ADCMs was decreased in IP_3R triple knock-out (*Iptr1/2/3*$^{-/-}$) T cells on either PLL or collagen IV (Fig. 5 D, F, G, H). No significant difference was detected

between WT T cells and *Iptr1/2/3*$^{-/-}$ T cells on a non-adhesive surface (Fig. 5 A, B, G, H). In summary, adhesion to collagen IV (or PLL) results in the activation of FAK, PLC-γ and Ca^{2+} release through IP_3Rs.

Using high-resolution STED microscopy, clustering and interaction of proteins involved in the signaling pathway underlying ADCM was determined at a spatial resolution of approximately 40 nm. Clustering was assessed by defining a cluster forming region, which corresponded to the radius of a fluorescence spot multiplied by 1.3 (fig. S1, A to D). All spots located in a cluster forming region were designated as belonging to one cluster. With this method we observed the formation of larger clusters of FAK, ORAI1 and IP_3R1 in WT T cells on adhesive collagen IV (or PLL) as compared to those on non-adhesive BSA coating (Fig. 6 A, B, C). For PLL, the number of spots in clusters of STIM1, STIM2, IP_3R2 and IP_3R3 were also investigated. The number of spots in clusters of STIM1, but not of STIM2, IP_3R2 and IP_3R3 was different in T cells on adhesive compared to those on non-adhesive coatings (fig. S2A, B, C, D). Furthermore, co-localization between ORAI1 and FAK or IP_3R1 increased significantly on adhesive PLL or collagen-IV vs non-adhesive BSA (Fig. 6 D, E). Again, a significant difference of co-localization between ORAI1 and STIM1 (fig. S2 E), but not between ORAI1 and STIM2, IP_3R2 or IP_3R3 (fig. S2 F, G, H) was detected in T cells under non-adhesive compared to those on PLL. These results suggest that FAK, IP_3R1, STIM1 and ORAI1 are involved in integrin signaling on adhesive ECM proteins and indicate that this signaling process is localized at least partially in ER-PM junctions.

Modeling IP_3R signaling at ER-PM junctions

Given the small lateral dimension of the ER-PM junction (~200 nm), current Ca^{2+} imaging techniques cannot optically resolve single Ca^{2+} channels at high spatiotemporal resolution. Therefore, to visualize the Ca^{2+} dynamics underlying ADCM formation taking place at ER-plasma

membrane (PM) junctions, we turned to computational modeling. We considered a three-dimensional ER-PM junction and a ring of surrounding IP_3Rs embedded in a cytosolic volume (Fig. 7 A). This model ((*24*), fig. S3 and table S1) is based on a study that investigated the interplay between ORAI channels and SERCA pumps during SOCE (*25*). We assumed a basal junctional Ca^{2+} concentration of 30 nM, an initial full ER Ca^{2+} store (400 μM) and the consecutive opening of up to eight immobile IP_3Rs (*26*) during a specified time period, τ_{open} (44 ms), which represents the average duration of a Ca^{2+} microdomain (Fig. 1B). The subsequent local depletion of ER Ca^{2+} concentration activates SOCE through the pre-existing co-localization of ORAI1/STIM2 (fig. S2 E) or of ORAI1/STIM2/STIM1 (fig. S2 E, F), leading to Ca^{2+} entry through the five ORAI channels localized at the PM, according to the Ca^{2+} concentration in the sub-PM ER. In our previous study on quiescent T cells on a weakly adhesive surface (*24*), we concluded that the concerted activity of 2 to 6 IP_3Rs and ORAI is necessary to achieve the junctional Ca^{2+} amplitude observed experimentally when considering the activation of SOCE involving STIM2 homotetramers (Fig. 7 B, blue line, which shows data from (*24*)). Here, we also considered SOCE activation relying on STIM2/STIM1 heterotetramers, which have a lower sensitivity to small decreases in luminal Ca^{2+} in comparison to STIM2 homotetramers. Because of this lower sensitivity, fluxes and thus Ca^{2+} increases in the junction are smaller when ORAI1 molecules are bound to STIM2/STIM1 heterotetramers (Fig. 7 B, yellow line). The average junctional Ca^{2+} concentration ($[Ca^{2+}]_J$) resulting from the opening of 5 up to 7 IP_3R channels (Fig. 7 B) amounts to 290 to 310 nM. The average $[Ca^{2+}]_J$ increases in a slightly sub-linear manner with the number of open IP_3Rs, up to the point at which the larger luminal depletion around the start of IP_3R's open channel pore triggers further opening of ORAI (Fig. 7 B). With increasing distances from the start of the channel's pore, the Ca^{2+} concentration increases from ~18 μM at the start of the pore of an

open IP_3R to 400 μM at the center of the sub-PM ER shown by cross-section of Ca^{2+} profiles in the junction (Fig 7 C). The concentration gradient around the pore of the channel is steep, with the half-maximal concentration being reached 0.15 nm away from the channel's pore (*24*). SOCE activation can thus only be due to STIM molecules located in close proximity to immobile IP_3Rs (*26*). As expected, the minimal amount of open IP_3Rs required to achieve a physiological Ca^{2+} microdomain increases from 2 to 5 under SOCE activation through STIM2/STIM1 compared to that of through only STIM2. In reality, due to the stochastic character of IP_3Rs openings at near local IP_3 concentrations, it is expected that successive microdomains result from the opening of variable numbers of receptors, whose average depends on the composition of the STIM multimers.

DISCUSSION

Adhesive interactions are crucial for the recognition of foreign pathogens and for T cell migration to inflamed tissue (*27*). Hence, T cell activation requires adhesive interactions between T cells and other cell types, such as antigen-presenting cells (APCs) or endothelial cells, and between T cells and ECM proteins (*4*). In this study, we investigated the molecular mechanism underlying ADCM formation in T cells, determined the integrins and their corresponding ECM proteins that were involved in adhesion-dependent T cell sensitization, and present a refined mathematical model describing ADCM formation.

Involvement of integrins in ADCM formation

Adherence of primary T cells to collagen IV by $\alpha_2\beta_1$ integrin or to laminin-1 by $\alpha_6\beta_1$ integrin resulted in increased numbers of local Ca^{2+} microdomains (Fig. 1 D, E, G, Fig.2 D, E, G, H). In contrast, Jurkat T cells respond with an increase in global $[Ca^{2+}]_i$ when acutely placed on the

human or mouse forms of various ECM proteins (*8*, *11*). This discrepancy may be due to the blast-like activated T cell nature of Jurkat T cells in which integrin signaling produces sufficient Ca^{2+}-mobilizing second messengers to evoke global Ca^{2+} signaling. In contrast, in primary T cells, the local Ca^{2+} response sensitizes the cell for further signaling, such as for re-activation in inflamed tissue as opposed to TCR signaling. In addition to collagen IV or laminin-1, adherence of T cells to PLL also resulted in Ca^{2+} microdomains. PLL is commonly used for cell attachment to slides (*28*), but in general no further effects on signaling have been observed. However, Jurkat T cells form much larger contacts with PLL-coated glass than uncoated glass, thereby reducing TCR mobility and enhancing TCR clustering (*29*), suggesting that not only do T cells attach to PLL, but that also signaling is evoked. Our data indicate that at least some of this signaling proceeds through $ß_1$ integrins.

Downstream signaling mechanisms of ADCM formation

Cell adhesion-dependent FAK activation has been reported for many cell types (*30*, *31*). In T cells, integrins are necessary for adhesion in vivo (*32*), either to other cells or to components of the extracellular matrix. In Jurkat T cells, integrin-dependent adhesion requires PLC-γ activation, IP_3-dependent Ca^{2+} signaling, SOCE activation, Src kinase activity, and cADPR signaling (*9*, *11*, *12*, *33*). Because integrins can be activated in either by outside-in activation by integrin ligands or by inside-out activation by ligation of cell-surface receptors (such as TCR/CD3 or chemokine receptors) (*32*), it is likely that activation of FAK in T cells adhering to ECM proteins proceeds through outside-in signaling but through inside-out signaling at later stages upon TCR/CD3/CD28 ligation, especially in response to weak TCR signals that generate elevated levels of phosphatidylinositol 4,5-bisphosphate (*34*), which is converted by PLCγ to IP_3. In accordance with

our finding of ADCMs being confined to ER-PM junctions, FAK-elicited Ca^{2+} signaling is observed only at lipid rafts (*35*). Only immobile IP_3Rs localized close to the PM respond to physiological agonists, termed 'licensed' IP_3Rs (*36*), which are closely associated with ER-PM junctions (*36*) and thus allow for effective SOCE activation through modest local sub-PM ER depletion (Fig. 8B). Our finding that genetic deletion of all *Stim1*, *Stim2*, or *Orai1* (*13*) prevents ADCMs is in line with such a mechanism. Mathematical simulations of ADCM formation based on a cluster of 5 ORAI1 channels at ER-PM junctions allowed us to predict that the observed Ca^{2+} signature (Fig. 1 A, B) quantitatively agrees with Ca^{2+} entry triggered by the localized release of ER Ca^{2+} through either 5 to 7 IP_3R when STIM1/2 heterotetramers are involved or 2 to 6 IP_3R when ORAI1 is bound to STIM2 homotetramers (Fig. 7 B). Because of their lower affinity for Ca^{2+}, STIM2 tetramers allow for immediate opening of ORAI1 to ~21% of their maximal conductance under the luminal Ca^{2+} initial condition, enough to reach Ca^{2+} concentrations in junctions corresponding to experimental observations. For STIM1/2 heterotetramers, ORAI1 channels shift to their lowest conductance state (~7% opening). A further ER depletion evoked by IP_3Rs is needed to trigger ORAI1 opening, up to ~21% of their maximal conductance, to reach Ca^{2+} concentrations in agreement with experimental observations (fig. S3). The model also predicts that because ADCMs are not associated with ER Ca^{2+} depletion, the increase in ER Ca^{2+} concentration at the mouth of a SERCA pump located ~20 nm away from an ORAI1 channel is limited (less than 1%). This can be explained by the relatively low activity of the SERCA pumps that are saturated at cytosolic Ca^{2+} concentrations around 350 nM (*24*).

Biological relevance of ADCMs

Blocking FAK by PF-562,271 resulted in decreased global free $[Ca^{2+}]_i$ responses after TCR stimulation in primary murine T cells on either PLL or collagen IV coatings, supporting the hypothesis that adhesion may sensitize the T cell by producing infrequent local ADCMs. Although PF-562,271 is a commonly used FAK inhibitor with an IC_{50} value of 1.5 nM for FAK, it also inhibits Pyk2 (IC_{50} value = 13 nM) and Fyn (IC_{50} value = 277 nM) in vitro (*37*). However, in intact cells, 5 to 25 µM of PF-562,271 applied for up to 24h hours results in altered cell migration and FAK phosphorylation in human embryonal rhabdomyosarcoma cells (*38*). In another study, primary $CD4^+$ T cells treated with up to 20µM PF-562,271 show altered phosphorylation of ZAP-70 and LAT after TCR activation (*39*). Here, we used 10µM of PF-562,271 with a pre-incubation time of only 5 min before image acquisition to ensure low intracellular concentrations and avoid possible side-effects during the measurements. Furthermore, we found that PF-562,271 reduced the number of ADCMs that occurred before TCR stimulation, when inhibition of ZAP-70 or LAT should have little, if any, effect.

NFAT translocation is crucial for T cell proliferation and in general is triggered by calcineurin activation upon elevated Ca^{2+} levels (*40*). NFAT-1 activation is tightly coupled to Ca^{2+} microdomains near open CRAC channels through direct tethering to the scaffolding protein AKAP79 (A-kinase anchoring protein 79) (*41*) and promotes gene expression (*42*). STIM2 promotes the activation of NFAT-1 through AKAP79 by recruiting ORAI1/STIM1 without substantially affecting Ca^{2+} signaling and ORAI1/STIM1 clustering (*43*). Our data demonstrate increased nuclear translocation of NFAT-1 in T cells adhering to collagen IV (Fig. 3, A to D) as soon as 7.5min after TCR stimulation in comparison to other studies looking at later timepoints (≥30min after TCR stimulation) (*44*). This finding suggests that ADCMs enable a fast and efficient activation once the TCR/CD3 complex is triggered and that ADCM sensitize T cells for activation

through TCR/CD3, consistent with a study showing that inhibition of FAK in primary $CD4^{+}$ T cells impairs T cell activation (*39*).

In addition to NFAT-1 translocation, ADCMs may also play an essential role in T cell motility. In neutrophils, cell motility is influenced by $[Ca^{2+}]_i$ transients when cells are attached to fibronectin or vitronectin (*45*). Furthermore, intracellular Ca^{2+} signaling during recruitment and migration of neutrophils has been visualized in vivo (*46*). In line with our results, inhibition of ORAI1 resulted in decreased Ca^{2+} signals during adhesion, and furthermore different functional states of neutrophils, similar to our model for T cells, have been proposed in relation to the Ca^{2+} response (*46*). Moreover, also in other myeloid leukocytes, such as macrophages, β_1 integrins are essential for cell motility (*47*).

Model of T cell sensitization

Together with other results (*13–16*), our findings can be summarized in a three-state model. The first state describes a fully quiescent T cell (Fig. 8A) in which local IP_3- or NAADP-evoked Ca^{2+} release or local STIM- and ORAI1-dependent Ca^{2+} entry are not activated. This state may resemble a situation when a cell 'drifts' in a large blood vessel without any contact to antigen-presenting cells (APC) or adhesive structures. The second state occurs upon integrin activation when T cells bind to adhesive structures such as laminin-1 or collagen IV of the basement membrane in transit to inflamed tissue. Low level sensitization of this particular T cell subset is mirrored by ADCM formation due to local activation of FAK, PLCγ, IP_3 signaling, local store-depletion by IP_3R, and Ca^{2+} entry through STIM/ORAI and P2X4 (Fig. 8B). In the third state, antigen recognition, especially in conjunction with co-stimulation (such as through CD28), represents the fully activated state characterized by rapid formation of NAADP (*48*). NAADP bound to HN1L/JPT2

(*15*) then triggers local Ca^{2+} release from the ER through RYR1 (*13*, *14*). The resulting local decrease of the luminal Ca^{2+} concentration then further stimulates SOCE through ORAI1 and STIM1/2 as well as P2X4 and P2X7 (Fig. 8C) (*13*). Our qualitative model (Fig. 8, A to C) was supported by a quantitative mathematical model that predicts that ADCMs require a concerted activity of 2 to 6 IP_3Rs and ORAI channels to achieve the junctional Ca^{2+} amplitude observed experimentally. Currently, such Ca^{2+} nanodomains cannot be spatially resolved by live cell Ca^{2+} imaging. However, super-resolution microscopy imaging techniques may soon be available to verify or disprove our prediction.

MATERIALS AND METHODS

Reagents

Synta66 was purchased from Aobious. STX564 was kindly provided by Barry V.L. Potter (Drug Discovery & Medicinal Chemistry, Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom). Murine ECM proteins were purchased from Invitrogen (laminin-1, natural, cat. no. 23017-015), Leinco technologies (ICAM1, recombinant, cat. no. I-587-50), Santa cruz biotechnology (collagen IV, natural, cat. no. SC-29010) and Biomol (collagen type VI, recombinant, cat. no. 154097.10). Monoclonal antibodies were purchased from Merck (anti-α_6 integrin, cat. no. MAB1378; anti-$\alpha_2\beta_1$ integrin, cat. no. MAB1998Z; anti-$\alpha_5\beta_1$ integrin, cat. no. MAB2575) and Antibodies online (anti-α-L integrin, cat. no. ABIN1176861). Inhibition of cell adhesion to respective proteins was listed as `application´ for the respective antibodies by the supplier. Anti-mouse CD3 mAb (cat. no. 553058) was purchased from BD Biosciences. Thapsigargin was purchased from Calbiochem/Merck Millipore. Fluo4-AM and Fura Red-AM were obtained from Life Technologies. Fura2-AM was purchased from Merck. Unless otherwise noted, chemicals, proteins or antibodies were obtained either from Sigma or Merck.

Animals

WT mice (C57BL/6J; *Mus musculus*) were bred and housed in the animal facility of University Medical Center Hamburg-Eppendorf (UKE). All animals were held under standardized conditions at a 12/12-hour light/dark cycle with food and water provided ad libitum. All mice experiments were approved by the Animal Welfare Officers of UKE and Behörde für Gesundheit und Verbraucherschutz Hamburg (ORG934). Mice in which all three IP_3R subtypes are knocked-out (*Itpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$ Tie2-Cre triple floxed) were originally provided by the group of Prof. Ju Chen from the Department of Medicine, School of Medicine, University of California San Diego(*49*). Further breeding and housing were performed in the animal facility of University Medical Center Hamburg-Eppendorf (UKE) by the group of Prof. Huber (Section of Molecular Immunology und Gastroenterology, I. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany) under same conditions as stated above for WT mice.

Isolation of $CD4^+$ T cells

Identification, image processing and analysis of spontaneous Ca^{2+} microdomains, which we called adhesion-dependent Ca^{2+} microdomains (ADCMs) in this paper), were done as previously described (*50*). Isolation of $CD4^+$ T cells was performed from murine spleens and lymph nodes, which were dissected from the mice and generally used directly for isolation or stored in cold medium until use (maximum 1 day). Lymph nodes and spleen were isolated in isolation medium (RPMI, 7.5 % newborn calf serum (NCS), 1% penicillin/streptomycin) using a plunger of a syringe and a cell strainer (40 µm Nylon), transferred to a 50 ml tube, and centrifuged at 4°C (300xg, for 5 min). The pellet was resuspended in 5 ml of cold ACK buffer (4.3 g ammonium chloride, 0.5 g

KHCO3, 0.0186 g Na2-EDTA in 400 ml H_2O, pH 7.2–7.4) for erythrocyte lysis and incubated for 3-5 min on ice. The lysis was stopped by the addition of 25 ml medium, and the lysate was centrifuged at 4°C (300xg, for 5 min). The pellet was resuspended in 2 ml PBS. Because the suspension contained B and T cells, isolation of $CD4^+$ T cells was performed using a negative selection kit (EasySep™Mouse T Cell Enrichment Kit, STEMCELL Technologies) according to the manufacturer's instructions.

Loading and slide preparation for imaging experiments

Briefly, freshly isolated murine $CD4^+$ T cells were loaded with Fluo4-AM (10 µM) and Fura-Red-AM (20 µM) for 50 min at room temperature. Typically, experiments were performed in Ca^{2+} measurement buffer containing 1mM Ca^{2+} (140 nM sodium chloride (NaCl), 5 mM potassium chloride (KCl), 1 mM magnesiumsulfate ($MgSO_4$), 1 mM calciumchloride ($CaCl_2$), 20 mM (4-(2-Hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES), 1 mM sodium dihydrogen phosphate (NaH_2PO_4), 5.5 mM Glucose- pH 7.4). For the different imaging experiments, chamber slides were coated using different coatings. Rubber O-rings were mounted onto slides with silicon grease so that the cells can then be placed on the slides.

For experiments with WT cells without integrin blocking, the loaded T cells were seeded on coverslips coated with either BSA alone (5 mg/ml) or PLL (0.1 mg/ml) incubated on top of the BSA coating for 5 min. Experiments examining the influence of $[Ca^{2+}]_{ex}$ on Ca^{2+} microdomains, were performed with T cells seeded on the previously described coatings, and the Ca^{2+} measurement buffer (1mM Ca^{2+}) and Ca^{2+} free buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$,

20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, 1 mM EGTA- pH 7.4) were used. Inhibition experiments using 1, 10 and 100µM Synta66 and STX564 were performed on BSA+PLL coating, and only the Ca^{2+} microdomains below the plasma membrane were analyzed. For the blocking experiments with specific anti-integrin mABs, WT cells were seeded onto BSA coating and WT and all anti-integrin mAB incubated cells were seeded onto BSA+PLL and analyzed directed after loading. Cells were incubated for 30 min at 37°C with anti-integrin mAb (10 µg/ml), which block binding between ECM proteins (laminin-1, fibronectin, collagen IV and collagen VI) and the corresponding integrin receptor. For the experiments on different ECM coatings, coverslips were coated with BSA and with ECM proteins (10 µg/ml) in PBS (137 mM NaCl, 7 mM Na_2HPO_4, 1 mM KH_2PO_4, 2 mM KCl, pH 7.4) for 1 h at 37°C and washed with PBS. To prevent denaturation of ECM proteins, the coverslips were kept in PBS until use. For experiments using pharmacological blockers, cells were preincubated for 5 min either with 0.2 % DMSO as control, 10 µM PF562,271 (FAK inhibitor), 10 µM U73122 (PLC inhibitor) or U73343 (control compound for U73122). Imaging was performed on BSA+PLL coated slides. Experiments comparing *Iptr1/2/3*$^{-/-}$ T cells and respective WT Cre negative controls were done with cells seeded on slides coated with BSA and BSA+PLL or with BSA and BSA + collagen IV as described above.

Seeding of CD4^{+} T cells on different coatings

The following seeding procedure was done for all experiments except for STED measurements. First, 10 µl of CD4^{+} T cell suspension containing approximately 100 000 cells was added to the center of the prepared slide and allowed to adhere for 3 min. For experiments with inhibitors, the cells were previously incubated in an Eppendorf tube with the final concentration and then added to the slide. 90 µl of Ca^{2+} buffer was added and cells were allowed another 2 min to adhere to the

slide. The slide was then mounted on a wide-field microscope, a suitable field of view was sought, and a bright-field image was acquired before starting video acquisition. Imaging was carried out at 100 x magnification with an exposure time of 25 ms in 14-bit mode using a Dual-View module (Optical Insights, PerkinElmer Inc.) to split the emission wavelengths (filters: excitation, 480/40; beam splitter (bs), 495; emission 1, 542/50; emission 2, 650/57). In general, measurements were started 6 min after seeding the cells. The total acquisition length was set to 3 min, with the first min acquiring basal cell activity with 1 frame per 5 s and the remaining 2 min with maximum frame rate (40 frames/s). To detect ADCMs, the first 15 s at maximum speed (1 min after acquisition start) were analyzed, meaning that timepoint "0 s" in the representative images corresponds to the first frame at maximum speed, approximately 7 min after seeding the cells onto the respective coatings. In general, Ca^{2+} microdomains were analyzed using a script implemented in MATLAB as previously described (*13*).

Imaging of global Ca^{2+} signals after TCR stimulation and postprocessing

Freshly isolated primary murine $CD4^+$ T cells were loaded with Fura2-AM (4µM) and incubated for 40 min at 37°C. Next, cells were washed and resuspended in Ca^{2+} buffer (140 nM sodium chloride (NaCl), 5 mM potassium chloride (KCl), 1 mM magnesium sulfate ($MgSO_4$), 1 mM calcium chloride ($CaCl_2$), 20 mM (4-(2-Hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES), 1 mM sodium dihydrogen phosphate (NaH_2PO_4), 5.5 mM Glucose- pH 7.4). Before imaging, cells were mounted on coverslips coated with BSA (5 mg/ml) and subsequently with either PLL (0.1 mg/ml) or collagen IV (10 µg/ml, diluted in PBS for 1 h at 37°C as described above). The seeding procedure was identical as for the Ca^{2+} microdomain experiments described above. Experiments were performed without FAK inhibition and after preincubation for 5 min with the

FAK inhibitor PF562,271 (10 µM). Imaging was performed with a Leica IRBE microscope using a 40x magnification and 25 ms exposure time. The microscope contained a Sutter DG-4 as a light source and an electron-multiplying charge-coupled device camera (C9100-13, Hamamatsu). Images were acquired in 16-bit mode and were therefore 512 × 512 pixels. The filter set (AHF Analysentechnik) for Fura2 measurements was excitation, HC 340/26, HC 387/11; beamsplitter, 400DCLP; emission, 510/84. Image acquisition was performed with Volocity software (version 6.6.2; PerkinElmer Inc.). Acquisition rate was 1 frame every 2 s, resulting in 450 frames in total (15 min acquisition time). Stimulation of T cells was performed after 1 min by addition of anti-CD3 antibody (1µg/ml) and after 11 min using thapsigargin (1.67µM) as a positive control. Data processing including background correction, splitting fluorescence channels and selection of cells (ROIs) was done with Fiji 2v.

Protein clustering and colocalization by STED imaging

Freshly isolated primary murine T cells were seeded on either BSA coating (5 mg/ml) only or a coating of BSA (5 mg/ml) and then either PLL (0.1 mg/ml) or collagen IV (10 µg/ml, diluted in PBS for 1 h at 37°C as described for Ca^{2+} microdomain imaging). Cells were fixed with 4% (w/v) paraformaldehyde (Alfa Aesar) for 15 min and permeabilized with 0.05% (v/v) saponin (Fluka) for 15 min. Unspecific binding sites were blocked with 10% (v/v) fetal bovine serum incubated overnight at 4°C. Primary antibodies [mouse anti-ORAI, 1:100 (13130-1-AP, Proteintech); rabbit anti-STIM1, 1:100 (D88E10, Cell Signaling); rabbit anti-STIM2, 1:600 (4917S, Cell Signaling) rabbit anti-FAK, 1:100 (ab131435, Abcam); rabbit anti-IP_3R1, 1:100 (PA1-901, Invitrogen); rabbit anti-IP_3R2, 1:100 (PA1-904, Invitrogen); rabbit anti-IP_3R3, 1:100 (ACC-116, alomone)] diluted in 3% (v/v) fetal bovine serum were incubated for 1 h at room temperature. Secondary antibodies

[anti-rabbit STAR RED, 1:200 (STRED-1001-500UG, Abberior Instruments) and anti-mouse Alexa Fluor 594, 1:200 (A11037, BioLegend)] were incubated for 1 h at room temperature. Coverslips were mounted with Abberior Mount Solid (Abberior) overnight. Images were acquired with the Abberior Expert Line four-channel easy3D STED equipped with a 775 depletion beam (Abberior Instruments), a Nikon 60x, 1.4-numerical aperture objective and a QUAD beam scanner. Alexa Fluor 594 was excited with a pulsed 561-nm diode beam, depleted with a pulsed 775-nm STED beam and detected with a 615±20-nm emission filter. Star red 640 was excited with a pulsed 640-nm diode beam, depleted with the 775-nm STED beam and detected with an avalanche photo diode (APD) with a 685±70-nm band pass filter. In all experiments, the pixel size was set to 20 nm. Co-localization analysis was performed with FIJI (version 1.52p) after a Gaussian Blur filtering of 0.6 using the trainable weka (waikato environment for knowledge analysis) segmentation plugin to get pixel-based segmentation. Subsequently, a more detailed segmentation of the detected proteins was generated by watershed segmentation. The areas and coordinates of the individual proteins in each image were calculated and quantified with the help of a Matlab script (based on the one described in (*51*). Cluster formation (definition of cluster formation in fig. S1) and co-localization between two different protein-spots were calculated.

NFAT translocation experiments

Primary mouse CD4$^+$ T cells were placed on slides coated with BSA (5mg/ml) alone, BSA (5 mg/ml) + PLL (0.1 mg/ml) or BSA + collagen IV (10 µg/ml). Cells were fixed and permeabilized either without or with stimulation (as indicated in the previous section). For stimulation, cells were incubated for 7.5 min and 15 min with biotin anti-CD3 (553060, BD Biosciences)/anti-CD28 (553296, BD Biosciences) labelled transparent streptavidin beads (TP190902KOM5, PolyAn). To

block non-specific binding sites, cells were incubated overnight with 10 % (v/v) fetal bovine serum. NFAT-1 was labelled with primary antibodies (rabbit anti-mouse, 1:200 (5861S, Cell Signaling Technology)) by incubation at room temperature for 1h. To remove the antibody, the cells were washed with PBS (4x) and incubated with secondary antibodies (anti-rabbit Alexa Fluor 488, 1:400 (A21206, Life technologies) again for 1h at room temperature. After washing with PBS (4x) the nucleus was labelled by DAPI staining (62247, ThermoFisher, 1:1000) for 10 min at room temperature. After washing (4x with PBS), the coverslips were mounted with Abberior Mount Solid (Abberior) overnight. Images were acquired using a high-resolution spinning-disk (Visitron) equipped with a CSU-W1 SoRa Optic (2,8x, Yokogawa), a 100x magnification objective (Zeiss) and a sCMOS camera (Orca-Flash 4.0, C13440-20CU Hamamatsu). DAPI was excited with a 405nm laser and detected with a 460/50 emission filter. Alexa Fluor 488 was excited with a 488nm laser and detected with a 525/50 emission filter. To analyze NFAT translocation, the ratio of the nuclear NFAT-1 signal compared to the cytoplasmic NFAT-1 signal was determined as percentages. Spots were highlighted by creating a pixel-based segmentation using the trainable weka segmentation plugin on Fiji (see the previous section) and the area (sum of detected spots) of all NFAT-1 spots in the nucleus and the cytoplasm was calculated. The DAPI staining was used to distinguish between the nucleus and the cytoplasm. The dynamic range (baseline to maximum response) of our NFAT-1 translocation assay encompassed a baseline at 33% and the maximum response of approximately 60%, as determined by fully activating SOCE with thapsigargin for 30 min (Fig. 3 D). This dynamic range was used to normalize our results.

Statistical analysis

Postprocessing was performed in MATLAB (version 2020a) as previously published (*50*). Data analysis was performed with Excel (Microsoft) and Prism 9 (GraphPad Software). Data sets were tested for normal distribution by Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie for P value. Because all data sets except that for the NFAT translocation experiments were not normally distributed, a non-parametric Kruskal-Wallis test with Dunn's correction for multiple testing was performed. For the statistical analysis of the NFAT experiments an ordinary one-way ANOVA was used. For statistical testing, a significance level of $\alpha = 0.05$ was adopted. An a priori power analysis to determine sample size was not performed. For protein distribution and co-localization, a weka (Waikato environment for knowledge analysis) segmentation was performed. A two-tailed Mann-Whitney U-test was carried out to reveal statistical significances on clustering and co-localization data.

Mathematical simulations

The model uses a previously proposed mathematical description of the ER-PM junction (*25*). It describes Ca^{2+} fluxes at ER-PM junction and in the portion of the ER adjacent to the junction (schematized in Fig. 7A) using diffusion equations and appropriate boundary conditions. To solve the partial ordinary equations in a system characterized by very steep gradients (*25*), we used Green's functions and the module Transport of Diluted Species in COMSOL Multiphysics (COMSOL Multiphysics® v. 5.4.). This software allows for fast and flexible simulations, as well as for a precise representation of the location and spatial extent of the ORAI1 channels and the SERCA pumps. Agreement between the two methods of resolution was checked, as well as the accordance of simulation results with the analytical calculations of the Ca^{2+} profile in an idealized STIM-ORAI microdomain (*52*, *53*). To model cell attachment-evoked microdomains, we assumed

basal Ca^{2+} concentrations of 30 nM in the junction and 450 μM in the ER, respectively. The boundary condition at the bulk of the ER was changed accordingly. ORAI1 opening at time τ_{center} is simulated by considering that Ca^{2+} ions flow through the channel at time t such that (τ_{center} - 0.5*τ_{open}) > t > (τ_{center} + 0.5*τ_{open}), at a rate across each channel that corresponds to the single channel current (2.1 fA). When two channels were supposed to open, each channel was set so that they mediated the same Ca^{2+} flux. τ_{open} was set to 43 ms, which corresponded to the average duration of a Ca^{2+} microdomain. For other values of t, the current through each ORAI1 channel equaled 0. The distance between ORAI1 channels was set to 47 nm, in agreement with (*54*). If not specified, the values of the parameters are the same as in (*25*).

Supplementary Materials

Figs. S1-S3

Table S1

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Author contributions:

Conceptualization: AHG, MW, BPD, AL, DCGM, AF
Methodology: LCHC, BPD, AL, DCGM, LMW
Investigation: MW, LCHC, AL, AK, DCGM, LU, ML
Visualization: MW, LCHC, AL, AK, BPD, LU, ML
Funding acquisition: AHG, AF, SH, RW, GD, BPD, MW
Project administration: AHG, MW, BPD, AF, AL
Supervision: AHG, AF, GD, MW, BPD, AL
Writing – original draft: MW, BPD, LCHC, DCGM, AL, AHG
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Figures

Fig. 1. Influence of PLL, integrin blocking and $[Ca^{2+}]_{ex}$ on Ca^{2+} microdomains formed in primary murine WT $CD4^{+}$ T cells.

(A) Left: example of a Ca^{2+} microdomain in a primary murine T cell on bovine serum albumin (BSA) + poly-L-lysine (PLL). Timepoint: frame 11 = 1.25s. Scale bar: 2 µm. Middle and right: Surface plot of the cell and the ROI indicated by the red square on the left. $[Ca^{2+}]_{ex}$: extracellular Ca^{2+} concentration. **(B)-(F)**: Representative examples of cells analyzed without blocking of integrins (B) and with integrin blocking using the indicated anti-integrin mABs (C)-(F). Scale bar: 2 µm. Microdomains are indicated by arrow heads. **(G)** Quantification of the number of signals in murine T cells treated with anti-αL, anti-α_6, anti-$\alpha_2\beta_1$ or anti-$\alpha_5\beta_1$ to block different integrins (mean ± SEM, the number of cells analyzed is indicated for each column). A total of 10 experiments were performed with 7 spleens. Experiments were performed in extracellular buffer containing 1 mM Ca^{2+}.* $p < 0.05$, ** $p < 0.01$ by Kruskal-Wallis test. **(H)** Quantification of the number of signals in murine T cells on slides coated with either BSA plus PLL coating or on BSA coating alone in Ca^{2+} buffer with 1 mM Ca^{2+} or nominally no Ca^{2+} (0 mM $[Ca^{2+}]_{ex}$,) (mean ± SEM, the number of cells analyzed is indicated for each column). **** $p < 0.0001$ by Kruskal-Wallis test. **(i)** Number of signals in T cells treated with Synta66 or STX564 (mean ± SD, n= 30 cells for 1 µM STX564, n=33 cells for 1 µM Synta66; n=53 cells for 10 µM STX564; n=50 cells for 10 µM Synta66; n=34 cells for 100 µM Synta66).

Fig. 2. Adhesion-dependent evoked Ca^{2+} microdomains formed in primary murine WT $CD4^{+}$ T cells seeded on different coatings.

(A) Left: Sample image of a Ca^{2+} microdomain in a primary murine T cell on BSA + collagen IV coating. Timepoint: frame 11 = 1.25s. Scale bar: 2 µm. Middle and right: Surface plot of the cell and the ROI indicated by the red square shown on the left. **(B)–(F)** Representative images of cells seeded on different

protein coatings. Microdomains are indicated by arrow heads. **(G)** Quantification of the number of signals for adhesion-dependent Ca^{2+} microdomains in murine T cells seeded on PLL (pos. control), laminin or collagen IV (mean ± SEM, the number of cells analyzed is indicated for each column). **(H)** Corresponding amplitude of Ca^{2+} microdomains on different coatings of cells from (G). A total of 9 experiments were performed with 7 spleens. All experiments were performed in extracellular buffer containing 1 mM [Ca^{2+}]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ by Kruskal-Wallis test.

Fig. 3. Influence of adhesion on nuclear translocation of NFAT after TCR stimulation in primary murine $CD4^{+}$ T cells.

(**A** to **C**) Representative images of basal NFAT signal (A) and at 7.5 min (B) or 15 min (C) after TCR stimulation using anti-CD3/CD28 coated beads on murine T cells seeded on BSA (basal: 59 cells; 7.5min: 53 cells; 15min: 42 cells), PLL (basal: 42 cells; 7.5min: 104 cells; 15min: 48 cells) and Col-IV (basal: 22cells; 7.5min: 47 cells; 15min: 35 cells). **(D)** Left: Ratio of the distribution of individual local NFAT signals between nucleus and cytosol shown over time as mean ± SEM (cell numbers given above). Middle: Estimation of the dynamic range of the assay. The maximal response was obtained with 1.67 µM thapsigargin incubated for 30 min. Right: Normalization of the data in graph on the left. § (PLL), $p < 0.05$, ## (Col IV), $p < 0.01$, and §§§§ (PLL) $p < 0.0001$ by ordinary one-way ANOVA with significances in comparison to BSA for the respective timepoint. For each condition, a total of 4 independent experiments were performed with 4 spleens.

Fig. 4. Pharmacological inhibition of FAK and PLC and the influence on ADCM and NFAT translocation in primary murine $CD4^{+}$ T cells.

(**A** to **D**) Representative images of cells showing the effect of vehicle (0.2 % DMSO) (A), the FAK inhibitor PF562,271 (B), the PLC inhibitor U73122 (C) or control compound for U73122 (U73343) (D) on the number of Ca^{2+} microdomains in primary murine WT $CD4^{+}$ T cells. Microdomains are indicated by arrow heads. **(E)** Quantification of the number of signals for Ca^{2+} microdomains in murine T cells treated as

indicated and seeded on BSA and PLL. **(F)** Mean tracings of global Ca^{2+} signals on PLL after TCR stimulation with anti-CD3 and FAK inhibition. **(G)** Total peak area after TCR stimulation of murine T cells treated as indicated. Ca^{2+} microdomain experiments were performed in extracellular buffer containing 1 mM [Ca^{2+}]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ by Kruskal-Wallis test. **(H)** Normalized NFAT-1 translocation in murine T cells treated with the indicated inhibitors and seeded on PLL and Col IV. Mean ± SEM, the number of cells analyzed is indicated for each column. **** $p < 0.0001$ by ordinary one-way ANOVA. (**I**) Representative images of NFAT translocation in murine T cells treated with the FAK inhibitor PF562,271. See Fig. 3C for representative images of cells in the absence of FAK inhibition. Pharmacological inhibition experiments: 5 experiments and 3 spleens; for global Ca^{2+} imaging analyses with TCR stimulation after FAK inhibition: 9 experiments and 9 spleens; and for NFAT translocation experiments: 4 experiments and 4 spleens.

Fig. 5. Influence of IP_3R knockout of 3 subtypes on ADCM formation.

(A)-(F) Representative images of either WT (A, C, E) or triple IP_3R KO (B, D, F) T cells analyzed on BSA + PLL (B, D), BSA + collagen-IV (E, F) or BSA alone (A, D). Microdomains are indicated by arrow heads. **(G, H)** Quantification of the number of signals for adhesion-dependent Ca^{2+} microdomains in (A) to (F) (mean ± SEM, the number of cells analyzed is indicated for each column). A total of 7 experiments were performed with 6 spleens (including one lymph node pair) to compare WT and triple IP_3R KO on PLL and BSA. A total of 6 experiments were performed with 4 spleens to compare cells seeded on collagen IV and BSA. All experiments were performed in extracellular buffer containing 1 mM [Ca^{2+}]. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ by Kruskal-Wallis test.

Fig. 6. Adhesion-dependent clustering of FAK, ORAI1 and IP_3R1 and co-localization in primary murine $CD4^+$ T cells.

(**A** to **E**) Super resolution STED images at approximately 40 nm were used to determine the clustering of FAK (A), ORAI1 (B), IP_3R1 (C) or co-localization between ORAI1 and FAK (D) or between ORAI1 and

IP_3R1 (E) on slides coated with either BSA (no adhesion), with PLL or collagen IV (adhesion). Left column: representative images of protein distribution and co-localization in primary murine T cells after weka (Waikato environment for knowledge analysis) segmentation. Scale bar: 2 μm. Right column: Magnification of ROIs showing clustered or co-localization (white) spots of proteins. Scale bar: 100 nm. Bar graphs: statistical analysis of average number of protein spots located in on cluster or co-localization (A to C) or percentage of proteins that co-localize (D, E). * p<0.05, ** p<0.01, **** p<0.0001 by two-tailed Mann-Whitney U-test.

Fig. 7. Simulation of Ca^{2+} profiles in the ER-PM junction and sub-PM ER.

(A) Schematic representation of the numerically simulated ER-PM junction and the sub-PM ER (extension from the internal ER) where C_C and C_S represent Ca^{2+} concentrations in the cytosol and in the sub-PM ER, respectively. Five ORAI channels (blue) and ten SERCA pumps (orange) are localized in the PM and the ER membranes, respectively. Eight IP_3R (green) are localized around the junction facing the PM. Upper panels are experimentally-obtained Ca^{2+} profiles of adhesion-dependent microdomains in T cells. See fig. S3 for the mathematical model. Scale bars: upper panel: 2 μm; middle panel: 500 nm. (**B)** Evolution of Ca^{2+} microdomain amplitude with the number of simultaneously open IP_3Rs in the ER-PM junction. The microdomains observed under conditions of Ca^{2+}-replete ER can be induced by the spontaneous opening of a few IP_3Rs near the ER-PM junction that in turn trigger the opening of ORAI1 channels bound to STIM2 homotetramers (blue curve) or to STIM2/1 heterotetramers (yellow curve). (**C)** Simulated Ca^{2+} microdomain resulting from the simultaneous opening of 7 IP_3Rs adjacent to the junction, 22ms after their opening. The opening of the IP_3Rs in turn induces the opening of ORAI1 channels in the ER-PM junctions due to local depletion of ER Ca^{2+}. Cross-section of Ca^{2+} profiles in the junction, in the cytosol adjacent to the junction and in the sub-PM ER, where local depletion of ER Ca^{2+} elicits the opening of the nearby ORAI1 channels. Vertical bars indicate the colour codes and the minimal and maximal concentrations

reached in the related panel. To simulate the presence of pre-formed aggregates of ORAI1-STIM2/1 (*13*), ORAI1 opening is assumed to occur immediately after depletion.

Fig. 8. Three-state model of Ca^{2+} microdomain formation in T cells.

(A) A quiescent T cell in which no Ca^{2+} microdomains are formed, such as a T cell in the blood stream of a large vessel without any contact with adhesive structures or antigen-presenting cells (APC). **(B)** A sensitized state in T cells, in which binding to adhesive structures like laminin-1, collagen IV or PLL results in activation of FAK-PLCy-IP_3 signaling, preformed clusters of STIM-ORAI1, and activation of P2X4, thereby leading to the formation of ADCMs. This state may occur when after diapedesis, T cells encounter laminin-1 and collagen IV of the basement membrane. **(C)** Full activation by antigen recognition and binding to adhesive structures, characterized by formation of NAADP in the first seconds upon TCR/CD3 stimulation and NAADP binding to HN1L/JPT2 that evokes initial Ca^{2+} microdomains from the RYR1. Furthermore, local depletion of the luminal Ca^{2+} concentration then stimulates SOCE through ORAI1 and STIM1/2 and purinergic signaling through P2X4 and P2X7, thereby leading to nuclear translocation of NFAT-1. Created with Biorender.