Microtubule associated protein-4 (MAP4) controls nanovesicle dynamics and T cell activation

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Key words: MAP4, T cell activation, vesicle dynamics, microtubules.

Summary Statement:

Microtubule associated protein-4 (MAP4) regulates early T cell signaling by controlling microtubule stability and CD3ζ-bearing nanovesicle dynamics. MAP4 also acts balancing earlier and late T cell activation events.
ABSTRACT

The Immune Synapse (IS) is a specialized structure formed at the contact area between T lymphocytes and antigen-presenting cells (APC), essential for the adaptive immune response. Proper T cell activation requires its polarization towards the APC, which is highly dependent on the tubulin cytoskeleton. Microtubule associated protein-4 (MAP4) is a microtubule (MT)-stabilizing protein that controls MTs in physiological processes such as cell division, migration, vesicular transport or primary cilia formation. In this study, we have assessed the role of MAP4 in T cell activation. MAP4 decorates the pericentrosomal area and MTs of the T cell, and it is involved in MT detyrosination and stable assembly in response to T cell activation. Also, MAP4 prompts the timely translocation of the microtubule organization center (MTOC) towards the IS and the dynamics of signaling nanovesicles that sustains T cell activation. However, MAP4 acts as a negative regulator of other T cell activation-related signals, including DAG production and IL2 secretion. Our data indicate that MAP4 acts as a checkpoint molecule that balances positive and negative hallmarks of T cell activation.
INTRODUCTION

The recognition of an antigen peptide on the surface of an antigen presenting cell (APC) triggers a signaling cascade leading to the formation of a polarized and specialized structure called the IS (Huppa and Davis, 2003). Its proper formation is tightly regulated by the interplay of the actin and tubulin cytoskeletons (Soares et al., 2013). MT network is polarized at the IS due to the translocation of the MTOC towards the contact area, leading to the rearrangement of the tubulin cytoskeleton by polymerization of new MTs (Martin-Cofreces et al., 2014).

MAPs are proteins that bind to MTs and regulate their dynamics, growth, catastrophe and association to other molecular complexes. MAP4 is the most abundant MAP in non-neuronal tissues. In vitro, MAP4 promotes MT assembly and stabilization (Nguyen et al., 1999; Nguyen et al., 1998). Moreover, it has been involved in some physiological processes such as cilia formation (Ghossoub et al., 2013) or myotube organization (Mogessie et al., 2015). Most studies regarding MAP4 have addressed its role in a cell division context, controlling the assembly of the mitotic spindle and the orientation of the centrosome (Chang et al., 2001; Samora et al., 2011).

In this work, we assessed MAP4 role in T cell activation and polarization. We have identified a new role of MAP4 in CD3ζ-bearing nanovesicle dynamics at the IS and T cell activation.

RESULTS AND DISCUSSION

MAP4 regulates MTs stabilization and MTOC translocation at IS.

In order to assess MAP4 location in CD4+ T cells during IS formation, MAP4 was immunostained in Jurkat T cells conjugated with SEE-pulsed Raji B cells (SEE-APCs) (Fig. 1A). MAP4 staining was similar to that of α-tubulin, decorating the MTOC. MAP4 dynamics was assessed upon T cell-APC contact formation in Jurkat T cells co-transfected with rodent MAP4 coupled to GFP and α-tubulin-mCherry and conjugated with SEE-APCs. Time-lapse confocal microscopy revealed a similar behavior for both proteins (Fig.S1 and Movie S1).

In most systems, MAP4 appears mainly co-localized with MTs (Chang et al., 2001; Cheng et al., 2010; Samora et al., 2011). Moreover, in T cells, most MAP4 appeared
concentrated around the MTOC. These results suggest that, in T cells, MAP4 may be important for the assembly of new MTs during IS formation. To address this, we first measured the ability of MTs to reassemble in control cells or cells depleted of MAP4 using specific siRNAs (MAP4KD) (Fig. 1B). T cells were treated with nocodazole (8 µM) to fully depolymerize MTs, and their re-assembly assessed upon washout of the inhibitor. Most MAP4KD cells displayed depolymerized tubulin, which concentrated around the nucleus (Fig 1C), indicating that MAP4 is required for the reassembly of MTs. To assess whether MAP4 participates in MT stabilization in response to TCR signals, we studied the de-tyrosination (Glu) of α-tubulin, a MT stability marker observed upon TCR activation, in T cells. Interestingly, the increase of de-tyrosinated tubulin upon TCR triggering was prevented in MAP4KD cells (Fig. 1D). However, no changes in the overall efficiency of conjugate formation were detected (Fig. 1E). Together, these results indicate that MAP4 is an important mediator of MT assembly, likely by controlling MT stability, in response to signals emanating from the IS.

MTOC reorientation is a hallmark of T cell activation during the formation of the IS that may depend on MT dynamics (Martin-Cofreces et al., 2014). Consequently, we next analyzed the distance of the MTOC to the IS at different time points of conjugation in control and in MAP4KD Jurkat T cells. A significant increase in this parameter was observed in MAP4KD cells compared to control cells shortly upon activation, disappearing at longer time points (Fig. 1F). Reconstitution of MAP4 KD cells with GFP-MAP4 construction rescued MTOC translocation (Fig. 1G). These results indicate that MAP4 controls the timely translocation of the MTOC to the IS.

MTOC at the IS supports the generation of a new MT network near the IS sustaining T cell signaling (Martin-Cofreces et al., 2012) and favoring the polarized secretion of vesicles to the interphase between the T cell and the APC (Huse et al., 2008). Other MAPs like HDAC6 or dynactin are also involved in MTOC polarization. While depletion of dynein/dynactin complex impaired the translocation of MTOC (Martin-Cofreces et al., 2008), HDAC6 inhibition seems mostly to accelerate it either in CD4+ (Serrador et al., 2004) or CD8+ T cells (Nunez-Andrade et al., 2016). Similarly, cells depleted for Par1b/MARK2 kinase showed a defect in MTOC translocation (Lin et al., 2009). Due to MARK proteins role in regulating MAP4 ability to bind MTs in other systems (Cheng et al., 2010), MARK2/Par1b could have a role on MAP4 effect over T cell MTs.
MAP4 modulates TCR signaling and dynamics of signaling nanovesicles at the IS.

To ascertain whether the defects in the tubulin cytoskeleton due to MAP4 decrease can lead to the impaired signaling at the T cell receptor (TCR signalosomes), we studied the pattern of phosphorylation of downstream molecules of the TCR such as CD3ζ, LAT, PKC0 and ERK, in SEE-activated control and MAP4KD Jurkat T cells (Fig. 2A). Quantitative analysis revealed a decrease in CD3ζ, LAT-Y191 and ERK phosphorylation and a slight decrease in LAT-Y132 phosphorylation in MAP4KD cells (Fig. 2B and S2). However, no changes in PKC0 phosphorylation were detected (Fig. S2). Likewise, CD3ζ phosphorylation in response to stimulation with anti-CD3/CD28 antibodies was impaired in MAP4KD cells (Fig. 2C, D). This reduction in CD3ζ phosphorylation may account, in part, for the defective MTOC polarization, as previously reported (Jenkins et al., 2009).

To assess the mechanism by which MAP4 regulates TCR early signaling, the dynamics of CD3ζ-bearing nanovesicles was analyzed by total internal reflection microscopy (TIRFm) in control and MAP4KD Jurkat T cells transfected with CD3ζ-Cherry and activated in a stimulatory surface coated with anti-CD3/CD28 antibodies. The analysis of the vesicle tracks showed a significant defect in the movement and displacement of the vesicles (Fig. 2E and Movie S2-S3). Surface levels analysis of TCRαβ and CD3ε in MAP4KD cells showed no differences (Fig. 2F). TCR sustained signal is highly dependent on its cycle of degradation and recycling (Monjas et al., 2004). However, no differences were detected either in terms of the internalization of TCR/CD3ε molecules or in their general recycling in control and MAP4KD cells upon activation (Fig. 2G). Our results suggest that the impairment in TCR early signaling is more likely due to defects in the polarized action of CD3ζ-bearing nanovesicles rather than differences in the whole rate of internalization/recycling of the TCR. Moreover, it has been proved that a pool of active phosphorylated-CD3ζ exists in the vesicular compartment, being crucial for sustaining TCR signaling and proper T cell activation (Yudushkin and Vale, 2010). Accordingly, although MAP4 reduction would probably not affect to the initial triggering of TCR signaling, it leads to a blockade of the movement of CD3ζ-nanovesicle pool (possibly phosphorylated), avoiding its mobilization towards the fusion areas and therefore the maintenance of TCR signaling. On the other hand, since
TCR-microcluster transport is also dependent on microtubules (Hashimoto-Tane et al., 2011), we cannot rule out an additional defect on TCR-microcluster aggregation.

**MAP4 absence enhances late activation markers and promotes diacylglycerol (DAG) imbalance.**

To determine whether inhibition of TCR signaling by MAP4 depletion affects TCR-dependent gene expression or cytokine secretion, we assessed the mRNA pattern of expression of later activation markers in control or MAP4KD Jurkat T cells activated with SEE-APCs. A significant increase in IL2 and CD69 mRNA expression was detected in MAP4-silenced cells (Fig. 3A). Correspondingly, similar results were obtained by analyzing IL2-secretion in activated Jurkat T cells or primary T lymphoblasts (Fig. 3B). The same trend was detected when analyzing CD69 surface expression in activated Jurkat T cells (Fig. 3C) and in primary T lymphoblasts (Fig. S3A).

To explain this increase of late activation markers, we explored additional signaling intermediates downstream of the TCR. A significant increase in PLCγ1 phosphorylation in MAP4KD cells was observed either in Jurkat T cells (Fig. 3D) or in primary T lymphoblasts (Fig. 3E). To functionally analyze its relevance, we examined the production of DAG by using PKC0-C1-GFP. Quantitative analysis of its accumulation at the IS revealed a significant increase in the DAG production in MAP4KD cells (Fig. 3F). PKC0 whole protein accumulation was unaffected in MAP4KD cells (Fig. S3B). Since MAP4KD cells activated by SEE-APCs showed an increase in NFAT and NF-κB transcriptional activity (Fig. 3G), as well as in nuclear NF-κB (p65) upon TCR activation (Fig. S3C), it is possible that PKC0 is more active, by a CD28-mediated increased turn-over between plasma membrane and cytoskeleton moieties (Huang et al., 2002).

Although PLCγ1 clustering depends on LAT, PLCγ1 phosphorylation occurs prior to LAT-Y132 phosphorylation (Houtman et al., 2005) and it is dependent on Itk (Andreotti et al., 2010). In fact, our results show no differences in PLCγ1 clustering in MAP4KD cells (Fig. S3D). Therefore, MAP4 might be regulating PLCγ1-Itk spatial association. Alternatively, the spatial regulation of CD148, the phosphatase responsible of PLCγ1 blockade (Baker et al., 2001), could also be affected. Strikingly, ERK phosphorylation was reduced in MAP4KD cells. This can be explained by the defect in LAT-Y191
phosphorylation, leading to a decrease in Sos-Grb2 complex recruitment and Ras pathway activation (Balagopalan et al., 2010).

In addition, local production of DAG by phosphorylated PLCγ1 (Y783) can control MTOC localization close to the plasma membrane upon TCR activation (Quann et al., 2009). Our data suggest that the delay in MTOC translocation boosts the production of DAG to overcome this defect in MAP4KD cells. In accordance, the blockade of MTOC translocation, by disruption of AKP450 centrosomal localization or over-expression of HDAC6, also increased DAG production (Fig. S4). Therefore, MAP4 acts as balancer for T cell activation and its final functional outcome.

MATERIALS AND METHODS

Cells

The human Jurkat-derived T cell line E6.1 (Vβ8+ TCR) and the lymphoblastoid B-cell line Raji were from ATCC. Human Cell lines were tested for mycoplasm contamination and authenticated through specific surface markers. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors as described (Mittelbrunn et al., 2011). These studies were performed according to the principles of the Declaration of Helsinki and approved by the local Ethics Committee for Basic Research at the Hospital La Princesa (Madrid); informed consent was obtained from all human volunteers.

Antibodies and reagents

Antibodies used in this study: anti-CD3ζ-pY83 (ab68236; 1:1000), anti-CD3ζ (ab190728 1:1000), anti-MAP4 (ab89650; 1:800 WB, 1:200 IF); anti-LAT-pY132(ab4476 1:1000); anti-LAT-pY191 (ab59197 1:1000) (Abcam); anti-α-Tubulin-FITC-conjugated (F2168; 1:100), anti-α-Tubulin (T6199; 1:2000 WB), anti-β-Actin (A2228; 1:1000) (Sigma); anti-PKC0 (610090; 1:100), anti-CD4 V450 (560346; 1:100 FACs), anti-CD3ε V500 (561416; 1:100), anti-Vβ8-FITC (1:100), anti-human CD28 (555725) (BD-Pharminen); anti-ERK1/2-pT202/Y204 (44285; Calbiochem; 1:100); anti-p65 (sc372; 1:1000) (Santa Cruz); anti-PKC0-pT538 (9377 S; 1:1000), anti-PLCγ1 (2822S; 1:1000), anti-PLCγ1-pY783 (#2821L; 1:1000), anti-ERK1/2 (91075; 1:100)

**Plasmids, transfection and qPCR**

Mouse GFP-MAP4 (Olson et al., 1995); Tubulin-mCherry (Vinopal et al., 2012), PKC0 C1 domain fused to GFP or m-Cherry (Carrasco and Merida, 2004) CD3ζ-mCherry (Martin-Cofreces et al., 2012), NFAT(9x)-Luciferase (Wilkins and Molkentin, 2004), NF-κB (5x)-Luciferase provided by Dr. MJ Calzada and pRenilla-CMV (Promega, E226), C-term-AKAP450-GFP (Robles-Valero et al., 2010) and HDAC6-GFP (Serrador et al., 2004). T cell lines were transfected with a pool of two specific double-stranded siRNA against human MAP4 (UAGGAGAGGAGAACCAGAU and CCAGAUUCUAUCCUACUGA) or a scramble negative control (CGUACGCAGAAUACUUCGA). For transfection and qPCR, we followed protocols as described (Blas-Rus et al., 2016). Primers sequence is indicated in Supplemental Table S1.

**T cell activation, cell lysis, nuclear/cytoplasmic fractioning and immunoblotting**

For antigen stimulation, Jurkat E6.1 cells were mixed with Raji B cells (1:5) pre-pulsed with 0.5µg/ml SEE (30 min) and allowed to conjugate for the indicated times. Then, cells were lysed and immunoblot was performed as described (Blas-Rus et al., 2016). For nuclear/cytoplasmic fractioning, cells were lysed, spun at 650 x g (15 min/4°C) and supernatant was recovered as the cytoplasmic fraction. Nuclear fraction was washed once with lysis buffer without NP-40 and lysed in loading buffer.
Cell conjugate formation, immunofluorescence and TIRFm

Cell conjugation preparation, immunofluorescence protocol, confocal and TIRFm imaging were performed as described (Blas-Rus et al., 2016). Specific conditions are described in corresponding figure legends. For MAP4 staining cells were fixed in 100% methanol (5 min/-20ºC) followed by PFA 2% (10 min/RT). Images were processed, quantified with Adobe Photoshop CS and ImageJ. MTOC translocation experiments images were analyzed with Imaris software.

Nocodazol treatment

Cells were treated with vehicle (DMSO) or nocodazol (8 µM) for 1 h, washed twice and let to recover for 1.5 h.

ELISA, flow cytometry and TCR internalization and recycling measurement

Jurkat E6.1 T cells were co-cultured with SEE-pulsed Raji B cells (1:1) for 24 h. For primary T cell lymphocytes, cells were stimulated with anti-CD3/anti-CD28 coated plates. Cells were used for flow cytometry (FACS) analysis and supernatant for IL-2 detection by ELISA (DyaClone). For FACS cells were incubated with primary and secondary antibodies (30 min/4ºC). Cells were washed and fixed in IC Fixation Buffer (eBioscience) (20 min/4ºC).

For TCR internalization measurement, Jurkat E6.1 cells were stimulated with anti-CD3ε (HIT3α) and CD28 antibodies-coated plates for indicated times. Cells were then fixed and stained for CD3ε (UCHT1). Cells were analyzed with a FACs Canto II Cytometer (BD) and FlowJo. Recycling experiments were performed as described (Finetti et al., 2009). Stimulation was performed with anti-CD3/anti-CD28 coated plates.

Luciferase assay

Cells were transfected with NFAT/NFκB-Luciferase construction plus Renilla plasmid (2µg + 0.4 µg per 10⁶ of cells, respectively) and activated with SEE-pulsed-Raji B cells (24 h). Protocol was performed accordingly to manufacturer instructions (Promega). Measurements were normalized to Renilla levels and protein quantity.
**Statistical analysis**

Data was analyzed with ROUT test, to detect outliers, and Shapiro-Wilk normality test to determine the application of parametric or non-parametric tests. A Student-t test (parametric) or U-Mann Whitney (non-parametric) analysis was used for pairs of non-dependent data. Kruskal-Wallis test was used for grouped analysis. Finally, when samples compared were activated under the same conditions (dependent samples) a paired analysis was used; either paired t-test (parametric) or Wilcoxon test (non-parametric). Analysis was performed with GraphPad Prism.

**Acknowledgements**

We thank Dr. Miguel Vicente Manzanares for critical reading of the manuscript and Aitana Sanguino Pascual for technical support.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

E.B.-M and F.S-M designed experiments, made figures and wrote the manuscript; E.B.-M; N. B.-R; N.B. M.-C collected and/or analyzed data.

**Funding**

This study was supported by grants SAF2014-55579-R from the Spanish Ministry of Economy and Competitiveness, INDISNET-S2011/BMD-2332 from the Comunidad de Madrid ERC-2011-AdG 294340-GENTRIS. The Centro Nacional de Investigaciones Cardiovasculares (CNIC, Spain) is supported by the Spanish Ministry of Science and Innovation and the Pro-CNIC Foundation. CIBER Cardiovascular from Instituto de Salud Carlos III and co-founding by FEDER. E.B.-M was granted by Pro-CNIC Foundation and Obra Social La Caixa.
REFERENCES


Figure 1. MAP4 decorates T cell MTOC and allows microtubular stability. (A) Images show conjugates of Jurkat T cells and SEE-pulsed or unpulsed Raji B cells (APCs; 30 min). Green, α-tubulin. Magenta, MAP4. Bright field image with CMAC in cyan (APCs) is also shown. Bar 10 µm. (B) MAP4 expression in scramble-transfected (control) and MAP4-silenced Jurkat T cells (MAP4KD). Actin is shown as loading control. (C) (Left panel) Images show the recovery of MTs in MAP4KD cells treated with vehicle (DMSO) or Nocodazol (8µM), washed and recovered for 1.5 h. Green, α-tubulin. Maximal projections are shown. The inset shows magnification of indicated cells. Bar 10 µm. (Right panel) Quantification of cells with recovered MTs from experiments as in the left panel (at least 340 cells were counted from two different experiments). (D) (Left panel) Immunoblot of de-tyrosinated tubulin (Glu) in control or MAP4KD cells. Cells were activated with SEE-APCs. α-tubulin and Smc1, loading controls. (Right panel) Quantification of detyrosinated tubulin (paired t-test; *,P<0.05. n=4). (E) Quantification of the percentage of control or MAP4KD T cells conjugated with SEE-APCs for 30 min. (1138 and 1146 cells, from three different experiments, non-significant (ns)) (F) (Left panel) Images show MTOC translocation in control and MAP4KD cells conjugated with unpulsed or SEE-APCs (30 min). Green, α-tubulin; magenta, β-actin. Bright field images including CMAC-labeled APCs are shown. Bar 10 µm. (Right panel) Quantification of the distance of MTOC to the contact area (µm) as in the left panel for the indicated times (Mann-Whitney test; ****,P<0.0001; Control n=216, 196, 95, MAP4KD n=231, 251, 105 from three different experiments). (G) Quantification of the distance of MTOC to the contact area (µm) in control/MAP4KD cells transfected with GFP or MAP4KD cells reconstituted with GFP-MAP4. (Kruskal-Wallis test; ****,P<0.0001; n= 201. 216, 165, from two independent experiments). All graphs represent Mean±s.d.
Figure 2. MAP4 sustains early T cell signaling. (A) Immunoblots showing phosphorylation of the indicated molecules in control or MAP4KD Jurkat T cells activated with SEE-pulsed Raji B cells (SEE-APCs) for the indicated times. α-tubulin,
GAPDH and β-Actin, loading controls. (B) Quantification of CD3ζ, ERK2, LAT phosphorylation as in A (n=6, 7, 6; paired t-test; *, p-value<0.05). (C) Immunoblot of CD3ζ phosphorylation in control or MAP4KD cells activated with crosslinked anti-CD3/CD28 antibodies for the indicated times. β-Actin, loading control. (D) Quantification of CD3ζ phosphorylation as in C (n=6, paired t-test, *, p-value<0.05). (E) (Left panel) Map of trajectories of CD3ζ-mCherry-bearing vesicles in control and MAP4KD cells spreading over anti-CD3/CD28-coated glass-bottom chambers. Maximal projection of a time-lapse (Δt=65 s) and initial bright field image (t=0) are shown. (Right panel) Quantification of the displacement length (µm) as in E (n=16 and 17; Student t-test; **, P<0.01). (F) Quantification by FACs of the surface basal levels of TCR (Vβ8) and CD3ε in control and MAP4KD Jurkat T cells (n=3). (G) Quantification of CD3ε internalization (left panel) and recycling (right panel) by FACS of control or MAP4KD cells activated with anti-CD3/CD28-coated plates. (n=5). All graphs represent Mean±s.d.
Figure 3. MAP4 modulates DAG production and late activation markers expression. (A) mRNA levels of IL2 and CD69 (n=8 and 6; Wilcoxon test; **, P<0.01; *, P<0.05) from control and MAP4KD cells activated with SEE-pulsed APCs (4 h). mRNA levels were normalized to a housekeeping gene (Actin or Gapdh) and to the non-stimulated cells. (B) IL2 secretion levels (pg per ml and 10^6 cells) measured by ELISA in control and MAP4KD Jurkat T cells activated with SEE-APCs (left panel; n=7; paired t-test; **, P<0.01) or primary T lymphoblasts activated with anti-CD3/anti-CD28 coated plates (right panel; n=4; paired t-test; *, P<0.05) for 24 h. (C) CD69 surface expression in control and MAP4KD cells activated with SEE-pulsed APCs (24 h). (n=6; paired t-test; **, P<0.01). (D) (Left panel) Immunoblot showing PLCγ1 phosphorylation in control and MAP4KD cells conjugated with SEE-APCs. Tubulin is shown as loading control. (Right panel) Quantification of PLCγ1 phosphorylation as in the left panel (n=6; paired t-test; *, P<0.05). (E) Immunoblot showing PLCγ1 phosphorylation in control and MAP4KD primary T lymphoblasts activated with crosslinked anti-CD3/CD28 antibodies. Tubulin is shown as loading control. Quantification under bands. (One representative gel out of three) (F) (Left panel) Images show conjugates of control and MAP4KD Jurkat T cells expressing PKC0-C1-GFP and activated with SEE-APCs (15 min). Actin (Magenta) and bright field image with CMAC in cyan (APCs) are shown. Bar 10 µm. (Right panel) Quantification of PKC0-C1-GFP accumulation as in left panel is shown (n=63 and 75 from three independent experiments; Mann-Whitney
(G) Quantification of NFAT (left panel) and NF-κB (right panel) activity by luciferase assay (n=6 and 5; paired t-test; *, P<0.05***, P<0.001). All graphs represent Mean±s.d.
Supplementary Figure 1. MAP4-GFP accompanies MTOC during its translocation. Time-lapse Z-stack maximal projection of Jurkat T cells transfected with MAP4-GFP (green) plus Tubulin-Cherry (magenta) and activated with SEE-pulsed Raji B cells. Images were taken each 43 s. Bright field is also shown (* APC cell). Scale bar 10 μm.
Supplementary Figure 2. MAP4 controls LAT-Y132 phosphorylation but not PKC0 activation. (Left panel) Immunoblots showing phosphorylation of the indicated molecules in control or MAP4KD Jurkat T cells activated with SEE-pulsed Raji B cells (SEE-APCs) for the indicated times. α-tubulin shown as a loading control. (Right panel) Quantification of LAT-Y132 and PKC0 phosphorylation as in left panel (n = 4 and 5).
Supplementary Figure 3. MAP4 regulates CD69 expression in primary T cells and p65 nuclear translocation. (A) FACs analysis of control and MAP4KD primary T lymphoblasts activated with anti-CD3/anti-CD28 coated plates. CD4+ cells were gated and normalized, GeoMean of fluorescence was analyzed (n=2). (B) Analysis of PKCθ accumulation at the IS in control or MAP4KD Jurkat T cells activated with SEE-pulsed APC cells for 30 min (n =67 and 68 from three independent experiments). (C) Immunoblot showing p65 nuclear translocation. Cytoplasmic/nuclear fractions from control and MAP4KD cells conjugated with SEE-APCs for the indicated times. GAPDH /Smo1 are loading controls for cytoplasmic/nuclear fractions. Quantification is shown under bands (n=1). (D) Analysis of PLCγ1 accumulation at the IS in control or MAP4 KD Jurkat T cells activated with SEE-pulsed APC cells for 30 min (n =69 and 69 from three independent experiments). All graphs represent the Mean±s.d.
Supplementary Figure 4. MTOC translocation controls DAG production. (Left panel) Z-maximal projection of Jurkat T cells transfected with GFP alone, C-term AKAP450-GFP or HDAC6-GFP (green) plus PKCθ-C1-mCherry (magenta) and activated with SEE-pulsed Raji B cells for 15 min. Bright field and CMAC in cyan (APC) image is also shown. Scale bar 10 μm. (Right panel) Quantification of PKCθ-C1-mCherry accumulation at the IS as in left panel. (n=73, 74 and 78 from three independent experiments; Mean±s.d.; Kruskal-Wallis test, ****p-value < 0.0001).
### Supplementary Table 1

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**Supplementary Table 1. Sequence of the primers used for qPCR.** Table indicating the sequence of the pair of primers used for qPCR analysis.
SUPPLEMENTARY MOVIES

Supplementary Movie 1. Life imaging of GFP-MAP4 dynamics at the IS. Jurkat T cells transfected with GFP-MAP4 (green) and Tubulin-Cherry (magenta) were conjugated with SEE-pulsed Raji-B cells. Images were acquired each 43 s. Video was mounted in ImageJ.
Supplementary Movie 2. Tracking of CD3ζ-bearing vesicles at the IS in control Jurkat T cells. Control Jurkat T cells were transfected with CD3ζ-mCherry and allowed to settle on anti-CD3/CD28 coated surfaces and recorded under TIRFm. Images were taken each 100 ms for 30 s (video mounted at 10 fps). Imaris tracking analysis and fluorescence images are shown.
Supplementary Movie 3. Tracking of CD3ζ-bearing vesicles at the IS in MAP4KDJurkat T cells. MAP4 KD Jurkat T cells were transfected with CD3ζ-mCherry and allowed to settle on anti-CD3/CD28 coated surfaces and recorded under TIRFm. Images were taken each 100 ms for 30 s (video mounted at 10 fps). Imaris tracking analysis and fluorescence images are shown.