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Microtubule associated protein-4 (MAP4) balances T cell activation through the regulation of positive and negative signals

Tesis doctoral

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Madrid, 2017

Francisco Sánchez Madrid, Doctor en Ciencias Biológicas y Catedrático de Inmunología de la Universidad Autónoma de Madrid,

CERTIFICA:

Que Eugenio Bustos Morán, licenciado en Biología por la Universidad Autónoma de Madrid, ha realizado bajo mi dirección el trabajo de investigación correspondiente a su Tesis Doctoral con el título:

Microtubule associated protein-4 (MAP4) balances T cell activation through the regulation of positive and negative signals

Revisado este trabajo, el que subscribe considera el trabajo realizado satisfactorio y autoriza su presentación para ser evaluado por el tribunal correspondiente.

Y para que así conste y a los efectos oportunos, firma el presente certificado en Madrid a 26 de Abril 2017.

Fdo. Prof. Francisco Sánchez Madrid

A mis padres, a mis hermanos y a mi sobrina

A mis amigos

A Rocío

“Sólo tú puedes decidir qué hacer

con el tiempo que se te ha dado”

Agradecimientos

Siempre he pensado que para que la Ciencia avance adecuadamente, debe triunfar la colaboración entre los científicos frente al afán competitivo que a veces se promueve desde las revistas. Como siempre dice Paco, la ciencia es impersonal, puesto que no es cosa de uno solo ni de unos pocos sino el resultado de la colaboración de mucha gente. En ese sentido, mi tesis al igual que el resto de trabajos científicos es el resultado de la influencia, la ayuda y el apoyo de mucha gente que me han permitido llegar a este punto.

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Summary

Summary

T cell receptor (TCR) recognition of a specific antigen, bound to the major histocompatibility complex of an antigen presenting cell (APC), triggers the activation of the T lymphocyte and promotes its effector function. T cell activation leads to the rearrangement of different proteins involved in the activation into a very specialized and polarized structure known as the Immune Synapse (IS). This structure generates the segregation of the TCR signalosome within the cSMAC, while excluding adhesion molecules, like integrins, to the pSMAC. TCR activation pathway involves a cascade of phosphorylation of many molecules. Among them, phospholipase gamma 1 (PLCγ1) is an important amplifier of the signal by generating two second messengers, inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), essential for T cell function. DAG production promotes the translocation of the microtubule organizing center (MTOC). MTOC polarization is accompanied by the displacement to the contact area of other important organelles for T cell activation like mitochondria, multivesicular bodies or Golgi Apparatus. In addition, it induces the nucleation of MT growth in the proximity of the contact, promoting the polarized secretion of vesicles essential for cytotoxic functions, like lytic granules, or for a proper activation of T cells (cytokines or recycling vesicles).

Microtubule associated protein-4 (MAP4) is a molecule that regulates the stability of microtubules and its assembly in different physiological processes such as cell division, primary cilia formation or myogenesis. Provided the importance of the MT network in T cell activation and IS proper architecture, we hypothesized that MAP4 could be modulating T cell activation. We found that MAP4 decorates T cell microtubules and MTOC, controlling their assembly and stability upon activation and regulating a timely translocation of the MTOC. Moreover, we proved that MAP4 promotes TCR phosphorylation and activation, as well as the activation of some downstream molecules. We demonstrated that MAP4 controls the movement of recycling CD3ζ-nanovesicles maintaining TCR signal. Unexpectedly, we discovered that MAP4 negatively modulates the activity of PLCγ1, DAG production and NFAT and NF-κB activity. In fact, MAP4 balances IL2 secretion and CD69 expression. These results point to a modulatory role of MAP4 acting as a balancer of T cell activation and effector function.

Resumen

Resumen

El reconocimiento por parte del receptor de células T (TCR) de un antígeno específico, unido al complejo mayor de histocompatibilidad de una célula presentadora (APC), controla la activación del linfocito T e induce su función efectora. La activación de la célula T promueve la reorganización de las diferentes proteínas señalizadoras en una estructura polarizada conocida como la Sinapsis Inmune (IS). Esta estructura promueve la agregación de la maquinaria de señalización asociada al TCR en el cSMAC, excluyendo a moléculas de adhesion, como las integrinas, al pSMAC. La activación del TCR implica la fosforilación de muchas moléculas. Entre ellas, la fosfolipasa C gamma 1 (PLCγ1) actúa como amplificador al generar dos segundos mensajeros, el inositol-1,4,5-trifosfato (IP3) y el diacilglicerol (DAG). También se produce la translocación del centro organizador de microtúbulos (MTOC), acompañado de la polarización de otros orgánulos importantes como las mitocondrias, los cuerpos multivesiculares o el aparato de Golgi. Induce, además, la nucleación de microtúbulos favoreciendo la secreción polarizada de vesículas con funciones citotóxicas (gránulos líticos) o para la activación de las células T (citoquinas o vesículas de reciclaje).

La proteína de asociación a microtúbulos-4 (MAP4) es una proteína que regula la estabilidad y ensamblaje de los microtúbulos en diferentes procesos fisiológicos tales como división celular, formación del cilio primario o miogénesis. Considerando la importancia del citoesqueleto de tubulina en la activación de las células T y en la formación de la IS, nuestra hipótesis es que MAP4 podría estar modulando este proceso. Hemos localizado a MAP4 en los microtúbulos y MTOC de la célula T, controlando su ensamblaje y estabilidad y regulando la translocación del MTOC. Además, hemos demostrado que MAP4 promueve la activación y fosforilación del TCR así como de algunas moléculas subyacentes. Hemos observado que MAP4 controla el movimiento de las vesículas de reciclaje que contienen CD3ζ, ayudando a mantener la señal del TCR. Inesperadamente, hemos descubierto que MAP4 regula negativamente la actividad de PLCγ1, la producción de DAG y la actividad de NFAT y NF-κB. De hecho, la proteína MAP4 equilibra la secreción de IL2 y la expresión de CD69. Estos resultados apuntan a un papel modulador de MAP4, actuando como un factor equilibrador en la activación y función efectora de las células T.

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List of abbreviations

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ADAP: Adhesion and Degranulation-promoting Adapter Protein

APC: Antigen Presenting Cell

Bcl-10: B cell leukemia 10 protein

BF: Bright Field

BSA: Bovine Serum Albumin

BTN3A1: Butyrophilin-3

Carma-1: Caspase recruitment domain-containing membrane-associated guanylate kinase protein-1

CD: Cluster of differentiation

Cdc: Cell division control protein

CLIP-170: Cytoplasmic Linker protein-170

CRAC: Ca^{2+} release-activated channels

cSMAC: central Supramolecular Activation Cluster

DAG: Diacylglycerol

DGK: Diacylglycerol kinase

DMSO: Dimethyl Sulfoxide

dSMAC: distal Supramolecular Activation Cluster

EB: End-Binding protein

ELK: Ets-like transcription factor

ERK: Extracellular signal-Regulated Kinase

GA: Golgi Apparatus

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GFP: Green Fluorescence Protein

GLK: Germinal-center kinase-like kinase

HDAC6: Histone Deacetylase 6

ITAM: Immunoreceptor Tyrosine-based Activation motifs

Ig: Immunoglobulin

IFN: Interferon

IFT: Intraflagellar Transport

IKK: IκB kinase

IL: Interleukin

IP3: Inositol-1,4,5-triphosphate

IRF3: Interferon regulatory factor 3

IS: Immunological Synapse

ITK: Interleukin-2-inducible T-cell kinase

kDa: KiloDalton

LAT: Linker of activated T cells

Lck: Lymphocyte-specific protein tyrosine kinase

mAb: Monoclonal Antibody

MALT1: Mucosa-associated lymphoma translocation protein-1

MHC: Major Histocompatibility Complex

MAP4: Microtubule associated protein-4

MAPK: Mitogen-Activated Protein Kinases

MNK: MAP kinase interacting kinase

MT: Microtubule

MTOC: Microtubule-Organizing Center

MVB: Multivesicular body

NCD: Nocodazole

NFAT: Nuclear factor of activated T cells

NF-κB: Nuclear factor kappa B

PBLs: Peripheral Blood Lymphocytes

PBMCs: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PDK1: 3-phosphoinositide-dependent protein kinase

PIP2: Phosphatidylinositol-4,5-bisphosphate

PKA: Protein Kinase A

PKC: Protein Kinase C

PKD: Protein Kinase D

PLCγ1: Phospholipase C, gamma 1

pSMAC: peripheral Supramolecular Activation Cluster

PTMs: Posttranslational modifications

SAPK: Stress-activated protein kinase

s.d.: Standard deviation

SEE: Superantigen E

siRNA: small interfering RNA

SLP-76: SH2 domain-containing leukocyte protein of 76 kDa

Sos: Son of sevenless

Smc1: Structural maintenance of chromosomes-1

SNX27: Syntaxin-27

STIM1: Stromal interaction molecule 1

TBK1: TANK binding kinase 1

TBS: Tris buffer saline

TCR: T Cell Receptor

TIRFm: Total Internal Reflection Fluorescence microscopy

VLA: Very Late Antigen

WB: Western Blot

ZAP70: ζ chain-associated protein kinase 70

Introduction

1. Introduction

Immunity constitutes a highly specialized system that protects the human body against pathogens and external threats. It is composed by several subtypes of cells, with different functions, as well as by an intricate communication network between them. The Immune System is formed mainly by two "branches": the innate immunity and the adaptive immunity (**Figure I1**). The innate immunity acts as the first barrier of defense against external threats. It responds normally recognizing general patterns or molecules present in the infectious microorganisms. Moreover, it also acts very fast counteracting the pathogens within the earliest times upon infection. Additionally, innate immunity activates the adaptive immune response.

The adaptive immunity, on the contrary, conducts its function at later times of the infection. However, it is highly specific, due to the clonal selection and proliferation only against particular antigens. Furthermore, it is responsible for the generation of a pool of memory cells that protect our body against a reinfection. Adaptive immunity axis relies mainly in the lymphocytes. B lymphocytes are responsible for the humoral immune response that depends on specific antibody generation. T cells, however, can contribute either to the regulation of the humoral immune response or directly to the cellular one.

T lymphocytes are composed by two main subsets: CD8+ and CD4+ T cells. CD8+ T cells participate in the cellular cytotoxic response, killing those cells from the host that have been infected by intracellular pathogens. Conversely, CD4+ T cells functions are more diverse and depend on the specific subset. They constitute an important link between the innate and adaptive immunity by enhancing the activation and recruitment of other leukocytes and therefore orchestrating the global response.

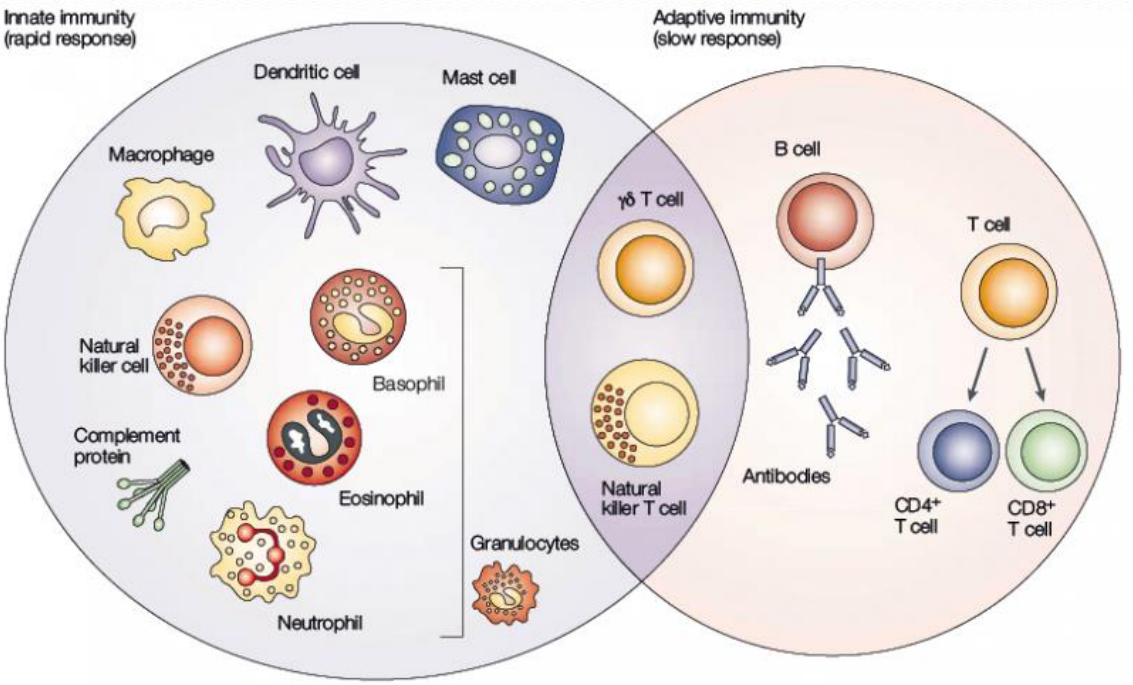

Figure I1. Immune system cell types. Innate immunity responds rapidly upon infection. It is formed by monocytes derived cells (dendritic cells and macrophages), mast cells, and granulocytes (basophils, eosinophils, neutrophils). Complement proteins are also an essential component of this type of immunity. The adaptive response is slower but more specific in its response against the pathogen. It is composed by both B lymphocytes and T lymphocytes. T lymphocytes are divided in two subsets of cells CD4+ (helper T cells) and CD8+ (cytotoxic T cells). Natural killer T cells (NK) and γδ T cells, due to their characteristics, form an intermediate group between innate and adaptive immunity cells. (Dranoff, 2004).

1.1 The Immune Synapse (IS)

Naïve T cell activation normally takes place initially in the lymph nodes or lymphoid associated organs through the formation of a structure called the immune synapse (IS). The IS is a highly specialized structure that is formed at the contact between a lymphocyte (either a B cell, a T cell or a NK cell) with the corresponding antigen presenting cell (APC). This interaction leads to a rearrangement of the

activatory molecules in different supramolecular aggregation clusters (SMACs) (Huppa & Davis, 2003). Although the triggering of the activation normally takes place in small microclusters, formed by the TCR and associated signalosome (Seminario & Bunnell, 2008), the maturation of this contact favors the congregation of these cell surface adhesion and signaling molecules into three described SMACs: central (cSMAC), peripheral (pSMAC) and distal (dSMAC) (Huppa & Davis, 2003) (**Figure I2**).

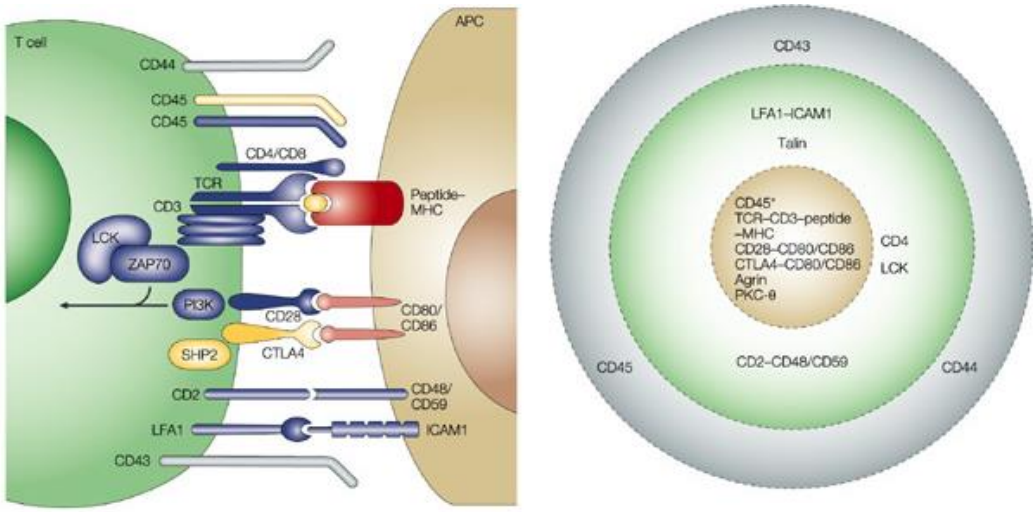

Figure I2. Immune Synapse structure. The IS is formed in the contact area between a T cell and an APC. It is constituted by three concentric SMACs. The cSMAC (*brown*) is composed by the TCR and correceptors like CD28. CD4/CD8 and CD2 are normally in the external limit of the cSMAC. cSMAC is also the place of aggregation of activation enhancers like PKC-θ and, later, of negative regulators like CTLA-4. The pSMAC (*green*) is formed by adhesion molecules like LFA1 and its linkers to the actin cytoskeleton (talin). Finally, the dSMAC (*grey*) is composed by glycosylated molecules like CD43 and CD45. CD45 is initially excluded to the dSMAC and later is concentrated in the cSMAC to negatively control T cell activation. (Huppa & Davis, 2003)

The cSMAC is mainly constituted by the T cell receptor (TCR) and the associated molecules (co-receptors) necessary for T cell activation like CD28, CD2, CD4 or CD8. The pSMAC, on the contrary, is formed by adhesion molecules like integrins (LFA1, VLA4) and also it constitutes the area where actin polymerization occurs (Huppa & Davis, 2003). This polymerization, in addition to myosin IIA function,

is necessary for the generation of a centripetal flux that enhances TCR displacement to the central region and the formation of the different SMACs (Ilani et al, 2009). The dSMAC refers to the region where molecules excluded from the IS are accumulated. This exclusion could be size dependent, for example due to the grade of glycosylation of the molecule (CD43), and also it is essential for the physical separation of the TCR from negative regulators like CD45 and other phosphatases (Huppa & Davis, 2003).

Although the precise role of the IS is still under debate, it is currently accepted that the IS acts as a mechanism of polarization that sustains TCR signaling more than triggering it. In fact, it has been suggested that the cSMAC is also a region for TCR degradation (Varma et al, 2006) and, therefore, that the balance between TCR recycling and degradation in this area promotes a fine tuned T cell activation. The other main function of the IS is to generate a polarized contact that redirects the traffic of cytokines and cytolytic granules to the cleft of the synapse. This way T cells polarize the secretion exclusively to the conjugated target cell avoiding "off-target" effects and also a dilution effect of the lytic molecules (Huse et al, 2008).

1.2 T cell receptor (TCR) signaling cascade

TCR triggering depends on the recognition by the variable region of the TCRαβ of a specific antigen bound to the major histocompatibility complex (MHC) of an APC. When the antigen is recognized and the stable binding established, CD3ε chain, which is associated to the TCR as CD3γε or CD3δε, undergoes a conformational change prior to any kind of phosphorylation (Gil et al, 2002). This conformational change leads to the exposition of a proline enriched region (PRS) that promotes the accumulation of Nck (Gil et al, 2002). The Nck recruited, then, associates with the SH2 domain-containing leukocyte protein of 76 kDa (SLP76) and with Vav controlling, downstream, the cascade responsible for actin polymerization (Bustos-Moran et al, 2016). In parallel, TCR engagement causes the exposure of CD3ζ immunoreceptor tyorisne-based activation motifs, ITAMs, which can be phosphorylated by the Src-family kinases Lck or Fyn. This phosphorylation allows the accumulation of proteins containing SH2-domains like the ζ-chain-associated protein kinase 70 (ZAP70). On the one hand, CD3ζ-ZAP70 binding favors the aggregation of CD4/CD8 closer to the TCR-MHC complex. CD4/CD8 establishes a lateral contact with the MHC (MHC class II in the case of CD4 or MHC class I for CD8) that stabilizes TCR-MHC contact and also enhances ITAM

phosphorylation, due to its intracellular binding to Lck (Gascoigne et al, 2011). On the other hand, when ZAP70 binds to the phosphorylated ITAMS, it is able to phosphorylate the linker of activated T cells protein (LAT). LAT is an adaptor protein that amplifies TCR signaling by promoting the accumulation of different molecules to the contact area. It is phosphorylated in several residues (Y191, Y171, Y226 and Y132) (Balagopalan et al, 2010). The combination of the different residues leads to the recruitment of several molecules. For example, Y191 and Y171 phosphorylation favors the stabilization in this area of SLP76 and other associated molecules like Gads, son of sevenless (Sos) or Grb1. Additionally, LAT Y132 phosphorylation allows the recruitment of phospholipase C gamma-1 (PLCγ1) and its phosphorylation by the interleukin-2-inducible T cell kinase (Itk) (Balagopalan et al, 2010). Once phosphorylated, PLCγ1 activates and catalyzes the transformation of the membrane phosphatidilinositol-4,5-biphosphate (PIP2) into inositol-1,4,5-triphophate (IP3) and diacylglycerol (DAG) (Bustos-Moran et al, 2016). On the one hand, the IP3 generated binds to specific receptors in the endoplasmic reticulum (ER) and induces the release of ER-stored calcium. Then, ER calcium release promotes the interaction of the stromal interaction molecule 1 (STIM1) with ORA1 membrane calcium channels and triggers calcium global wave. Eventually, calcium intracellular increase promotes the activation of the nuclear factor of activated T cells (NFAT), a transcriptional factor essential for interleukin-2 (IL2) generation and T cell proliferation (Kummerow et al, 2009). On the other hand, DAG activates protein kinases C (PKCs) (serine/threonine kinases). PKCs are important proteins both for centrosome polarization and cytoskeleton rearrangement (Quann et al, 2011) and to the activation of transcription factors like the nuclear factor-κB (NF-Kb) complex (Paul & Schaefer, 2013). PKC, in conjunction with DAG, activates protein kinase D-2 (PKD2), which acts as another amplifier of TCR cascade mediating the secretion of cytokines that promotes T cell function and proliferation like IL2 or interferon γ (IFNγ) (Navarro et al, 2014; Navarro et al, 2012). Additionally, DAG promotes the polarization and activation of Ras-GRP, a protein essential in the activation of Ras and the triggering of the mitogen-activated protein kinase (MAPK) cascade (Navarro & Cantrell, 2014). MAPK cascade results in the phosphorylation of the extracellular signal-regulated kinases (ERK1/2). Later ERK1/2 activate several transcription factors like ELK-1, SAP-1 and SAP-2, leading to the expression of genes such as c-Fos or Jun. Furthermore, ERK1/2 also phosphorylates MNK1 and MNK2,

which participate in the activation of the eukaryotic translation initiation factor eIF4-E needed for the anabolism necessary for the effector function and clonal expansion (Navarro & Cantrell, 2014) (**Figure I3**).

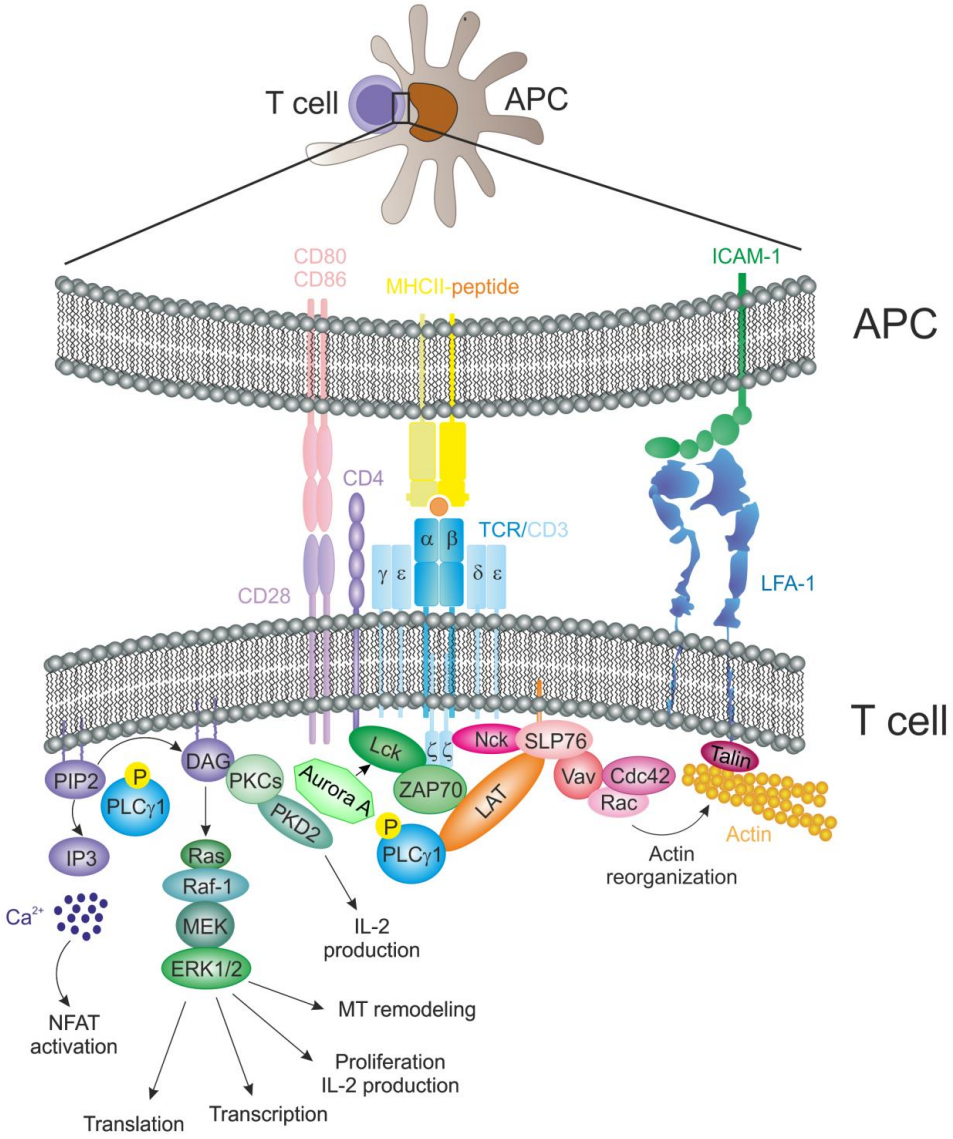

Figure I3. TCR signaling cascade. TCR recognition of the peptide bound to the corresponding MHC leads to the exposure of the ITAMs, which are phosphorylated by Lck. ITAMs phosphorylation allows the binding of ZAP70 (SH2 domains) which activates LAT. LAT adaptor protein amplifies the signal by recruiting PLCγ1, which generates IP3 and DAG as second messengers. While IP3 induces calcium wave and NFAT activation, DAG activates both PKCs and Ras, inducing PKD2 and MAPK pathways. Finally, Nck interaction with SLP76 and Vav promotes the recruitment of actin polymerization factors like Cdc42 or Rac at the pSMAC. (Bustos-Moran et al, 2016)

1.3 Microtubule organizing center (MTOC) polarization

One of the hallmarks of T cell activation is the rearrangement of the tubulin and actin cytoskeleton. Tubulin cytoskeleton reorganization at the IS depends mainly on the polarization of the MTOC towards the contact area (Martin-Cofreces et al, 2014). This polarization has been proved to be essential as reinforcement for T cell activation and sustained signaling (Martin-Cofreces et al, 2008). The reorientation of the MTOC also promotes the polarization of other important organelles for T cell proper activation such as mitochondria, Golgi apparatus or multivesicular body (MVB) (Martin-Cofreces et al, 2014). MTOC polarization leads to the polarized secretion of associated vesicles allowing a paracrine signaling of the cytokines secreted and a directional transmission of exosomes towards the APC. In fact, a proper reorientation of the centrosome is essential for the polarized secretion of the lytic granules towards the target cells, avoiding the "off-target" consequences of a non-polarized release (Huse et al, 2008; Martin-Cofreces et al, 2014).

The mechanism underlying MTOC polarization has been thoroughly studied in the literature. However, some details in the mechanism still remain unclear. One of the main factors involved in MTOC reorientation is DAG production (Quann et al, 2009). During early TCR activation DAG is produced and accumulated at the contact area with the APC. This favors the recruitment of proteins containing DAG-responsive domains like PKCs (Quann et al, 2011). Among the family of PKCs, there are three subsets: conventional PKCs (cPKCs), which are DAG and calcium dependent, novel PKCs (nPKCs), which are only dependent on DAG and, finally, atypical PKCs (aPKCs), which depend neither on calcium nor on DAG. Since calcium signaling is not essential for MTOC polarization, most of the studies have focused on nPKCs (Quann et al, 2011). When DAG is accumulated at the IS, three of the PKCs are recruited: PKCε, PKCη and PKCθ. PKCε and PKCη are firstly recruited to the IS, due to their higher affinity for DAG, and they are responsible for the posterior recruitment of PKCθ. Both PKCε and PKCη have overlapping functions since only the double knockout mice rendered a defect in MTOC polarization. Once recruited to the IS PKCs have been observed to promote the proper localization of dynein and non-muscle myosin IIA,

regulating by this way the polarization of the centrosome (Huse et al, 2013; Quann et al, 2011) (**Figure I4**).

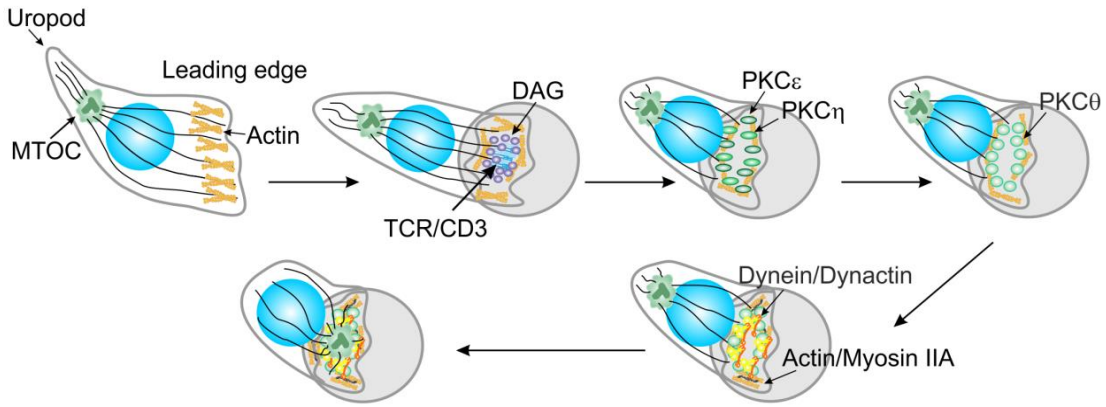

Figure I4. MTOC polarization mechanism. Initially, a migrating T cell has the MTOC placed at a structure known as uropod. T cell engagement with the corresponding APC enhances the production of DAG. This leads to the recruitment of PKCε and PKCη to the contact area. The recruitment of both nPKCs promotes the accumulation of PKCθ at the IS. Then, clustered PKCs regulate, by phosphorylation, the proper localization and activity of dynein/dynactin complex and myosin IIA, which generate the forces necessary for MTOC polarization. Modified from (Bustos-Moran et al, 2016)

The two principal molecular motors underlying MTOC polarization are dynein complex and myosin IIA. Dynein complex is a multimeric complex composed by two heavy chains that bind to MTs and contain the motor domain, and two light chains that stabilize the complex and allow further interactions. Dynein complex is associated with dynactin, a multisubunit complex that enhances its processivity (Kardon & Vale, 2009). The docking of the dynein/dynactin complex at the IS is favored by the adhesion and degranulation promoting adapter protein (ADAP). This protein clusters at the pSMAC in association with the integrin ring (Combs et al, 2006). ADAP is able to bind both to dynein complex and to MTs at the same time, generating the tension along the MT necessary to attract the MTOC and dock it to the contact area (Combs et al, 2006). Although dynein-dependent mechanism has been suggested to not be essential, since dyenin depletion or inhibition in mouse cells was not enough to block MTOC

polarization (Hashimoto-Tane et al, 2011), other studies detected a complete blockade of MTOC polarization when dynein was knocked-down or blocked with p50-dynamitin overexpression (Martin-Cofreces et al, 2008).

The other molecular motor involved in MTOC polarization seems to be myosin IIA. Chemical inhibition of this protein reduced modestly MTOC polarization. Strikingly, the combination of myosin IIA inhibition and dynein complex depletion rendered a much higher effect on MTOC translocation (Liu et al, 2013). Based on this, a model has been proposed for MTOC polarization suggesting that dynein complex would dock to the pSMAC, due to its interaction with ADAP, and attract the MTOC by pulling from the MTs. Meanwhile a pool of myosin IIA, placed at the opposite site, will push the centrosome from behind towards the contact area. In parallel, DAG production and nPKCs activity would control by phosphorylation the action and localization of both molecular motors to promote MTOC proper translocation (Huse et al, 2013).

Finally, in accordance with the idea that MTOC polarization is a reinforcement of TCR signaling instead of one of its triggering signals, it has been proved that either ITAM phosphorylation (Lowin-Kropf et al, 1998) or LAT and SLP-76 activation are essential for a proper polarization of the MTOC (Kuhne et al, 2003). Accordingly, Lck has been seen to be more important to maintain the MTOC docked to the IS area than to its complete polarization per se (Tsun et al, 2011), while Fyn, another Src-family protein functionally similar to Lck, impairs MTOC polarization when blocked (Martin-Cofreces et al, 2006). Therefore, TCR proper triggering is necessary, in addition to DAG signal, for MTOC timely translocation and docking.

1.4 Microtubule associated protein-4 (MAP4) structure

Microtubule associated proteins (MAPs) are proteins that bind to the MTs regulating their stability and growth by several mechanisms: controlling the rate of growth/shrinkage, the frequency of rescue events or some MT posttranslational modifications (PTMs) (Bowne-Anderson et al, 2015). Microtubule associated protein-4 is a protein firstly described in HeLa cells as protein bound to the microtubules with a Mw of 210 kDa (Bulinski & Borisy, 1980a). Later, it was demonstrated that MAP4 was expressed along different kind of cellular tissues, being considered as an ubiquous MAP in mammal proliferating and differentiated cells (Bulinski & Borisy, 1980b; Parysek et

al, 1984). MAP4 protein is encoded by one single gene that generates several isoforms due to alternative splicing (West et al, 1991).

The structure of MAP4 contains two acidic regions separated by a long basic domain responsible for the binding of MAP4 to the microtubules by electrostatic forces. The N-terminal region is composed by an acidic conserved domain consisting of a variable number of 14-aminoacid degenerated repeats, with KDM as consensus sequence. The function of this domain is not well known, but, according to its structure (flexible), it could act as a projection domain. This acidic region is followed by a basic domain (a third of the protein) containing both a serine-proline (S-P) rich domain, which it is thought to stabilize the binding to the MTs, and also a PGGG MT-binding domain, which is conserved along other MAPs like tau or MAP2. Finally, the C-terminal region is also well conserved and formed by acidic amino acids (West et al, 1991) **(Figure I5)**.

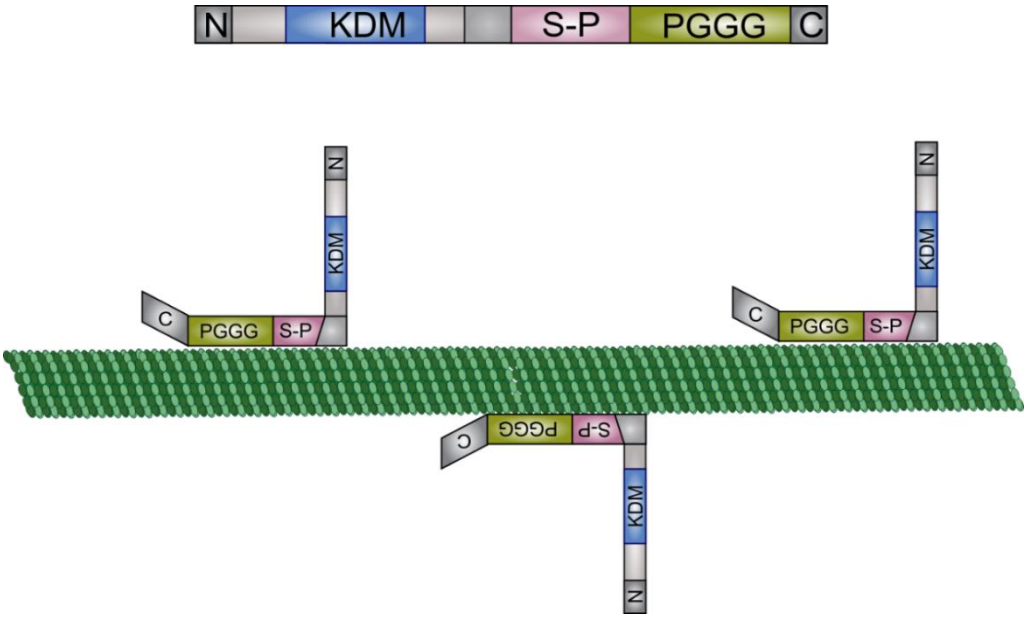

Figure I5. MAP4 structure and domains. MAP4 structure is formed by a basic region delimited by two extreme acidic regions. The N terminal domain is formed by an acidic region constituted by the repetition of the KDM motif. The C terminal domain is also acidic and well conserved. Finally, the basic region is responsible for the binding to the MTs. It is formed by a proline enriched domain (S-P) followed by a variable number of repetitions of the tau and MAP2 homolog PGGG domain. MAP4 is bound to the MTs by the basic domain, while the N-terminal acidic domain is projected outside.

A study with chimeras of the different MAP4 domains fused to GFP revealed that both the S-P rich domain and the PGGG repetition are necessary for a proper binding to the MTs. (Olson et al, 1995). Conversely, the N-terminal acidic domain and the conserved C-terminal acidic region would modulate the strength of the binding due to the negative charges (Iida et al, 2002; Olson et al, 1995).

In vitro characterization of the functions of MAP4 in MT stability generated, at first, some kind of controversy. Two studies, one overexpressing MAP4 (Barlow et al, 1994) and another one blocking its function by microinjection of anti-MAP4 antibodies (Wang et al, 1996) showed non-detectable effect on MT stability or assembly. Strikingly, a parallel study of overexpression of either the full length MAP4 or some of their domains rendered a change on MT stability (Olson et al, 1995). Accordingly, two studies with different approaches (MAP4 overexpression or silencing) agreed that MAP4 strongly regulates both MT stability and assembly by favoring the tubulin polymer fraction and regulating some posttranslational modifications like tubulin detyrosination (Nguyen et al, 1997; Nguyen et al, 1999).

1.5 MAP4 physiological functions

MAP4 has been studied in many physiological processes related with cell polarization and microtubule reorganization. The majority of the studies have been conducted in cell division since this system has a high impact on microtubule rearrangement for mitotic spindle formation. A first study described the phosphorylation of MAP4 in two residues (Ser 696 and Ser 787) during cell division by cyclin B-cdc2 complex affecting to cell division progression (Ookata et al, 1997). Moreover, mutation of these two residues leads to defects in mitotic cycle progression due to a delay in G2/M phase (Chang et al, 2001). Accordingly, depletion of *Xenopus* MAP4 homolog (XMAP4) in blastomeres caused disruptions in the assembly of mitotic spindles (Shiina & Tsukita, 1999). Finally, a molecular study unveiled the mechanism underlying these defects (Samora et al, 2011). In this work, the authors proved that MAP4 depletion generates defects in the M-plaque formation and proper alignment of the chromosomes, as well as an improper z-orientation of the spindle pole and morphology. Samora et al., demonstrated that MAP4 interaction with molecular motors (like dynein complex), at

the cortical microtubules, controls proper mitotic spindle orientation. MAP4 absence allowed an enhancement of cortical forces that disrupted spindle orientation, blocked cell cycle progression and explained the defects observed in cell division in other systems (Samora et al, 2011). (**Figure I6**).

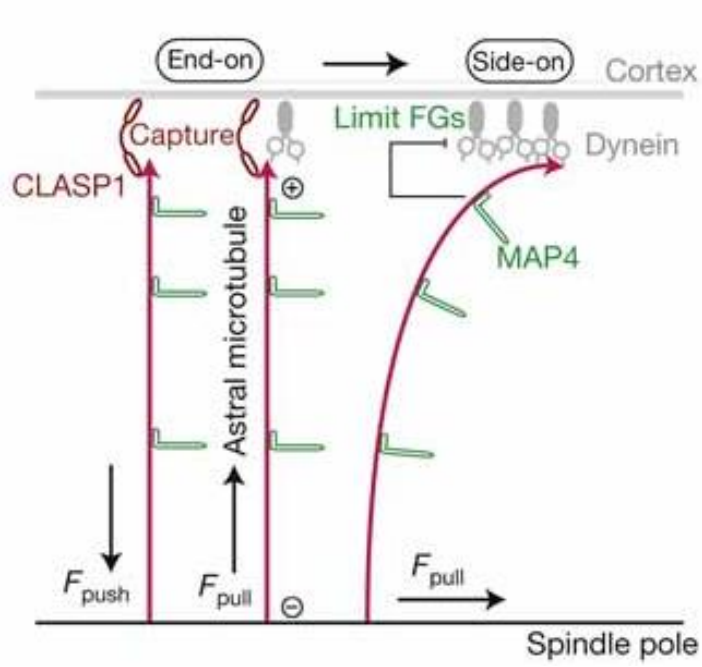

Figure I6. MAP4 regulation on cell division. During mitotic spindle formation astral microtubules are first attached to the cortex through the interaction of CLASP1 with cortical proteins. Then, force generation (FG) by dynein molecular motors pull the microtubules changing from an End-interaction to a Side-interaction. This side-interaction causes a limitation of the forces generated since dynein gliding is reduced due to MAP4 MT decoration. In short, MAP4 presence limits side MT FGs, providing a proper mitotic spindle orientation. Modified from (Samora et al, 2011)

In line with this, MAP4 has a role in vesicle movement by affecting to the gliding of molecular motors. Analysis of MAP4 overexpression has shown defects in the recycling pathway of some molecules as well as in the anterograde transport of Golgi Apparatus and lysosomal movement. This reduction in vesicle displacement is caused either by a steric impediment for a proper gliding of the motor MAPs along the MT or by a competition for the binding sites, but not by changes in MT stability

(Bulinski et al, 1997). Moreover, XMAP4 overexpression in Xenopus melanophores rendered a defect in the vesicle transport mediated by dyneins (towards the minus end) while favouring the movement of kinesin-dependent granules (towards plus end), by interacting with $p150^{Glued}$ and increasing kinesin processivity (Semenova et al, 2014). In accordance, it has been shown that MAP4 plays a role in the perinuclear transport of butyrophilin-3 (BTN3A1), a factor that mediates type I interferon response by activating the complex formed by TANK-binding kinase 1 (TBK1) and interferon regulatory factor-3 (IRF3). In response to nucleic acid detection, MAP4 is phosphorylated (S696), leading to the disruption of its interaction with the MTs and BTN3A1-TBK1 complex. This allows BTN3A1-TBK1 complex to bind to dynein and move towards the perniculear region, where it catalyses IRF3 phosphorylation and its consequent gene regulation (Seo et al, 2016).

Additionally, the role of MAP4 during primary cilia formation has been demonstrated, proving a competion with septin complex (septin 2, 7 and 9) for MT binding and blocking the elongation of the cilia (Ghossoub et al, 2013). Finally, two specific muscle MAP4 isoforms (mMAP4 and oMAP4) have been observed to be essential for the formation of the myotube and cell muscle differentiation participating in the rearrangement of the muscular MTs into a parallel array (Mangan & Olmsted, 1996; Mogessie et al, 2015).

Furthermore, due to its regulation over MTs and molecular motors, MAP4 has also been studied in some pathologies involving the tubulin cytoskeleton such as HIV infection (Gallo & Hope, 2012), cardiac hypertrophy (Cheng et al, 2010; Chinnakkannu et al, 2010), acute lung injury inflammation (Li et al, 2015) and bladder cancer metastasis (Ou et al, 2014).

1.6 MAP4 regulation by posttranslational modifications (PTMs)

Similarly to other MAPs, MAP4 binding affinity to the MTs is mainly regulated by phosphorylation. The addition of a negative charge from the phosphate to the basic region reduces the electrostatic forces responsible for the binding to the MT. MAP4 phosphorylation has been thoroughly studied along the literature. Several phosphorylation sites have been detected by mass spectrometry (mainly serines and threonines) according to Phosphosite data base (Hornbeck et al, 2015). However, just a

few sites have been tested by mutagenesis or complementary approach. S696 and S787 have been studied in the context of cell division and inflammation (Chang et al, 2001; Li et al, 2015; Seo et al, 2016), while feline MAP4 S924 and S1056 (corresponding to human S941 and S1073, respectively) phophorylations have proved to be enhanced in cardiac hypertrophy (Chinnakkannu et al, 2010). In accordance, the specific kinase responsible for MAP4 phosphorylation varies depending on the system. Several kinases like MARK2 (Cheng et al, 2010; Ebneth et al, 1999), cyclin B/cdc2 kinase complex (Ookata et al, 1997) or protein kinase A (PKA) (Ou et al, 2014) have been observed to phosphorylate MAP4 in different context. Therefore, MAP4 phosphorylation seems to be highly variable depending on the system studied. Additionally, a recent study has suggested a regulation of MAP4 by acetylation. Several residues within MAP4 sequence have been detected to be acetylated and this has been correlated with a reduction in MAP4 affinity for the MTs (Hwang et al, 2016).

Objectives

2. Objectives

MAP4 has been involved in several physiological processes, controlling MT stability and cell polarity. We postulate that MAP4 could be modulating T cell MT dynamics and therefore affecting to its proper activation.

In order to challenge our hypothesis, our aims were:

1. Analyze the localization of MAP4 during IS formation and its role in T cell microtubule stability and dynamics.

2. Assess the effect of MAP4 reduction on T cell early signaling and T cell effector function.

Materials and Methods

3. Materials and methods

3.1 Reagents and antibodies

Fibronectin (FN), poly-L-lys (PLL), dimethyl sulfoxide (DMSO), nocodazole (NCD) and methanol were from Sigma-Aldrich. *Staphylococcus aureus* enterotoxin E (SEE) from Toxin Technologies. Cell tracker CMAC (7-amino-4 chloromethylcoumarin; 0.1 µM), Fluo-3AM, Prolong Diamond anti-fade mounting medium and phalloidin conjugated to Alexa-568 or 647 (1:100) were from Invitrogen. GHOST Dye Violet 510 was from TOMBO Biosciences. Dual Luciferase Reporter Assay System was from Promega. Human IL2 (10 µg/ml) from Jackson ImmunoResearch. IC Fixation buffer was from eBiosciences. Paraformaldehide (PFA) was from Electron Microscopy Sciences. Mojosort Human CD4 T cell isolation kit was from Biolegend.

Antibodies used are indicated in the table below:

Primary Antibodies				
Antibody	**Host species**	**Specificity**	**Manufacturer**	**Application**
Anti-CD3ζ-pY83	Rabbit	CD3ζ-pY83	Abcam	WB
Anti-CD3ζ	Rabbit	CD3ζ	Abcam	WB
Anti-MAP4	Rabbit	MAP4	Abcam	WB and IF
Anti-LAT-pY132	Rabbit	LAT-pY132	Abcam	WB
Anti-LAT-pY191	Rabbit	LAT-pY191	Abcam	WB
Anti-α-Tubulin-FITC	Mouse	α-tubulin	Sigma-Aldrich	IF

Anti-α-Tubulin	Mouse	α-tubulin	Sigma-Aldrich	WB and IF
Anti-β-Actin	Mouse	β-Actin	Sigma-Aldrich	WB
Anti-PKCθ	Rabbit	PKCθ	BD-Pharmigen	WB
Anti-CD4 V450	Mouse	CD4	BD-Pharmigen	FACS
Anti-CD3ε V500	Mouse	CD3ε	BD-Pharmigen	FACS
Anti-Vβ8-FITC	Mouse	TCR (Vβ8)	BD-Pharmigen	FACS
Anti-human CD28	Mouse	CD28	BD-Pharmigen	Activation
Anti-ERK1/2-pT202/Y204	Rabbit	ERK1/2-pT202/Y204	Calbiochem	WB
Anti-p65	Rabbit	p65	Santa Cruz	WB
Anti-PKCθ	Goat	PKCθ	Santa Cruz	IF
anti-PKCθ-pT538	Rabbit	PKCθ-pT538	Cell Signalling	WB
anti-PLCγ1	Rabbit	PLCγ1	Cell Signalling	WB and IF
anti-PLCγ1-pY783	Rabbit	PLCγ1-pY783	Cell Signalling	WB
anti-ERK1/2	Mouse	ERK1/2	Cell Signalling	WB
anti-SMC1	Mouse	SMC1	Cell Signalling	WB
Anti-human CD3ε (HIT3α)	Mouse	CD3ε	Biolegend	Activation
Anti-GAPDH	Mouse	GAPDH	Biolegend	WB
Anti-CD19-Percp-Cy5.5	Mouse	CD19	Immunotools	FACS

Anti-CD69-FITC	Mouse	CD69	Immunotools	FACS
Anti-detyrosinated tubulin (Glu)	Rabbit	Detyrosinated (Glu) α-tubulin	Dr. M.A. Alonso	WB

Secondary Antibodies				
Antibody	**Host species**	**Specificity**	**Manufacturer**	**Application**
GAM-488/568/647	Goat	Mouse IgG	Invitrogen	IF
GAR-488/568/647	Goat	Rabbit IgG	Invitrogen	IF
DAG-488/568/647	Donkey	Goat IgG	Invitrogen	IF
GAM-HRP	Goat	Mouse IgG	Pierce	WB
GAR-HRP	Goat	Rabbit IgG	Pierce	WB
GAM	Goat	Mouse IgG	Jackson Laboratories	Activation

3.2 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 routinely and authenticated through specific surface markers. Cell lines were cultured in "complete medium" consisting of RPMI 1640 + GlutaMAX-I + 25 mM Hepes (Gibco-Invitrogen) supplemented with 10 % fetal bovine serum (FBS) (Hyclone, Thermofisher).

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors by separation on a Biocoll gradient (Biochrom) according to standard procedures. Monocytes were first separated from PBMCs by a 30 min adherence step in RPMI supplemented with 10% (FBS) at 37ºC. Non –adherent cells were obtained afterwards, and CD4+ cells were isolated with the Mojosort Human CD4 T cell isolation kit from Biolegend following manufacturer instructions. CD4+ T cells were checked after isolation by FACs to ensure a purity higher than 90%. To generate T cell lymphoblasts, cells were activated for 48 hours with anti-CD3ε and anti-CD28 (10 µg/ml and 8 µg/ml, respectively) coated plates. Then, cells were washed from the stimulus and grown in the same medium as cell lines but supplemented with human IL2 (50 U/ml). Lymphoblasts generated were used at day 7-8 post-activation. All 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.

3.3 Plasmids, siRNAs and transfection

Mouse GFP-MAP4 plasmid was a kind gift from Dr. Miguel Vicente (Olson et al, 1995); PKCθ-C1 domain fused to GFP or mCherry was provided by Dr. Isabel Mérida (Carrasco & Merida, 2004; Gharbi et al, 2011); NFAT (9x)-Luciferase was a gift from Dr. Juan Miguel Redondo (Wilkins & Molkentin, 2004) and NF-κB (5x)-Luciferase construction was kindly provided by Dr. Maria José Calzada. pRenilla-CMV plasmid was from Promega. CD3ζ-Cherry, C-term-AKAP450-GFP, HDAC6-GFP and tubulin-mCherry were described previously (Martin-Cofreces et al, 2012; Robles-Valero et al, 2010; Serrador et al, 2004; Vinopal et al, 2012).

T cells were transfected with a pool of two specific double-stranded siRNAs against human MAP4. (UAGGAGAGGAGAACCAGAU and CCAGAUUCU AUCCUCAUCU) or a scramble negative control (CGUACGGGAAUACUUCGA). Both were used at a final concentration of 3,5 µM.

For transfection, T cell lines were centrifuged 5 min at 1200 rpm and washed with HBSS (Hank´s balance salt solution; Lonza) and resuspended in Opti-Mem I (GIBCO-Invitrogen) in a cell concentration of $15x10^6$ cells per 400 µl. Plasmid was

added, at a concentration of 1.5 µg per 10^6 cells, and electroporation was performed with a gene-pulser III system from BioRad (240 mV, 975 mΩ). After transfection, cells were cultured in the medium described for cell lines. Experiments were performed 24 h post-transfection for DNA transfection and 72 h post-transfection for siRNA transfection.

3.4 T cell activation

For Jurkat T cell line antigen stimulation, Raji B cells were pulsed with 0.5 µg/ml of SEE for 30 min at 37ºC. Then, SEE was washed and Raji cells were mixed with Jurkat T cells (ratio 1:5). Cells were pulsed at low speed to favor the formation of conjugates and allowed them to activate at 37ºC for the indicated times.

For human T cell lymphoblast stimulation, cells were incubated either with anti-CD3ε and anti-CD28 (5 µg/ml and 3 µg/ml, respectively) coated plates at 37ºC or with the soluble antibodies crosslinked with a secondary antibody for the indicated times. In this late scenario, cells were pre-cooled at 4ºC for 20 min and, then, antibody mix was added and let to bind for 20 min at 4ºC. Later, cells were washed twice with cold HBSS and the hamster anti-mouse secondary antibody was added for 15 min at 4ºC. Finally, cells were warmed at 37ºC and activated for the indicated times.

3.5 Cell lysis, fractioning and immunoblotting

Cells were normally lysed for 20 min at 4ºC in 50 mM Tris-HCl pH 7.5 with 1% NP40, 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1.5 mM $MgCl_2$, and phosphatase and protease inhibitors. Lysates were spun at 21000 x g for 10 min at 4ºC to remove debris and nuclei. Supernatant was mixed with Laemli solution and β-mercaptoethanol (final concentration 0.15 M) and boiled for 10 min at 100ºC.

For nuclear/cytoplasmic fractioning cells were lysed at 4ºC in 10 mM Tris-HCl pH 7.5 wih NaCl 10 mM, MgCl2 3 mM, EGTA 0.1 mM and NP40 0.05%. Then, cells were spun at 650 x g for 15 min at 4ºC and supernatant was recovered as the cytoplasmic fraction and mix with Laemli solution and β-mercaptoethanol. Cell pellet

(nuclear fraction) was washed once with lysis buffer without NP40 and resuspended in Laemli solution and β-mercaptoethanol. Both fractions were boiled at 100ºC for 10 min.

For immunoblotting cells were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with TBS (Tris-buffered saline) containing 0.2% TWEEN and 5 % BSA, membranes were blocked with primary antibodies (O/N at 4 ºC) and peroxidase-labeled secondary antibodies (30 min RT) and detected with the ImageQuant LAS-4000 chemiluminiscence and fluorescence imaging system (Fujifilm).

3.6 Cell conjugate formation, immunofluorescence and IS analysis

Raji B cells were washed once with HBSS and loaded with the CMAC cell tracker (10 μM) and with SEE for 30 at 37 ºC. T cells (1.5×10^5 cells) were mixed with the APC (1:1) and plated onto Poly-L-Lys-coated slides (50 μg/ml; 1h at 37ºC). Cells were allowed to conjugate and settle for the indicated times at 37 °C, and then fixed. In the case of MAP4 staining, cells were fixed in 100% methanol 5 min at 4ºC, washed and followed by PFA 4% 10 min at RT. In the rest of stainings, cells were fixed with 2% paraformaldehyde and 0.12mM sucrose in PHEM (60 mM PIPES, 25 mMHepes, 5 mM EGTA, 2 mM $MgCl_2$), and permeabilized for 5 min at room temperature with 0.2% Triton X-100 in immunofluorescence solution (PHEM containing 3% BSA, 100 μg/ml γ-globulin and 0.2% azide). Cells were blocked for 30 min with immunofluorescence solution and stained with the indicated primary antibodies, followed by Alexa Fluor 488, 568 or 647-labeled secondary antibodies, alexa-conjugated phalloidin (5 μg/ml) or FITC-conjugated anti-α-tubulin (0.1 μg/ml). Cells were mounted on Prolong Diamond and analyzed with a Leica SP5 confocal microscope (Leica) fitted with a HCX PL APO 40x or 63x oil objective.

Images were processed and assembled using Image J software (http://rsbweb.nih.gov/ij/) and Photoshop software. For quantification in individual ISs, we used a home-made plugin for Image J software (http://rsbweb.nih.gov/ij/) called '*Synapse Measures*'. By comparing fluorescence signals from multiple regions of the T cell, APC, IS, and background fluorescence, the program yields accurate measurements of localized immunofluorescence. A detailed description of *Synapse Measures*, including the algorithms used, is described (Calabia-Linares et al, 2011).

3.7 Nocodazole treatment

Jurkat T cells were treated with the vehicle (DMSO) or nocodazole (8 µM) for 1h, and then washed twice with HBSS at RT. Finally, cells were plated in complete medium and let to recover at 37ºC for 1.5 h. The last 30 min of recovery, cells were settled in Poly-L-Lys-coated slides and then fixed as described.

3.8 Time-lapse confocal and total internal reflection fluorescence (TIRF) video microscopy

For time-lapse confocal video microscopy Raji B cells were used as APCs ($5x10^5$). Once SEE-pulsed or unpulsed, they were allowed to adhere to fibronectin-coated coverslips in Attofluor open chambers (Molecular Probes-Invitrogen) at 37 ºC in a 5% CO2 atmosphere. The cells were maintained in 1 ml HBSS (1% fetal bovine serum, 25 mM HEPES). T cells were added (1:1 ratio) and a series of fluorescence and differential interference contrast frames were captured using a TCS SP5 confocal laser scanning unit attached to an inverted epifluorescence microscope (DMI6000) fitted with an HCX PL APO 63x/1.40-0.6 oil objective. Images were acquired and processed with the accompanying confocal software (LCS; Leica) and Image J software (http://rsbweb.nih.gov/ij/).

For TIRF microscopy (TIRFm), T cells transfected with CD3ξ-mCherry were allowed to settle onto glass bottom dishes (No 1.5 Mattek; Ashland, MA, US) coated with anti-CD3 (10 µg/ml) and anti-CD28 (8 µg/ml). Recording was initiated 5 min after cells were plated, and cells were visualized with a Leica AM TIRF MC M system mounted on a Leica DMI 6000B microscope coupled to an Andor-DU8285_VP-4094 camera fitted with a HCX PL APO 100.0x1.46 OIL objective. Images were processed with the accompanying confocal software (LCS; Leica). The laser penetrance used was 200 nm for laser of 561 nm, using the same objective angle. Time-lapse settings were optimized for each type of experiment and are specified throughout the text. Synchronization was performed with the accompanying Leica software, and images were processed with Imaris, matlab and Image J software (http://rsbweb.nih.gov/ij/).

3.9 Quantitative real-time PCR (qPCR)

RT-PCR was performed with 1 µg of RNA isolated with Trizol RNA reagent (Invitrogen) from Jurkat T cells. mRNA levels of *IL2* and *CD69* genes were measured by triplicate using the Power SYBR Green PCR master mix from Applied Biosystems. Expression levels were normalized to the expression of glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) or *Actb (actin)* as indicated.

Primer sequences are listed below:

Primer sequence		
Gene	**Forward primer 5´-3´**	**Reverse primer 3´-5**
IL2	AAGTTTTACATGCCCAAGAAGG	AAGTGAAAGTTTTTGCTTTGAGCTA
CD69	CAAGTTCCTGTCCTGTGTGC	GAGAATGTGTATTGGCCTGGA
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
Actb	CCAACCCGCGAGAAGATGA	CCAGAGGCGTCAAGGGATAG

3.10 ELISA and flow cytometry (FACs)

Jurkat T cells were co-cultured with SEE-pulsed Raji B cells (1:1) for 24 h. For primary T cell lymphoblasts, cells were stimulated with anti-CD3 and anti-CD28 coated plates. Cells and supernatant were recovered after 24 h (Jurkat) or 16 h (primary T cells). Cells were used for CD69 flow cytometry analysis and supernatant was used for IL2 detection by ELISA (DyaClone) following manufacturer´s instructions.

For FACs analysis, cells were first blocked at 4ºC for 20 min in blocking solution [1% BSA, 1% FBS, 100 µg/ml γ-globulin and 0.02% sodium azide in phosphate saline buffer (PBS)]. Then, cells were stained with the corresponding primary conjugated antibody 30 min at 4ºC, washed twice with cold PBS and fixed in

IC Fixation Buffer (eBioescience) for 20 min at 4ºC. FACs analysis was conducted in a FACs Canto II cytometer (BD).

3.11 TCR internalization and recycling measurement

For TCR internalization measurement, Jurkat T cells were stimulated with anti-CD3ε (clone HIT3α) and CD28 antibodies-coated plates for the indicated times. Cells were then fixed as described, stained for CD3ε (clone UCHT1) and analyzed in the cytometer.

For recycling experiments, Jurkat T cells were stimulated with anti-CD3ε (clone HIT3α) and CD28 antibodies-coated plates for 60 min. Then, cells were washed twice in cold HBSS to remove the stimulus and plated in complete medium to allow the recycling. At the indicated times, cells were fixed and stained for CD3ε (clone UCHT1) and TCR (Vβ8) and analyzed in the cytometer.

3.12 Luciferase assay

Jurkat T cells were transfected with NFAT-Luciferase or NF-κB-Luciferase construction plus pRenilla-CMV plasmid (2 µg + 0.04 µg per 10^6 cells, respectively). After 24h, cells were activated with SEE-pulsed Raji B cells for another 24h. Then, cells were washed twice with HBSS and lysed with the lysis buffer from the commercial kit (Dual Luciferase Assay Reporter). Luciferase measurement was conducted following the kit provider´s instructions and using a Fluostar Omega luminometer (BMG Labtech). Measurements were normalized to Renilla levels and protein quantity (measured by BCA assay).

3.13 Calcium measurement

Cells were washed twice with HBSS, resuspended in calcium buffer (10 mM Hepes, 1% FBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$ in HBSS) and stained with Fluo-3AM (5 µM) 30 min at 37ºC. Then, cells were washed once with HBSS and resuspended again in calcium medium for their measurement at FACs. Cell basal fluorescence was recorded for 1 min and then commercial crosslinked anti-CD3 + anti-CD28 (StemCell) was used to stimulate the cells (following manufacturer's instructions). Calcium wave

was measured for 5 min and then ionomycin was added and recorded for 1 min as a positive control. Calcium measures were analyzed by FlowJo analyzing the fluorescence at the peak of the calcium wave or at the plateau and normalizing to the initial basal level of fluorescence.

3.14 Statistical analysis

First, data was analyzed with a ROUT test (Q = 1.00 %) to detect outliers. Then a Shapiro-Wilk normality test was applied to determine the application of parametric or non-parametric tests. Accordingly, a Student-t test (parametric) or U-Mann Whitney (non-parametric) analysis was used for pairs of non-dependent data. Krukal-Wallis test was used for non parametric grouped analysis. Finally, when samples compared were dependent samples a paired analysis was used; either paired t-test (parametric) or Wilcoxon test (non-parametric). All statistic analysis was performed with GaphPad Prism software.

Results

4. Results

4.1 MAP4 localization at the IS

To assess MAP4 localization in CD4+ T cells upon IS formation, MAP4 was immunostained in Jurkat T cells conjugated for 30 min with SEE-pulsed Raji B cells (SEE-APCs) **(Figure R1)**. When compared to α-tubulin staining, MAP4 distributed with a similar pattern, decorating both the MTOC and also the MTs in T cells. Surprisingly, non apparent changes were detected in MAP4 localization between activated *vs* non-activated T cells.

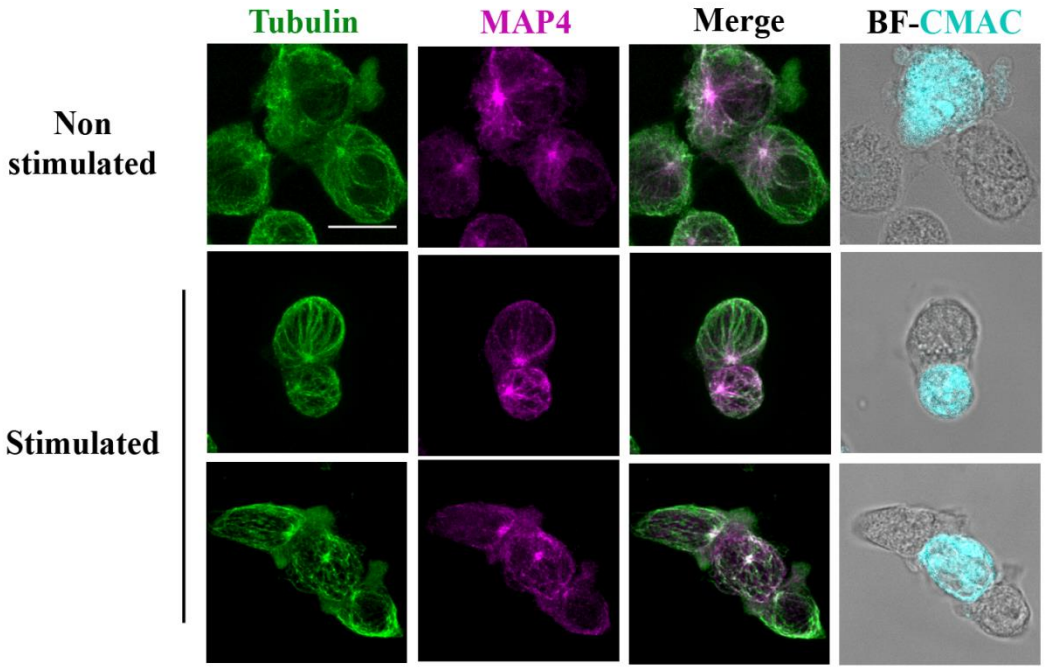

Figure 1. MAP4 decorates T cell MTOC and MTs. Maximal Z projection of a confocal stack of conjugates of Jurkat T cells with unpulsed or SEE-pulsed (two representatives images) Raji B cells (APCs; 30 min). Cells were fixed and stained for α-tubulin (green) and MAP4 (magenta). Bright field image with CMAC in cyan (APCs) is also shown. Scale bar of 10 µm.

Next, to analyze the dynamic localization of MAP4 upon T cell-APC contact formation, Jurkat T cells co-transfected with rodent MAP4 coupled to GFP and α-tubulin-mCherry were conjugated with SEE-APCs. Time-lapse confocal microscopy revealed a similar behavior for both proteins **(Figure R2 and Movie S1).** The results highlighted MTOC decoration of MAP4. In fact, MAP4 seems to accompany the centrosome along its translocation towards the contact area.

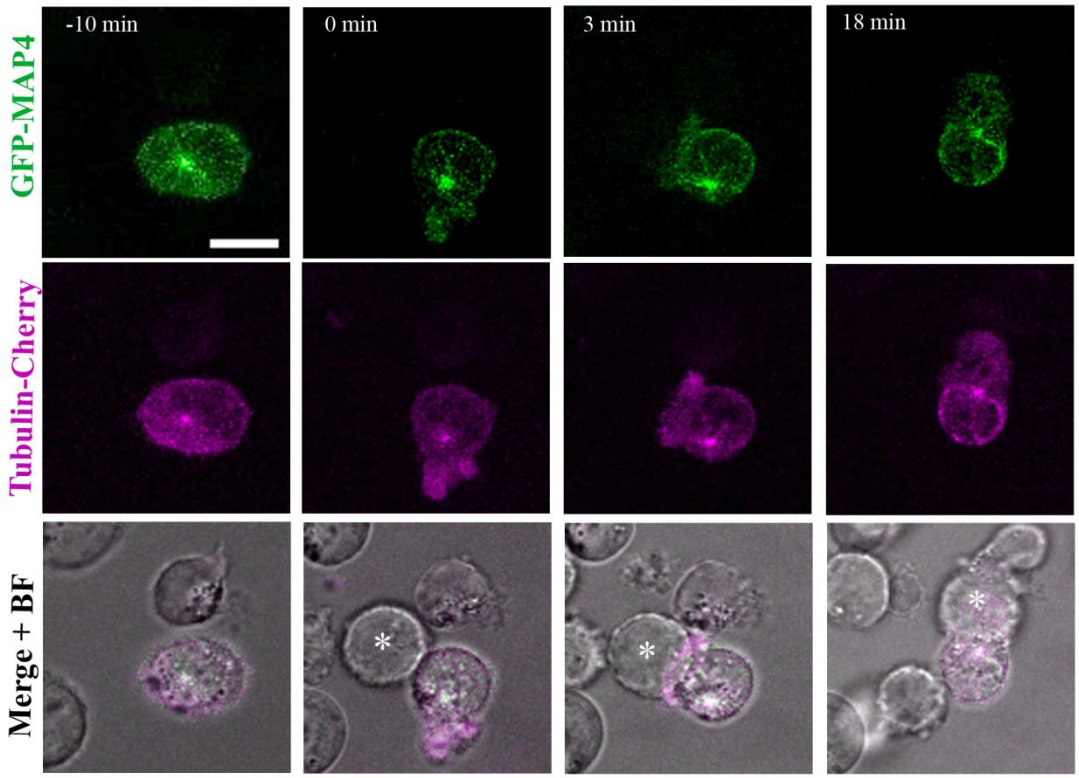

Figure R2. MAP4-GFP accompanies MTOC during its translocation. Time-lapse Z-stack maximal projection of Jurkat T cells transfected with GFP-MAP4 (green) plus Tubulin-Cherry (magenta) and activated with SEE-pulsed Raji B cells. Images were taken each 43 s. Bright field is also shown (* APC cell). Scale bar 10 µm.

These results prove that MAP4 is expressed in T cells and decorates T cell MTOC and microtubules during its activation.

4.2 MAP4 role on T cell MT dynamics and MTOC translocation

Since MAP4 seems to be mainly accumulated at T cell MTOC, we hypothesized that MAP4 could 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 (MAP4 KD) **(Figure R3A)**. First, T cells were treated with nocodazole (8 µM) or vehicle (DMSO) for 1 h to fully depolymerize MTs. Then, cells were washed twice and its MT re-assembly capacity was analyzed after 1.5 h of recovery. While most control cells proved to be able to completely recover their MT array, the majority of MAP4 KD cells displayed their tubulin fully depolymerized, showing a ring-shaped concentration around the nucleus **(Figure R3B and C)**, indicating that MAP4 is required for the assembly of new MTs.

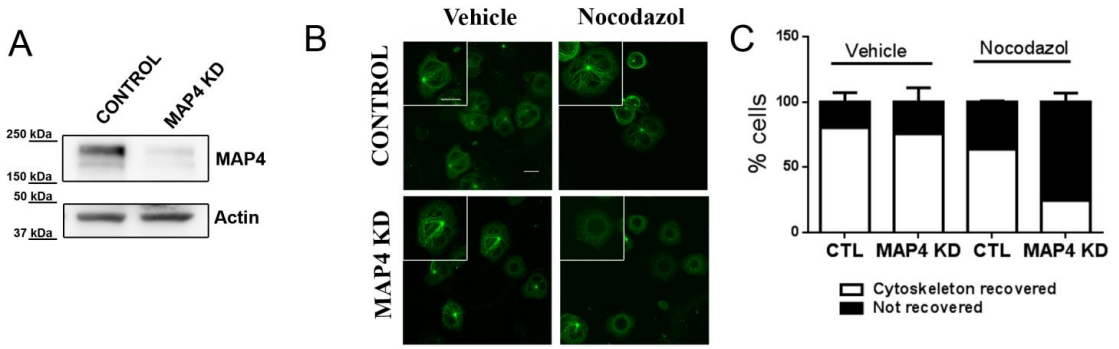

Figure R3. MAP4 KD T cells show a defect in MT assembly. (A) Immunoblot of MAP4 expression in scramble-transfected (control) and MAP4-silenced Jurkat T cells (MAP4 KD). Actin is shown as a loading control. **(B)** Maximal Z projection of confocal stacks showing the recovery of MTs in MAP4 KD cells treated with vehicle (DMSO) or Nocodazole (8µM), washed and recovered for 1.5 h. Cells were fixed and stained for α-tubulin (green). The inset shows magnification of one cell. Scale bar of 10 µm. **(C)** Quantification of cells with recovered MTs from experiments as in (B) (at least 340 cells were counted from two different experiments). Graph represents Mean ± s.d.

Additionally, to study the role of MAP4 in MT stability in a more physiological context, we analyzed whether MAP4 participates in MT stabilization in response to TCR signals. α-tubulin de-tyrosination (Glu) was measured as a marker of MT stability upon TCR activation (Andres-Delgado et al, 2012). Interestingly, the increase of de-tyrosinated tubulin detected in control cells upon TCR triggering was prevented in MAP4 KD cells activated with SEE-pulsed APCs. **(Figure R4A and B).**

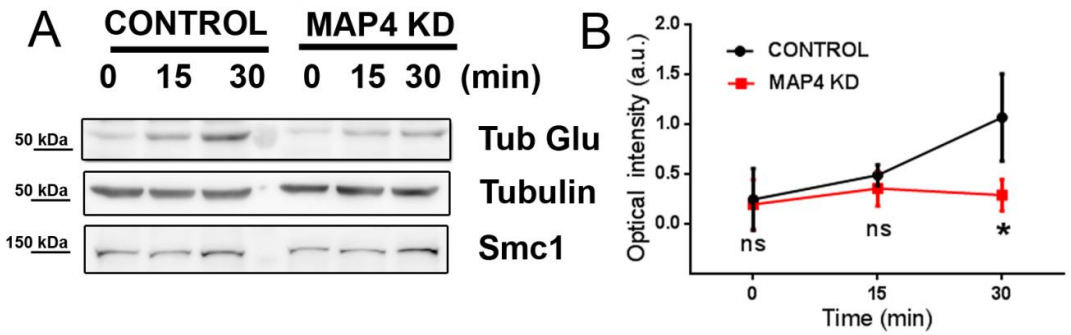

Figure R4. MAP4 controls T cell microtubule stability upon TCR activation. (A) Inmunoblot of de-tyrosinated tubulin (Glu) in control or MAP4 KD cells. Cells were activated with SEE-APCs for the indicated times. α-tubulin and Smc1 were used as loading controls. **(B)** Quantification of detyrosinated tubulin as in (A) (paired t-test; *,P-value < 0.05; n=4). Graph represents Mean ± s.d.

As a control, the percentage of conjugates formed by MAP4 KD cells was analyzed. Control and MAP4 KD Jurkat T cells were conjugated with SEE-pulsed Raji B cells for 30 min and the ratio between T cells conjugated over the whole number of T cells was measured. As shown in **Figure R5** no changes in the overall efficiency of conjugate formation were detected.

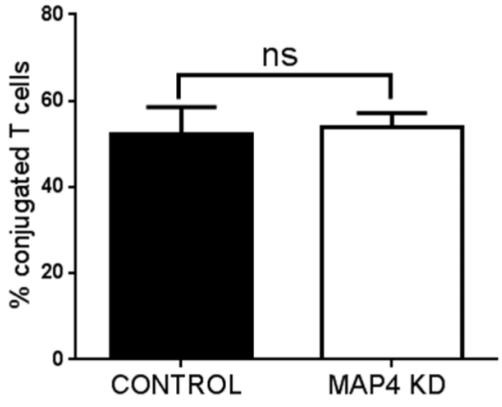

Figure R5. MAP4 deficiency does not affect to conjugate formation. Quantification of the percentage of control or MAP4 KD T cells conjugated with SEE-APCs for 30 min. T cells conjugated were counted and the number normalized to the whole number of T cells [1138 and 1146 cells, from three different experiments, Wilcoxon test, non-significant (ns)]. Graph represents Mean ± s.d.

MTOC reorientation is a hallmark of T cell activation during the formation of the IS that highly depends 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 MAP4 KD Jurkat T cells. A significant increase in the separation of the MTOC to the contact area was observed in MAP4 KD cells compared to control cells, especially shortly upon activation **(Figure R6A and B)**. This defect in MTOC polarization seems to be more a delay than a total impairment since the differences between control and MAP4 KD cells disappeared at longer time points **(Figure R6B)**.

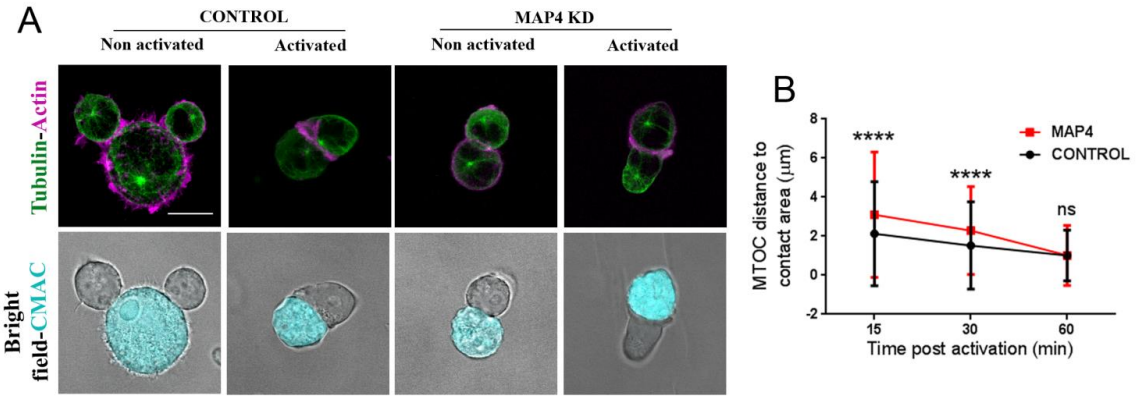

Figure R6. MAP4 controls a timely translocation of MTOC. (A) Maximal Z projection of confocal stacks showing MTOC translocation in control and MAP4 KD cells conjugated with unpulsed or SEE-APCs (30 min). Cells were fixed and immunostaining of α-tubulin (green) and β-actin (magenta) was performed. Bright field images including CMAC-labeled APCs (cyan) are shown. Scale bar of 10 µm. **(B)** Quantification of the distance of MTOC to the contact area (µm) as in the (A) for the indicated times [Mann-Whitney test; ****; P-value < 0.0001; Control n=216 (15 min), 196 (30 min), 95 (60 min); and MAP4 KD n=231 (15 min), 251 (30 min), 105 (60 min) from three different experiments]. Graph represents Mean ± s.d.

In order to exclude side effects of siRNA approach and to support the specific role of MAP4 in this process, MAP4 KD cells were reconstituted with the rodent GFP-MAP4 construction (not affected by the pool of siRNAs used); rescuing a proper MTOC translocation **(Figure R7)**.

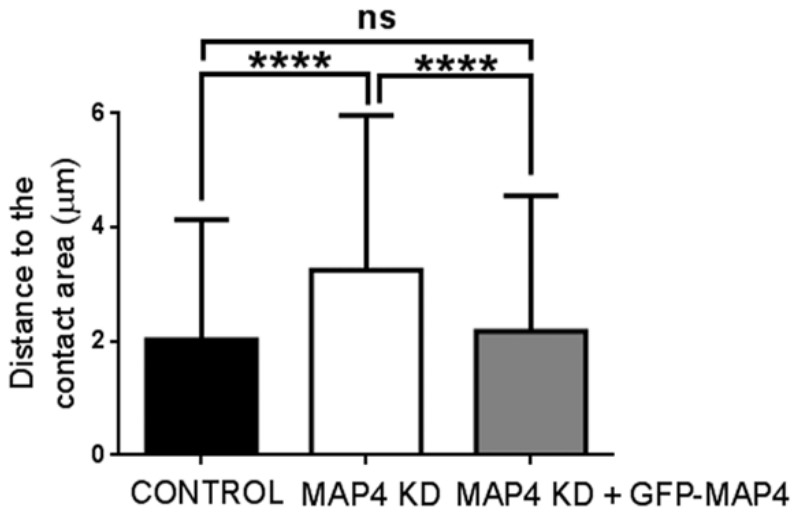

Figure R7. GFP-MAP4 rescues the defect in MTOC polarization of MAP4 KD cells. Quantification of the distance of MTOC to the contact area (µm) in control or MAP4 KD cells transfected with GFP versus MAP4 KD cells reconstituted with GFP-MAP4. [Kruskal-Wallis test;****; P-value < 0.0001; n= 201 (control), 216 (MAP4 KD), 165 (MAP4 KD + GFP-MAP4), from two independent experiments)]. Graph represents Mean ± s.d.

Together, these results indicate that MAP4 is an important mediator of MT assembly, most probably by controlling MT stability, in response to signals emanating from the IS. Consequently, MAP4 controls the timely translocation of the MTOC to the IS.

4.3 MAP4 effect on TCR early signaling pathway

In order to ascertain whether the defects observed in the tubulin cytoskeleton due to MAP4 decrease can lead to a change in the signaling at the T cell receptor level (TCR signalosomes), we studied the pattern of phosphorylation of TCR-downstream molecules such as CD3ζ, LAT, PKCθ and ERK, in control and MAP4 KD Jurkat T cells activated with SEE-pulsed Raji B cells **(Figure R8).** Immunoblot of these molecules and its quantitative analysis revealed a significant decrease in CD3ζ, LAT-Y191 and ERK 1/2 phosphorylation **(Figure R8A and B)**.

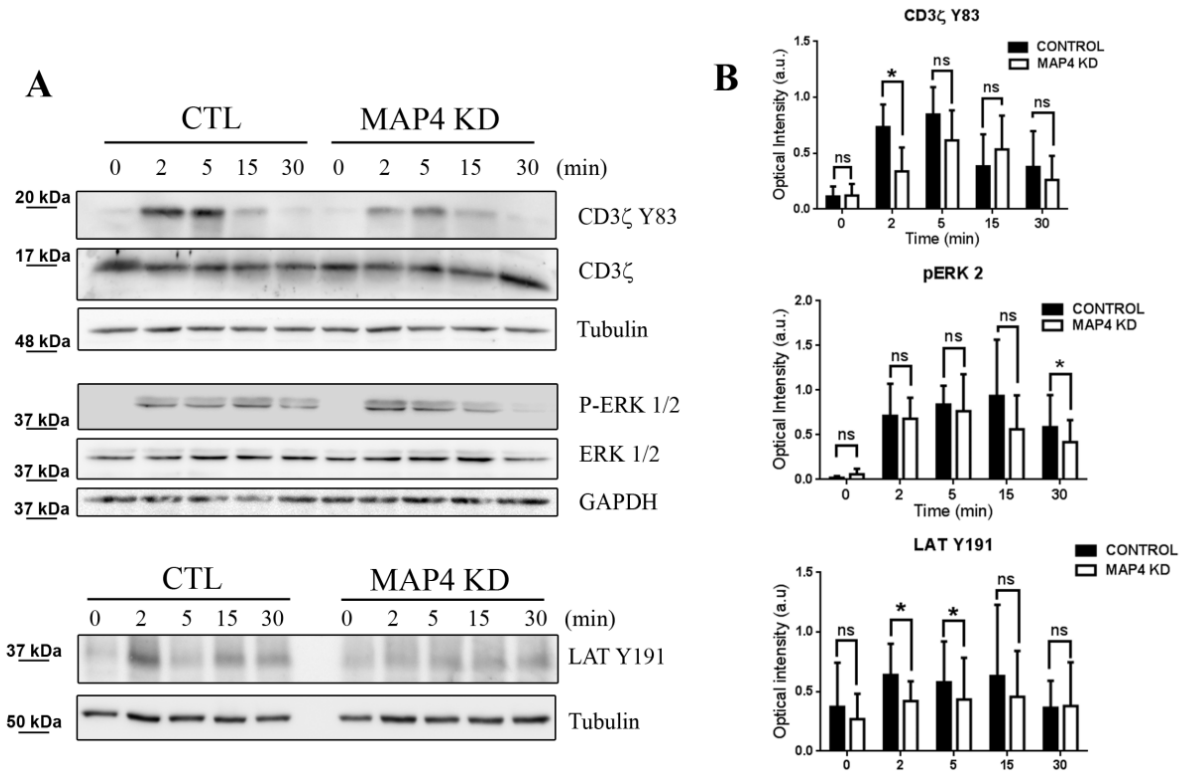

Figure R8. MAP4 sustains early T cell signaling. (A) Immunoblots showing phosphorylation of CD3ζ, ERK 1/2 and LAT Y191 in control or MAP4 KD Jurkat T cells activated with SEE-pulsed Raji B cells (SEE-APCs) for the indicated times. α-tubulin and GAPDH are shown as loading controls. **(B)** Quantification of CD3ζ, ERK2, LAT-Y191 phosphorylation as in (A) (n=6, 7, 6, respectively; paired t-test; *, P-value<0.05). Graphs represent Mean ± s.d.

Surprisingly, other phosphorylation residues triggered upon TCR activation like another LAT phosphorylation site (Y132) or T538 activatory phosphorylation of PKCθ rendered no apparent changes in activated MAP4 KD cells, as shown by the immunoblots and their corresponding quantification **(Figure R9A and B)**.

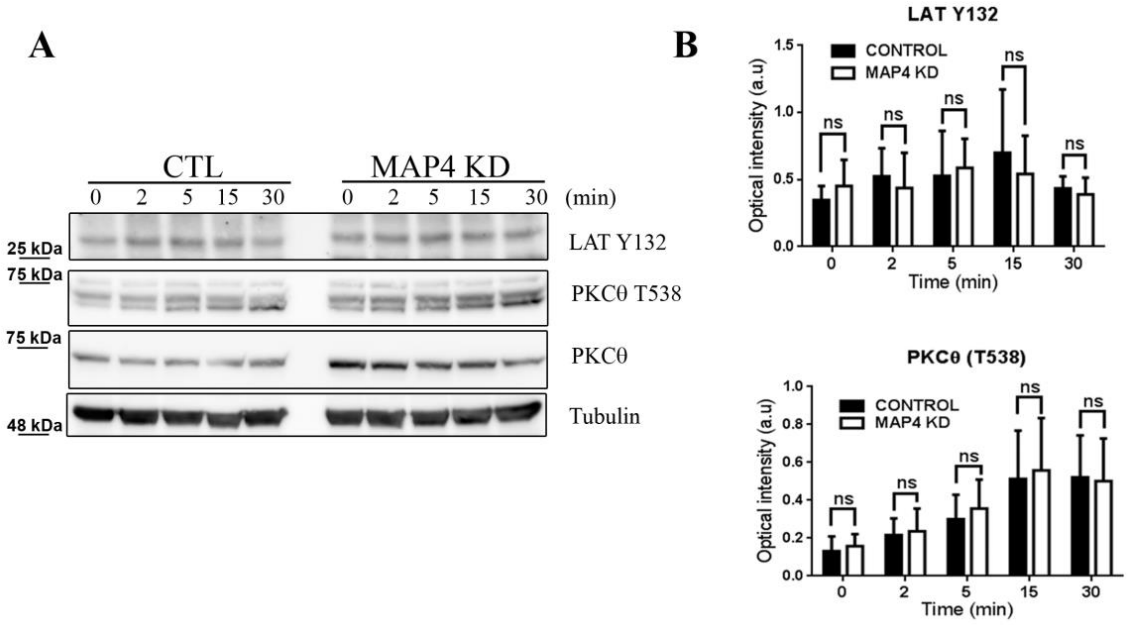

Figure R9. LAT-Y132 or PKCθ phosphorylation is not affected by MAP4 reduction. **(A)** Immunoblots showing phosphorylation of LAT (Y132) and PKCθ (T538) in control or MAP4 KD Jurkat T cells activated with SEE-pulsed Raji B cells (SEE-APCs) for the indicated times. α-tubulin is shown as a loading control. **(B)** Quantification of LAT -Y132 and PKCθ phosphorylation as in (A) (n= 4 and 5; paired-t test; ns) Graphs represent Mean ± s.d.

Additionally, to confirm the phosphorylation defects observed in MAP4 KD cells with another kind of stimulus, control or MAP4 KD T cells were activated for the indicated times with crosslinked anti-CD3 + anti-CD28 human monoclonal antibodies and CD3ζ Y83 phosphorylation was analyzed. This polarizing system generated similar results, showing also a reduction of CD3ζ phosphorylation in MAP4 KD cells **(Figure R10A and B)**.

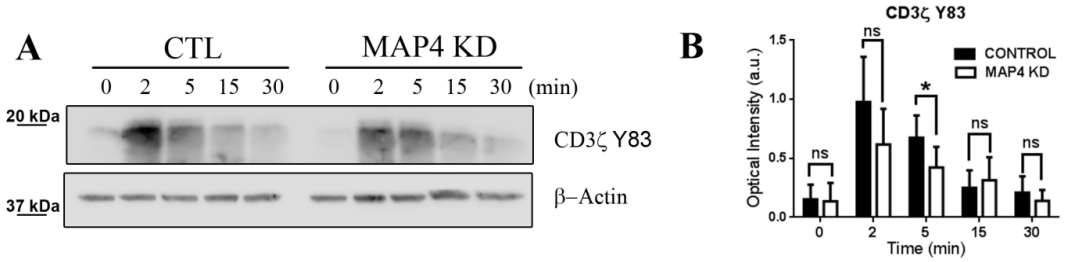

Figure R10. MAP4 also controls CD3ζ phosphorylation upon monoclonal antibody stimulation. (A) Immunoblot of CD3ζ phosphorylation in control or MAP4 KD cells activated with crosslinked anti-CD3 + anti-CD28 antibodies for the indicated times. β-Actin is shown as a loading control. **(B)** Quantification of CD3ζ phosphorylation as in (A) (n=6, paired t-test, *, p-value < 0.05). Graph represents Mean ± s.d.

As a control to rule out a possible effect of MAP4 knocking-down on the basal TCR/CD3 levels of the T cells transfected, TCR (Vβ8) and CD3ε surface levels were analyzed by flow cytometry **(Figure R11).** No significant differences were detected on the surface levels of both molecules between control and MAP4 KD cells.

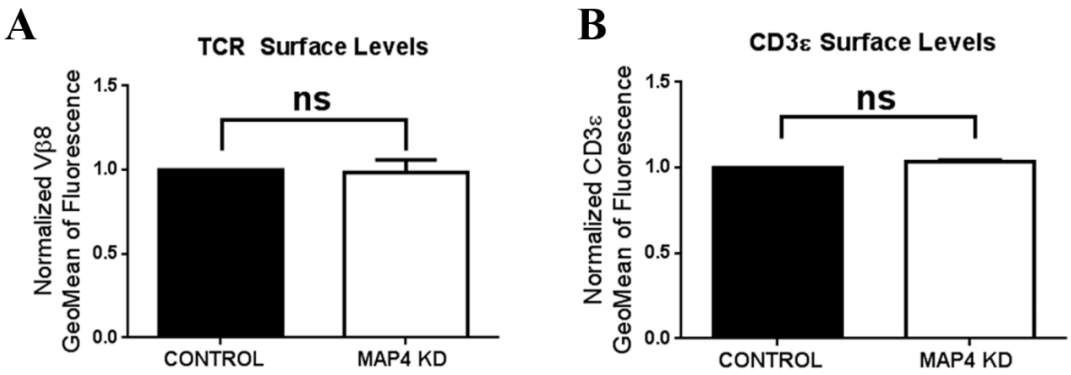

Figure R11. MAP4 KD has similar TCR/CD3 surface basal levels. Quantification by FACs of the surface basal levels of TCR (Vβ8) **(A)** and CD3ε **(B)** in control and MAP4 KD Jurkat T cells (n=3; Mann-Whitney test; ns). Graphs represent Mean ± s.d.

In short, MAP4 seems to act as a positive modulator of TCR signalosome by affecting to the pattern of phosphorylation of TCR-associated proteins, acting at different levels of the cascade.

4.4 MAP4 regulation over CD3ζ-bearing nanovesicle dynamics and TCR/CD3ζ membrane exchange

In order to assess the mechanism underlying MAP4 regulation on TCR signalosome, the dynamics of CD3ζ-bearing nanovesicles was analyzed. CD3ζ actively traffics through the endosomal compartments towards the IS (Yudushkin & Vale, 2010). This vesicle traffic is highly dependent on microtubule and sustains T cell activation at the IS (Martin-Cofreces et al, 2012; Soares et al, 2013). To analyze their dynamics, total internal reflection fluorescence microscopy (TIRFm) was used, since CD3ζ-bearing vesicles move within TIRFm range, and use the MTs as "trails" to displace. To this end, control and MAP4 KD Jurkat T cells transfected with CD3ζ-Cherry were settled and activated in a stimulatory surface coated with anti-CD3 + anti-CD28 antibodies. Images were taken every 100 ms with a laser penetrance of 200 nm and the trajectories of the vesicles were tracked **(Figure R12A, and Movie S2 and S3)**. The quantitative analysis of the vesicle tracks through IMARIS software showed a significant defect in the movement and displacement of the vesicles **(Figure R12A and B)**.

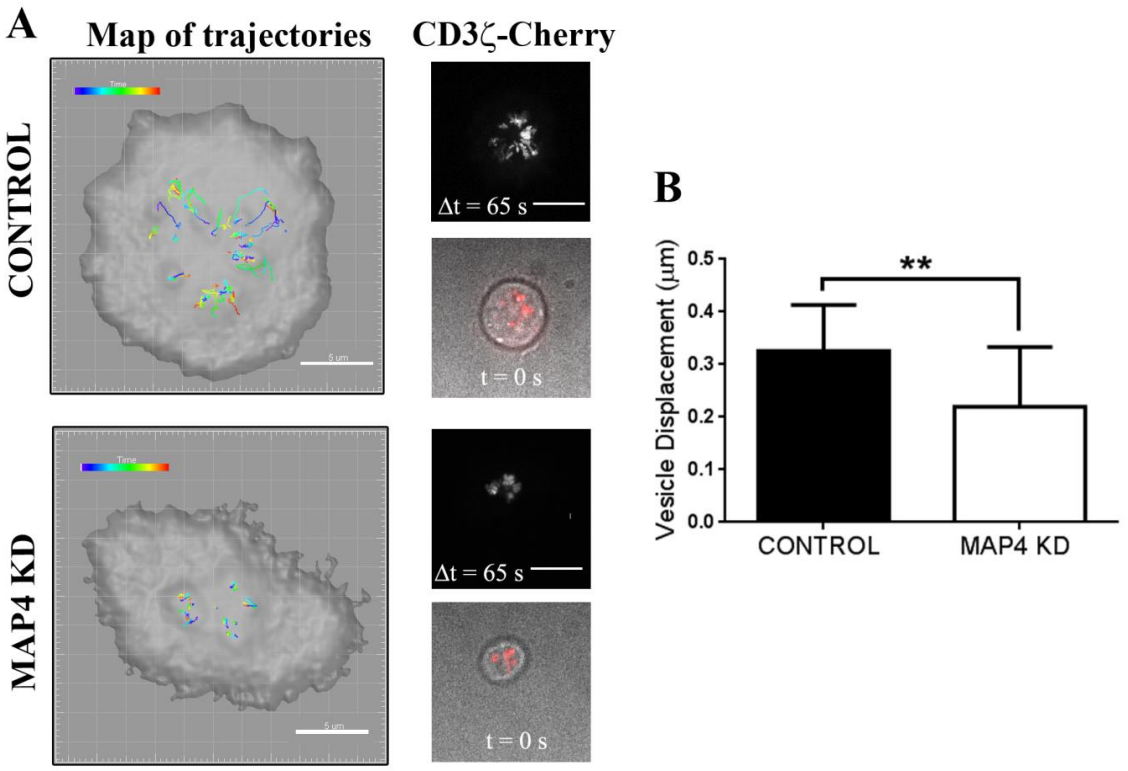

Figure R12. MAP4 blockades CD3ζ-bearing nanovesicle movement. (A) Map of trajectories of CD3ζ-mCherry-bearing vesicles in control and MAP4 KD cells spreading over anti-CD3/CD28-coated glass-bottom chambers. Images were taken every 100 ms under TIRF microscope with a 561 nm laser penetrance of 200 nm. Vesicles were tracked with Imaris software for 65 s and a maximal projection of the tracks is shown. A maximal projection of the fluorescent vesicles for time-lapse (Δt = 65 s) and an initial bright field image (t=0) are also shown. **(B)** Quantification of the displacement length (μm) as in (A) (n=16 and 17 from three independent experiments; Student t-test; **, P<0.01). Graph represents Mean ± s.d.

Since TCR sustained signal is also highly dependent on its cycle of degradation and recycling (Monjas et al, 2004), we decided to analyze the membrane exchange of CD3 and TCR molecules by analyzing its internalization and recycling rate. On the one hand, for internalization assay, control and MAP4 KD T cells were activated with anti-CD3 + anti-CD28 coated surfaces for the indicated times. Then, cells were fixed and

CD3ε surface levels were analyzed by FACs. Nevertheless, no significant differences were detected in terms of CD3ε internalization **(Figure R13)**.

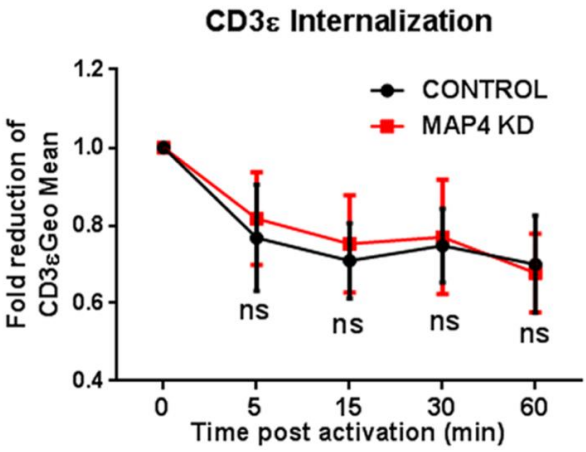

Figure R13. CD3ε internalization remains unaffected in MAP4 KD. Quantification by FACs of the rate of internalization by CD3ε surface levels in control and MAP4 KD Jurkat T cells activated with anti-CD3 + anti CD28 coated plates for the indicated times. [n=5; Wilcoxon test, non-significant (ns)]. Graph represents

On the other hand, in order to analyze TCR/CD3ε global recycling, control and MAP4 KD T cells were activated with anti-CD3 + anti-CD28 coated surfaces for 60 min. Then, stimulus was removed and cells were allowed to recycle for the indicated times. Finally, cells were fixed and TCR (Vβ8) and CD3ε surface levels were analyzed by FACs. However, no changes were observed in the general recycling rate in control and MAP4 KD cells upon activation **(Figure R14)**.

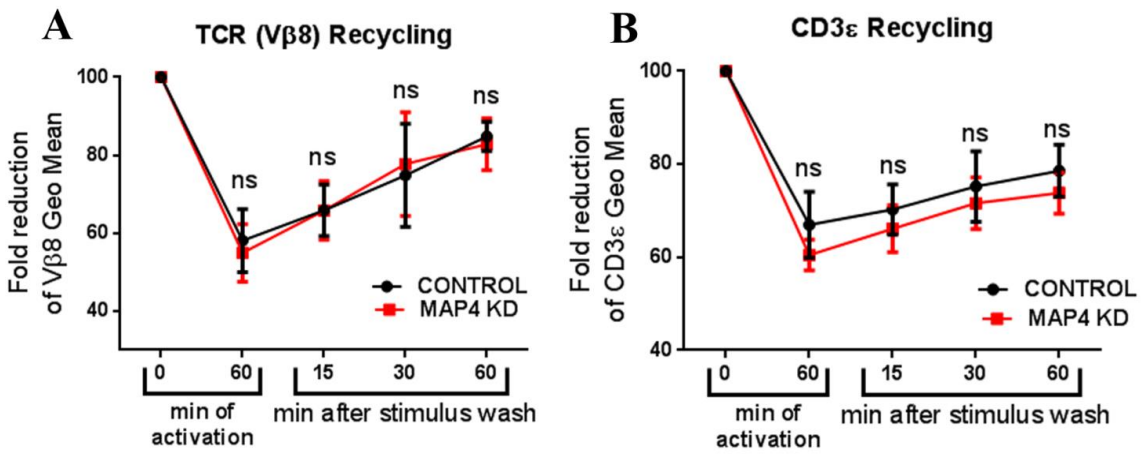

Figure R14. MAP4 does not affect to TCR/CD3 general recycling. Quantification by FACs of TCR (Vβ8) **(A)** and CD3ε **(B)** rate of recycling by surface levels of control and MAP4 KD Jurkat T cells activated with anti-CD3 + anti CD28 coated plates for 60 min and let to recycle for the indicated times. [n=5; paired t-test, non-significant (ns)]

These results suggest that MAP4 affects, more likely, to the polarized action of CD3ζ-bearing nanovesicles rather than to the whole TCR internalization/recycling rate, promoting the continuous supply of CD3ζ necessary to maintain TCR signaling.

4.5 MAP4 function in later activation markers and effector function

Since we had observed some defects in the phosphorylation cascade downstream the TCR, we wondered whether the expression pattern of genes controlled by T cell activation such as CD69 or IL2 could also be affected. To analyze mRNA pattern of expression of later activation markers, control or MAP4 KD Jurkat T cells were activated with SEE-pulsed APCs for 4h. Afterwards, whole mRNA was isolated and retro-transcribed. Quantitative analysis of IL2 and CD69 expression, by qPCR, rendered a significant increase in IL2 and CD69 mRNA expression in MAP4-silenced cells (**Figure R15**).

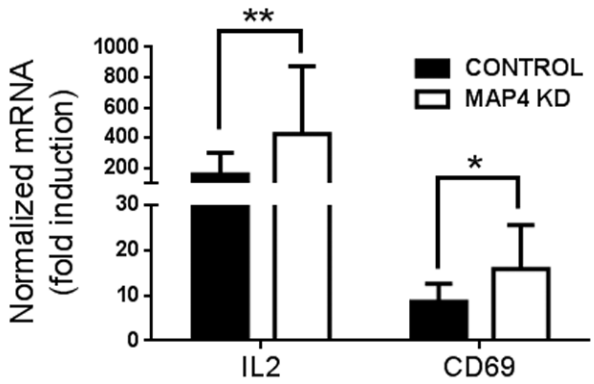

Figure R15. MAP4 reduction enhances T cell activation markers. mRNA levels of *IL2* and *CD69* from control and MAP4 KD cells activated with SEE-pulsed APCs (4 h). mRNA levels were normalized to a housekeeping gene (*Actb* or *Gapdh*) and to the levels of the corresponding target mRNA in non-stimulated cells. (n=8 and 6; Wilcoxon test; **,P-value < 0.01; *,P<0.05). Graph represents Mean ± s.d.

In order to corroborate these results and study whether cytokine secretion was similarly affected, IL2 secretion was analyzed. Control and MAP4 KD Jurkat T cells

were activated for 24 h with SEE-pulsed APCs and the quantity of IL2 accumulated in the resulting supernatant was analyzed by ELISA. In accordance, a significant increase in IL2 secretion was obtained due to MAP4 reduction **(Figure R16A)**. Likewise, we decided to test this effect detected in primary T cells. Human CD4+ T lymphocytes isolated from buffy coats were activated and grown to generate T lymphoblasts. After their polarization, T lymphoblasts were knocked-down for MAP4 and their IL2 secretion capacity was analyzed after 16 h of activation with anti-CD3 + anti-CD28 coated plates. Interestingly, cytokine secretion analysis in primary T cells rendered a similar result, with a significant increase in IL2 secretion in MAP4 KD cells. **(Figure R16B)**.

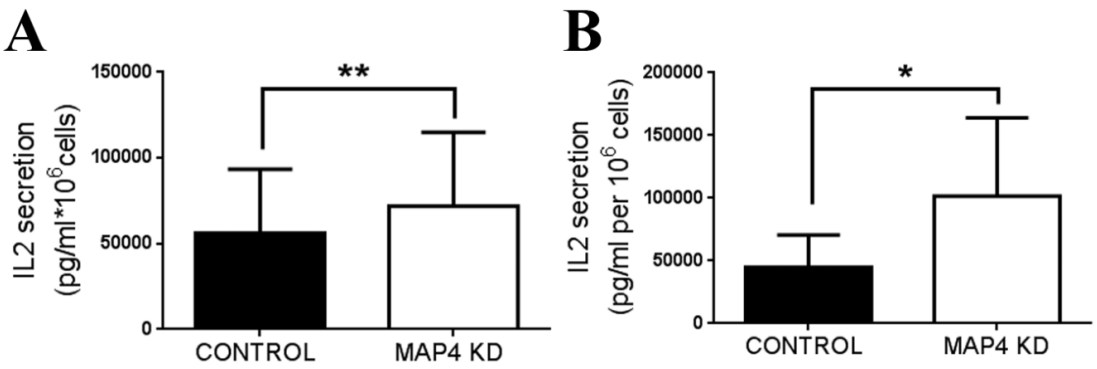

Figure R16. MAP4 modulates TCR-dependent cytokine secretion. (A) Analysis of IL2 secretion levels (ρg per ml and 10^6 cells) measured by ELISA in control and MAP4 KD Jurkat T cells activated with SEE-APCs for 24 h (n=7; paired t-test; **, P-value < 0.01) **(B)** Analysis of IL2 secretion levels (ρg per ml and 10^6 cells) measured by ELISA in primary T lymphoblasts activated with anti-CD3 + anti-CD28 coated plates for 24 hours (n=4; paired t-test;*, P-value < 0.05). All graphs represent Mean ± s.d.

In parallel, to analyze CD69 surface expression, control or MAP4 KD Jurkat T cells were activated for 24 h with SEE-pulsed APCs. Cells were stained for CD69 and analyzed by FACs. In accordance, to the gene expression analysis, membrane surface CD69 was significantly augmented in MAP4 KD cells. **(Figure R17A)**. Similarly,

MAP4 KD primary T lymphoblasts activated with anti-CD3 + anti-CD28 coated plates (16h) showed an increase in CD69 surface expression. **(Figure R17B)**.

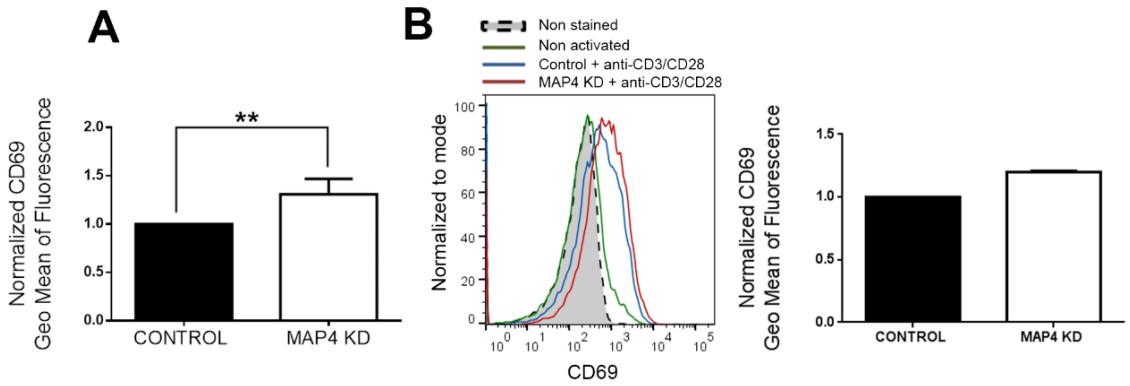

Figure R17. MAP4 knocking-down enhances CD69 surface expression. **(A)** FACs analysis of CD69 surface expression in control and MAP4 KD cells activated with SEE-pulsed APCs (24 h). Geometric Mean (GeoMean) of Fluorescence is shown (n=6; paired t-test; **, P-value < 0.01). **(B)** FACs analysis of control and MAP4 KD primary T lymphoblasts activated with anti-CD3 + anti-CD28 coated plates. CD4+ cells were gated and normalized, GeoMean of fluorescence was analyzed (n=2). All graphs represent Mean ± s.d.

4.6 MAP4 modulation on PLCγ1 activity and consequent diacylglycerol and calcium production

Due to the differences detected in the TCR early cascade of activation and the pattern of expression of T cell activated genes when MAP4 is reduced, we considered to explore additional signaling intermediates downstream of the TCR. When studying the activatory phosphorylation of PLCγ1 (Y783), a significant increase was observed in MAP4 KD cells activated with SEE-pulsed APCs, specially at later times (5 and 15 min) **(Figure R18A and B)**. However, no changes in PLCγ1 accumulation at the IS were detected in conjugates of MAP4 KD cells with SEE-pulsed APCs analyzed by confocal microscopy **(Figure R18C)**. This enhancement of PLCγ1 activity was supported by the detection of a similar increase in PLCγ1 phosphorylation in MAP4 KD

human primary T lymphoblasts activated with crosslinked anti-CD3 + anti-CD28 antibodies **(Figure R18D)**.

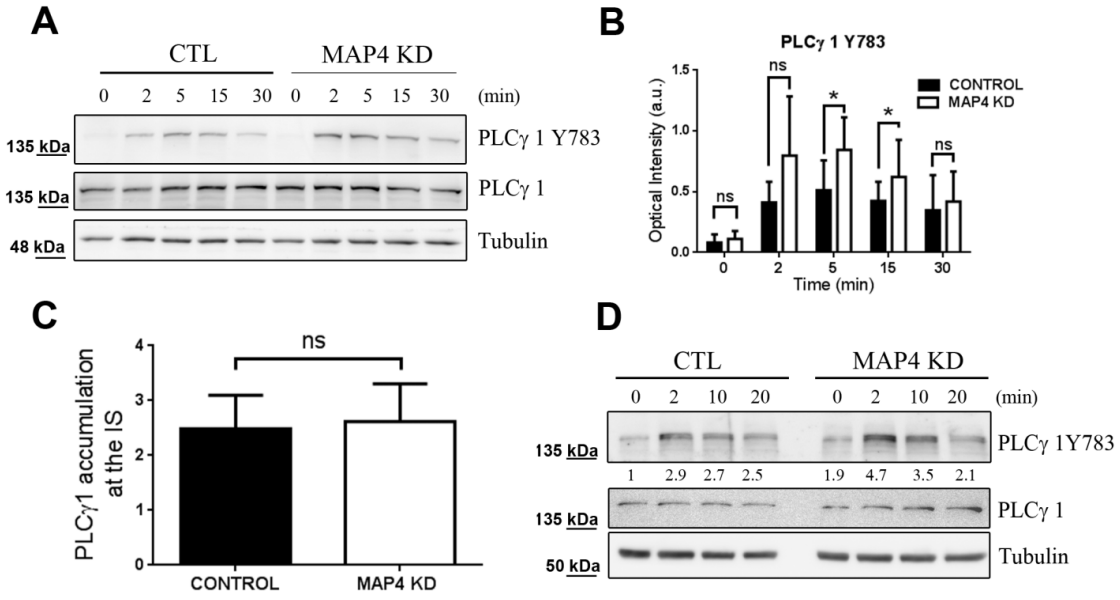

Figure R18. PLCγ1 activity is augmented due to MAP4 reduction. (A) Immunoblot showing PLCγ1 phosphorylation in control and MAP4 KD cells conjugated with SEE-APCs for the indicated times. Tubulin is shown as loading control. **(B)** Quantification of PLCγ1 phosphorylation as in the (A) (n=6; paired t-test; *, P-value < 0.05). **(C)** 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 66 from three independent experiments, Mann-Whitney test, ns). **(D)** Immunoblot showing PLCγ1 phosphorylation in control and MAP4 KD primary T lymphoblasts activated with crosslinked anti-CD3 + CD28 antibodies. Tubulin is shown as loading control. Quantification is shown under bands. (One representative gel out of three). Graphs represent Mean ± s.d.

When PLCγ1 activates, it catalyzes the conversion of membrane phosphatidilinositol 4,5-biphosphate (PIP2) into second messengers: inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). On the one hand, IP3 is responsible for the release of calcium from the endoplasmic reticulum (ER) and the consequent global calcium wave. On the other hand, production of DAG activates several PKCs necessary for MTOC translocation and gene expression (Bustos-Moran et al, 2016). In order to

functionally analyze the relevance of the increase in PLCγ1 phosphorylation detected in MAP4 KD, we decided to examine two of these downstream effectors of PLCγ1 activity: DAG production and calcium signaling.

In order to analyze DAG production at the IS, a construction containing PKCθ-C1 DAG-responsive domain was used. Control and MAP4 KD Jurkat T cells were transfected with PKCθ-C1-GFP plasmid and conjugated with SEE-pulsed APCs (15 min). Then, GFP accumulation at the IS was studied by confocal microscopy **(Figure R19A)**. Quantitative analysis of PKCθ-C1-GFP clustering at the IS revealed a significant increase in DAG production in MAP4 KD cells **(Figure R19B)**.

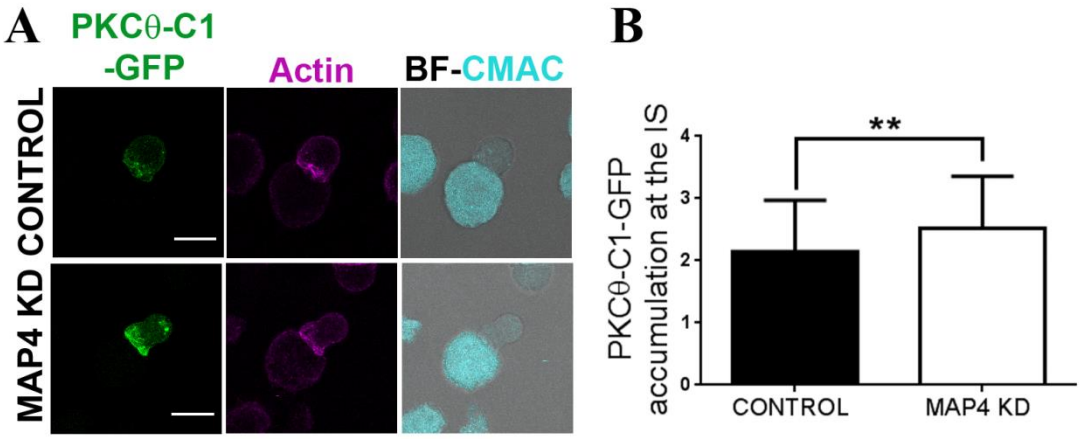

Figure R19. MAP4 controls DAG production at the IS (A) Maximal Z projection of confocal stacks showing conjugates of control and MAP4 KD Jurkat T cells expressing PKCθ-C1-GFP and activated with SEE-APCs (15 min). Cells were fixed and immunostained for actin (Magenta). Bright field images with CMAC in cyan (APCs) are shown. Scale bar 10 µm. **(B)** Quantification of PKCθ-C1-GFP accumulation at the IS as in (A) (n=63 and 75 from three independent experiments; Mann-Whitney test; **, P-value < 0.01).

Surprisingly, when the accumulation of PKCθ whole protein (in comparison with its C1 domain) was studied in MAP4 KD cells conjugates, no apparent effect was detected **(Figure R20)**.

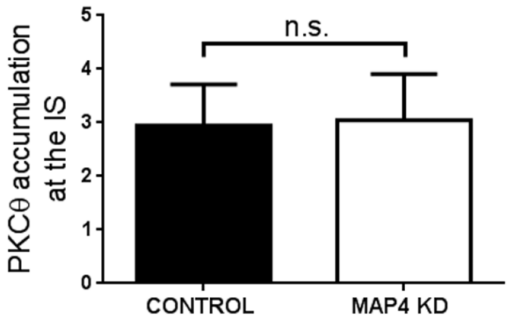

Figure R20. MAP4 does not affect to PKCθ IS clustering. Analysis of PKCθ accumulation at the IS in control or MAP4 KD Jurkat T cells activated with SEE-pulsed APC cells for 30 min (n =67 and 68 from three independent experiments, Mann-Whitney test, ns). Graph represents Mean ± s.d.

Accordingly, we decided to analyze the functional relevance of this DAG enhanced production on the activation of NF-κB complex. First, we studied the nuclear translocation of p65 (also known as RelA), one of NF-κB subunits, by nuclear/cytoplasmic fractioning of control and MAP4 KD cells activated with SEE-pulsed APCs. Subcellular fractioning analysis showed an increase in the proportion of nuclear p65 upon activation in MAP4 KD cells **(Figure R21A)**. Secondly, the transcriptional activity of NF-κB was assessed through a luciferase assay, using a construction constituted by five NF-κB-responsive sites fused to the gene of *luciferase*. Control and MAP4 KD cells were transfected with the NF-κB-Luciferase plasmid and activated with SEE-pulsed Raji B cells for 24 h. Consistently with p65 increased nuclear translocation, when measuring luciferase production, a significant increase in NF-κB transcriptional activity was detected in MAP4 KD compared to the control **(Figure R21B)**.

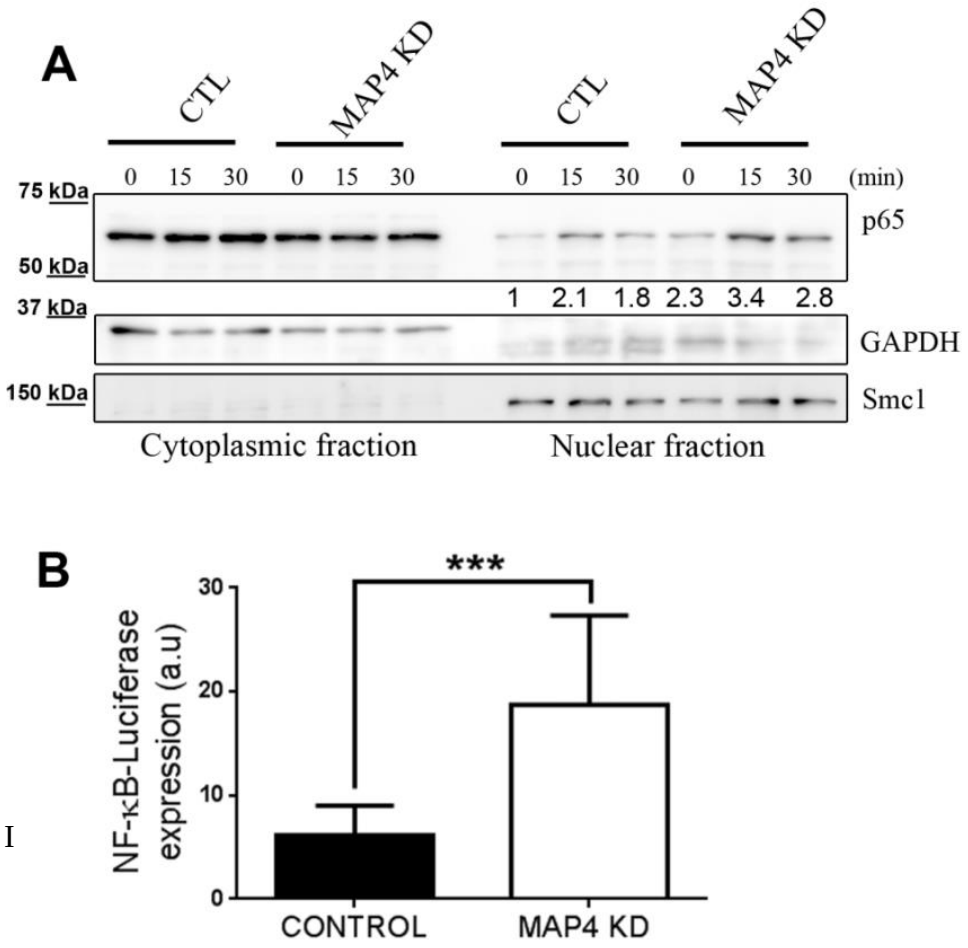

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Figure R21. MAP4 silencing promotes NF-κB transcriptional activity. (A) Immunoblot showing p65 nuclear translocation. Cytoplasmic/nuclear fractions from control and MAP4 KD cells conjugated with SEE-APCs for the indicated times. GAPDH and Smc1 are shown as loading controls for cytoplasmic/nuclear fractions, respectively. Quantification is shown under bands. **(B)** Quantification of NF-κB activity by a luciferase assay in control or MAP4 KD Jurkat T cells transfected with NF-κB (5x)-Luciferase construction and activated with SEE-pulsed APCs (24 h). (n= 5; paired t-test; *, P-value <0.001). All graphs represent Mean ± s.d.

In parallel, as another dowstream effector of PLCγ1 activity, calcium wave generated upon TCR activation was measured. Control and MAP4 KD cells stained with Fluo3-AM were activated with a pre-crosslinked anti-CD3 + anti-CD28 and

calcium changes were measured by FACs. Strikingly, flow cytometry analysis revealed no changes in the global calcium uptake between control and MAP4 KD cells **(Figure R22A and B)**.

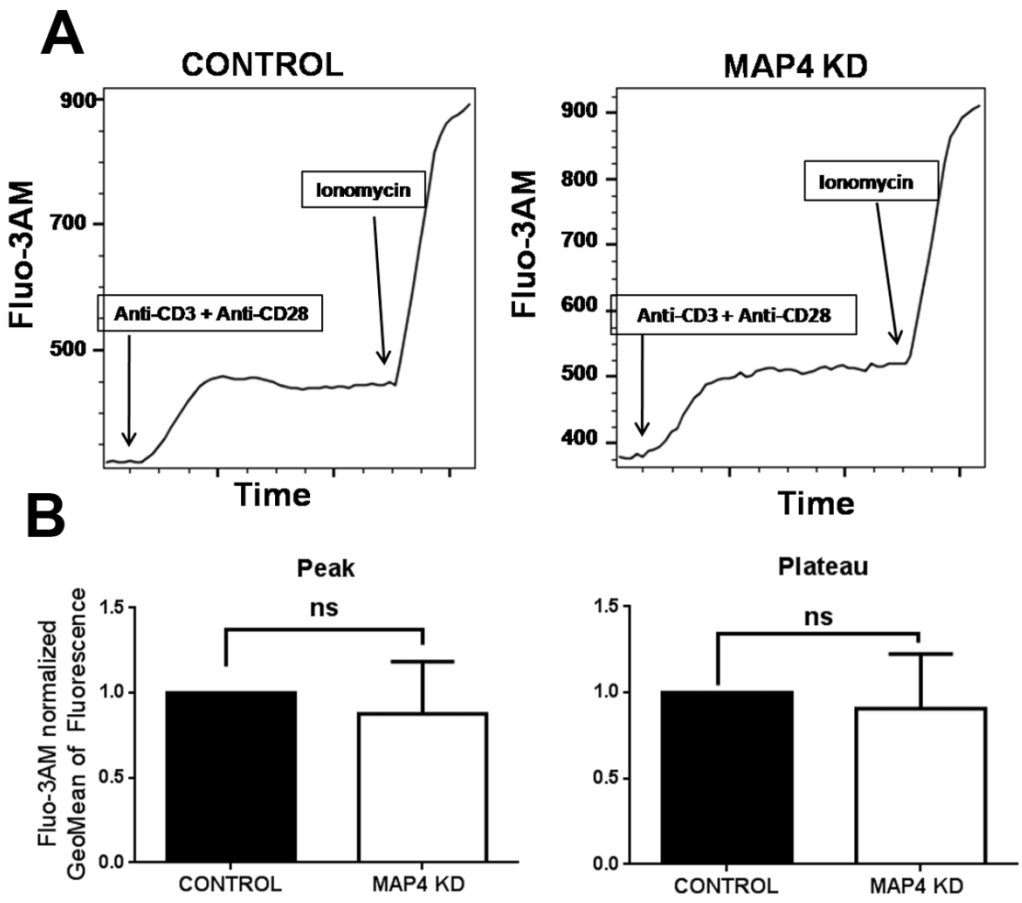

Figure R22. MAP4 KD cells show no difference in the global wave of calcium. (A) Kinetics of calcium, measured by Fluo-3AM, of control or MAP4 KD Jurkat T cells activated with anti-CD3 + anti-CD28 (STEM CELL). Baseline was recorded for 1 min, stimulated and recorded for 5 min and then re-stimulated with Ionomycin and recorded for 1min. **(B)** Quantification of the GeoMean of fluorescence of Fluo-3AM at the peak or plateau of the calcium wave. GeoMean was normalized to the baseline. (n=6; one sample t-test, ns). Graphs represent Mean ± s.d.

Nevertheless, since FACs measurement of calcium wave was not a good indicative of local calcium changes, but of the global calcium uptake of the cell, we decided to measure NFAT transcriptional activity as a reporter of local calcium. Aiming

this, we transfected control or MAP4 KD cells with a construction containing nine NFAT-responsive sites fused to *luciferase* gene. Luciferase assay, upon activation, resulted in a significant increase in NFAT transcriptional activity in MAP4 KD cells upon activation **(Figure R23)**.

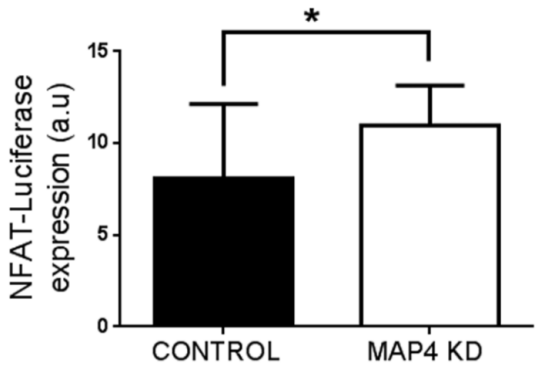

Figure 23. MAP4 controls NFAT transcriptional activity. Quantification of NFAT activity by a luciferase assay in control or MAP4 KD Jurkat T cells transfected with NFAT (9x)-Luciferase construction and activated with SEE-pulsed APCs (24 h). (n= 6; paired t-test; *, P-value <0.001). Graph represents Mean ± s.d.

Finally, since MTOC translocation was delayed in MAP4 KD and MTOC polarization is dependent on DAG production (Quann et al, 2009), we hypothesized that the delay in MTOC translocation could boost the production of DAG to overcome this defect in MAP4 KD cells. Additionally, MTOC delay in polarization could be blocking the proper transport of DAG negative regulators like DGKs to the contact area. To further test this hypothesis, we decided to assess for DAG production in systems in which MTOC polarization was impaired independently of MAP4. For that purpose we transfected Jurkat T cells with HDAC6-GFP or C-term-AKAP450-GFP constructions (since both plasmids have been previously proved to blockade MTOC polarization) (Robles-Valero et al, 2010; Serrador et al, 2004) and measured DAG production. Strikingly, the analysis of PKCθ-C1-mCherry accumulation at the IS revealed a significant increase of DAG production in cells transfected with each one of both constructions **(Figure R24 A and B)**.

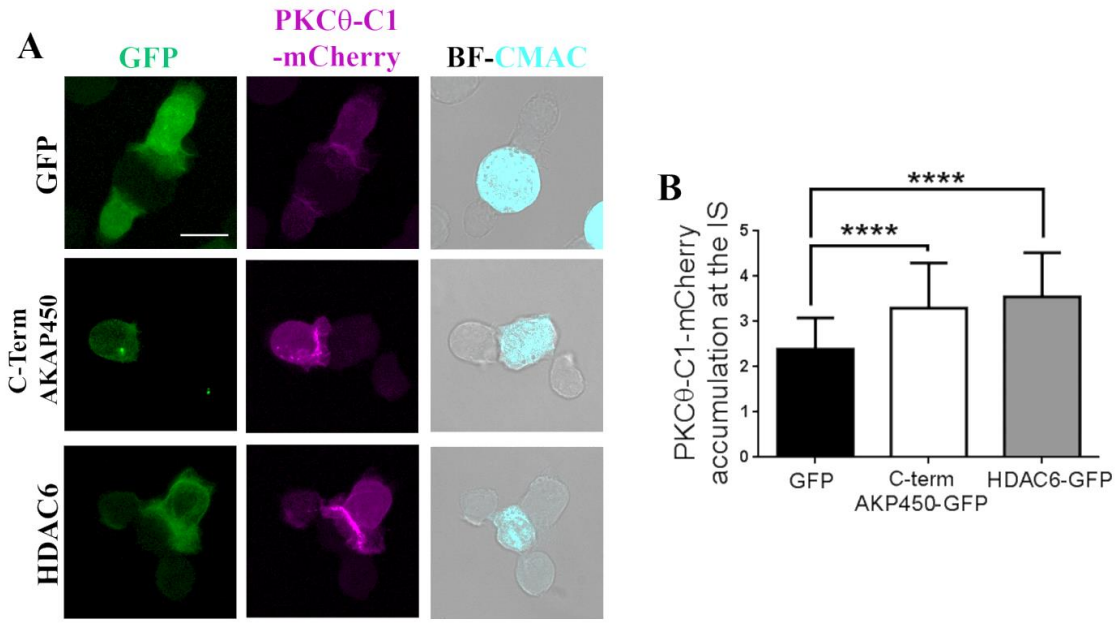

Figure R24. MTOC translocation controls DAG production. **(A)** Z-maximal projection of confocal stacks showing Jurkat T cells transfected with GFP alone, C-term AKAP450-GFP or HDAC6-GFP (green) plus PKCθ-C1-mCherry (magenta) and activated with SEE-pulsed Raji B cells for 15 min. Bright field and CMAC in cyan (APC) image is also shown. Scale bar 10 µm. **(B)** Quantification of PKCθ-C1-mCherry accumulation at the IS as in (A). (n=73, 74 and 78 from three independent experiments; Mean±s.d.; Kruskal-Wallis test; ****, P-value < 0.0001).

Together these results suggests that MAP4 modulates PLCγ1 activity and its silencing induces an enhancement on DAG production at the IS, boosting the activation of associated transcription factors like NFAT or NF-κB and thereby promoting, lately, an increase in the expression of T cell activation markers.

Discussion

5. Discussion

In this work, we have studied the role of MAP4 during T cell activation and immune synapse formation. We have proved that MAP4 decorates T cell microtubules and MTOC during the formation of the IS, accompanying the centrosome towards its polarization. Moreover, we have demonstrated that MAP4 controls T cell microtubule assembly capacity and the stability of the tubulin cytoskeleton upon TCR activation, promoting a timely translocation of the MTOC towards the contact area. In addition, we have identified a novel role of MAP4 controlling TCR activation and some of the molecules downstream this cascade. Furthermore, we have proved that MAP4 controls the displacement of the submembrane pool of CD3ζ-bearing nanovesicles, necessary for the maintenance of the signal. Finally, we have found that, strikingly, MAP4 negatively regulates T cell effector function and gene expression by controlling the local production of DAG and calcium and therefore affecting to the transcriptional activity of NF-κB and NFAT.

5.1 MAP4 localization at the IS and tubulin cytoskeleton regulation

MAP4 localization has been assessed in different systems and in response to different signals. In most of the studies MAP4 staining rendered a uniform pattern similar to the tubulin staining and decorating mainly the microtubules along all their length (Chang et al, 2001; Chinnakkannu et al, 2010; Nguyen et al, 1997) or with a punctuated staining following the microtubules pattern (Samora et al, 2011). Although some centrosomal staining was detected in other cell types, the majority of MAP4 protein was localized along the microtubules. In the case of T cells, we have detected a major staining of the centrosome and the decoration of just some microtubules, not all the tubulin array. Since MAP4 has been proved to be essential for the assembly of new microtubules either in vitro (Aizawa et al, 1991) or in vivo (Nguyen et al, 1999), this enhanced localization at the centrosome could be explained by a predominant role of

MAP4 regulating the assembly of new microtubules upon MTOC translocation, more than by a general stabilization of the whole array of MTs. In fact, the detection of a defect in MT assembly in T cells depleted for MAP4 supports this idea.

Although we would have expected a change in the localization of MAP4 upon TCR activation, maybe due to changes on its phosphorylation, we did not detect any apparent difference in the pattern between activated or non-activated T cells. This is consistent with previous observations where mutation of phosphorylatable sites of MAP4 rendered differences in the ability of MAP4 to stabilize microtubules or promote its assembly but do not alter its localization at the microtubular array (Chang et al, 2001). According to this study, MAP4 localization would not be changed due to its phosphorylation but the strength of the binding with the MTs would be different, affecting to the ability of MAP4 to stabilize the MTs and promote their assembly. Another possibility is that MAP4 localization could exchange from the pericentrosomal area to the microtubules upon TCR activation. In this regard, it has been seen that hypoxia-induced MAP4 phosphorylation promotes its translocation from the microtubules to the mitochondria to induce an apoptotic response (Hu et al, 2014). Considering this, a possibility would be that T cell activation could lead to subtle changes in the proportion of MAP4 in the MTs compared with the centrosome, priming a more stabilizing or MT assembly function depending on its subcellular localization.

Besides MT assembly, MAP4 also seems to be regulating the stability of a subset of MTs, detyrosinated MTs, upon TCR activation. Tubulin detyrosination has been demonstrated to be necessary for a sustained T cell activation, since it modulates the proper polarization of the MTOC towards the contact area (Andres-Delgado et al, 2012). Tubulin acetylation is another posttranslational modification (PTM) that has been proved essential for T cell activation and IS formation. However, our preliminary data did not show any effect on this modification in cells depleted for MAP4. This is consistent with previous works that have seen an effect of MAP4 only on the levels of detyrosinated tubulin (Nguyen et al, 1997).

Tubulin PTMs are modifications that can be detected in different subpopulation of microtubules. Their function remains unclear yet, although they have normally been associated with populations of stable (acetylated, detyrosinated) or dynamic (tyrosinated) microtubules (Janke & Bulinski, 2011). Additionally, PTMs have been

proved important for the binding of different MAPs. For example, tyrosination is a modification that allows the association of end-binding stabilizing proteins containing a CAP-Gly domain like CLIP-170 or p150 (Peris et al, 2006). In fact, the changes observed on the pattern of these PTMs during several processes like cell cycle progression or differentiation suggests the existence of a "tubulin code" that would encode the different microtubule subpopulations depending on their PTMs (Janke & Bulinski, 2011). In accordance with this hypothesis, the fact that MAP4 is regulating only some tubulin PTMs and that its reduction does not seem to affect to the whole array of MTs supports a differential role of the subsets of microtubules during IS formation. In line with this, it would be very interesting to further assess the function of this "tubulin code" during T cell activation and its regulation by other MAPs.

5.2 MAP4 effect on a timely MTOC translocation

As our results prove, MAP4 reduction causes a defect in MTOC translocation. This defect seems to be a delay more than a total impairment, which suggests that MAP4 helps to prompt a timely translocation of the centrosome. This conclusion is further supported by the rescue of the phenotype when overexpressing the murine isoform of MAP4.

Other MAPs have been studied in the context of centrosome polarization rendering similar phenotypes. Histone deacetylase-6 (HDAC6), for example, is a protein involved in tubulin deacetylation and it has been reported to impair the translocation of the centrosome towards the contact area either in CD4+ (Serrador et al, 2004) or CD8+ cells (Nunez-Andrade et al, 2016). Additionally, the dynein/dynactin complex was demonstrated to be responsible for the pulling forces needed to attract the centrosome towards the IS (Combs et al, 2006; Martin-Cofreces et al, 2008). In fact, its depletion or the overexpression of dynamitin (p50), a blocker of the complex, resulted in a defect in MTOC translocation (Martin-Cofreces et al, 2008).

A recent study has suggested a biphasic model for MTOC translocation, with an initial approximation to the IS and its posterior docking (Yi et al, 2013). In this study, the pulling forces were suggested to be a consequence of the capping of the MT plus ends to the cortical region and the posterior depolymerization of the MTs attached, in a capture-shrinkage way. In this sense, over-stabilization of the MTs would blockade

these forces. However, this model also supports that an initial MT polymerization is necessary for the capping of the microtubules to the membrane (Yi et al, 2013). Our data support this model, since MAP4 reduction does not totally impair MTOC translocation but delay it, probably because the defects in MT assembly slow down the initial capture of the MT ends at the plasma membrane but do not affect to the posterior shrinkage pulling force. In fact, in other systems, like in mitotic spindle orientation, where centrosome pulling forces mainly depend on lateral gliding of the microtubule through the anchored dynein/dynactin complex, MAP4 reduction rendered totally opposite effects, promoting this gliding and the attraction of the centrosome (Samora et al, 2011).

In addition, part of the defect in MTOC polarization could be both a consequence of the defects observed in early CD3ζ ITAM phosphorylation, and also a cause for the reduction on the maintenance of T cell activation.

Finally, Par1b/MARK2, a protein that has been found to phosphorylate MAP4 in other systems (Cheng et al, 2010), was proved to be important in the acquisition of the polarity during IS formation and for centrosome polarization in T cell activation (Lin et al, 2009). Therefore, this kinase could be accounting for the effect of MAP4 on MTOC translocation. After promoting the polymerization and capture of the MT ends in the cortical region, MAP4 could be phosphorylated by Par1b in order to allow the posterior shrinkage force necessary for centrosome translocation.

5.3 MAP4 regulation of early TCR signaling

We have demonstrated an effect of MAP4 on early T cell activation by maintaining the normal levels of CD3ζ ITAM phosphorylation, as well as the activation of some of the molecules downstream the TCR. Our results showed that MAP4 knocking-down generates a reduction in the levels of ITAMs phosphorylation. Nevertheless, this blockade was partial more than a total reduction of the phosphorylation levels. This suggests that MAP4 acts more like a modulator of the signal than a “key” protein in the TCR cascade.

ITAM phosphorylation has been seen to be driven by Lck upon activation. TCR engagement to the MHC-bound antigen leads to the exposition of the ITAM tails allowing its phosphorylation by Lck. This favors the recruitment of SH2 containing

proteins like ZAP70, which phosphorylate other proteins, like LAT, amplifying the signal (Bustos-Moran et al, 2016). The TCR complex contains up to 10 ITAM regions by the combination of CD3 –ε, -γ, -δ (one each) and –ζ (three) (Love & Hayes, 2010). Although it is thought that the phosphorylation of the different ITAMs is somehow redundant and helps to potentiate the signal, a differential binding affinity has been shown for some proteins like ZAP-70 (Love & Hayes, 2010). Therefore, it would be interesting to study the effect of MAP4 in the phosphorylation of other ITAMs in order to elucidate differential patterns of molecule binding.

In addition, CD3ζ reduced phosphorylation leads to a reduction in LAT Y191 phosphorylation, without affecting significantly to LAT Y132 phosphorylation. The identity of the kinase that phosphorylates LAT in vivo still remains unclear although some in vitro assays have suggested a possible phosphorylation by ZAP70 (Zhang et al, 1998), Itk (Perez-Villar et al, 2002) or Lck (Jiang & Cheng, 2007). However, a work, which aimed to unveil the early phosphorylation kinetics of molecules downstream the TCR, showed that LAT Y191 phosphorylation occurs in parallel to ZAP70 phosphorylation, but prior to LAT Y132 phosphorylation (Houtman et al, 2005). Therefore, this work supports the notion that ZAP70 is the kinase responsible for LAT Y191 phosphorylation. Our results agree with this view, since CD3ζ reduced phosphorylation would lead to less ZAP70 activation and therefore LAT Y191 phosphorylation.

The fact that LAT Y132 is not significantly reduced in our MAP4 knock-down cells, combined with Houtman et al., observations that LAT Y132 phosphorylation is lagged upon TCR activation (Houtman et al, 2005), suggests the importance of other mechanisms underlying the phosphorylation of LAT Y132 residue, which might not be so highly affected by MAP4. Nevertheless, it would be interesting to study the pattern of phosphorylation of other LAT residues (like Y171 or Y226) in order to convey this hypothesis.

Finally, ERK 1/2 phosphorylation was also reduced at longer time points of activation in cells knockdown for MAP4. ERK 1/2 protein is activated by Ras kinase pathway. Ras is a GTPase that gets active by the action of two guanosil exchange factors (GEFs): Ras-GRP and Sos (Genot & Cantrell, 2000). Ras-GRP is dependent on DAG production since DAG favors its accumulation near the membrane and

approximation to Ras (Genot & Cantrell, 2000). Sos is constitutively bound to Grb2 and its activation depends on the recruitment of Grb2 by LAT phosphorylation. A combination of a pair of phosphorylated sites (between Y171, Y191 and Y226 residues) is responsible for Grb2 binding to LAT (Balagopalan et al, 2010).

The overlapping functions of both GEFs have been controversial. A study in Jurkat T cells proposed a model in which the interplay of Ras-GRP and Sos was necessary for Ras fully activation (Roose et al, 2007). According to the model, Ras-GRP would be accounting for Ras-GDP transformation into Ras-GTP and, then, Ras-GTP would bind to Sos1 and allosterically activate it. Active Sos would then act as a feedback loop to maintain Ras activatory pathway and the phosphorylation of downstream effectors like ERK 1/2 (Roose et al, 2007). However, a work in human primary T lymphoblasts seems to disagree with this model, suggesting that, in primary lymphoblasts, Ras-GRP would be the only activator of Ras pathway upon TCR activation. Conversely, Sos would enhance this cascade only in response to mitogenic signals like CD25 ligation with IL2 (Warnecke et al, 2012).

In our case, since DAG seems to be increased upon TCR activation as a result of MAP4 knocking-down, we would have expected also an increase in ERK 1/2 phosphorylation. Nevertheless, providing the role of Sos positive feedback loop over ERK 1/2 phosphorylation, maybe the decrease in LAT Y191 phosphorylation, which was observed in MAP4 KD cells, could lead to a reduced recruitment of Grb2-Sos complex and therefore a decrease in Ras activation. In accordance, the effect on ERK 1/2 is detected at longer time than in the case of CD3ζ or LAT Y191 phosphorylations. This difference could be due to changes in the implication of Ras-GRP or Sos GEF function at different times of T cell activation kinetics. Consequently, MAP4 might not be affecting to Ras initial activation by Ras-GRP, but a possible impairment in Sos localization could prevent the maintenance of Ras activation due to the absence of the Sos-dependent positive feedback loop (Roose et al, 2007).

5.4 CD3ζ-bearing nanovesicles movement and CD3/TCR membrane exchange

CD3 cycle of recycling and degradation is essential for T cell activation. In a basal state TCR/CD3 molecules are internalized and recycled again to the membrane (Liu et al, 2000). However, TCR engagement with the corresponding antigen-MHC,

also triggers some signals that lead to its endocytosis and delivery to the lysosomes for its degradation (Liu et al, 2000; Valitutti et al, 1997). This reduction of surface TCR requires the polarized secretion to the IS of an intracellular pool of vesicles containing TCR/CD3 molecules, which help to maintain the activation (Das et al, 2004).

In our work, we have detected a defect in the movement of this intracellular compartment of CD3ζ-bearing nanovesicles. We postulate that this defect in the movement of the vesicles leads to a reduction in the rate of polarized recycling towards the contact area, suggesting a mechanism to explain the decrease in CD3ζ phosphorylation detected upon activation. In fact, this hypothesis is supported by the discovery of a pool of phosphorylated CD3ζ in the endosomal compartment (Yudushkin & Vale, 2010). Whether CD3ζ-nanovesicles observed belong to this phosphorylated pool or not, however, would require further experimentation.

Furthermore, the defects observed in ITAM phosphorylation do not seem to be caused by an alteration in the basal internalization/recycling rate since the surface basal levels of CD3ε and TCR remained unaffected by MAP4 depletion. Moreover, neither the rate of internalization nor the general recycling upon activation are affected in MAP4 knocking down cells. This suggests that the effect observed is restricted to the polarized compartment that maintains CD3ζ levels at the IS.

In fact, the polarized recycling towards the contact area has been proved to be dependent on microtubule transport (Das et al, 2004). Another MAP protein, the end-binding protein 1 (EB1) has been demonstrated to be essential for the movement of vesicles to the vicinity of the IS, by coupling the movement of CD3ζ and LAT-bearing vesicles with the growth of the microtubules from the MTOC (Martin-Cofreces et al, 2012). According to this, MAP4 depletion could be impairing the growing of new microtubules from the centrosome avoiding nanovesicle movement towards the IS and therefore its fusion. In addition, since MAP4 has been also proved to affect to vesicle traffic in other systems due to its effect on kinesin and dynein-driven transport (Bulinski et al, 1997; Semenova et al, 2014; Seo et al, 2016), we cannot rule out that the effect on vesicle displacement observed is caused by a similar mechanism. In addition, polarized recycling is dependent on intraflagellar transport proteins that normally act in the vesicle trafficking of the primary cilia (Finetti et al, 2009). Providing that MAP4 interacts with septin complex controlling their binding to primary cilia microtubules

(Ghossoub et al, 2013; Kremer et al, 2005), another possibility is that MAP4 is affecting, somehow, to the functionality of the IFT complex (similarly to septins) and therefore regulating CD3ζ polarized recycling (**Figure D1**).

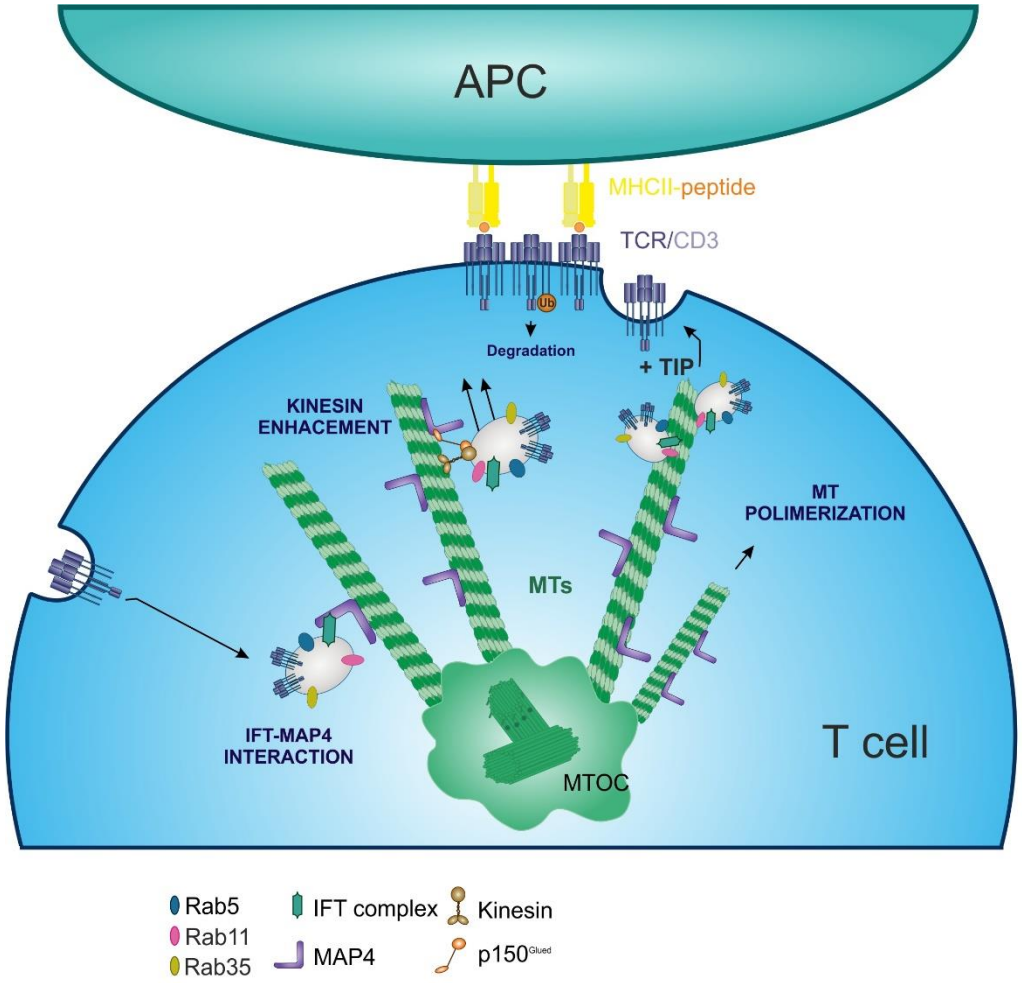

Figure D1. Model for MAP4 control on TCR/CD3ζ-bearing vesicles dynamics. TCR engagement to the MHC-bound peptide leads to the activation of the TCR and its posterior degradation by ubiquitination. Polarized recycling of TCR/CD3 vesicles is necessary for the maintenance of the signal. MAP4 can be affecting to this trafficking by several mechanism. It can be interacting with IFT complex and therefore affecting to its functionality. Another possibility is that, since MAP4 increases the processivity of kinesin transport through its interaction with p150, it might enhance plus end directed transport. Finally, MAP4 MT assembly activity could favor the growth of MTs bearing these vesicles (associated to EB1) and allow their approximation to the fusion area.

Although we did not monitor MTOC translocation in the TIRFm approximation, we consider that the defect in CD3ζ-bearing vesicle dynamics does not seem to be due to MTOC translocation delay, since the number of vesicles detected at the area was not affected and the defects in the vesicle displacement were maintained along time. Besides similar defects in nanovesicle movement have been observed in proteins, like Aurora A, which, although does not affect to MTOC translocation, has an effect on microtubule nucleation and growing (Blas-Rus et al, 2016).

Finally, our model suggests that MAP4 would be enhancing the polarized transport of the CD3ζ-bearing vesicle pool to the IS to help to maintain the signal as a positive regulator. Nevertheless, CD3ζ activation is dependent both in CD3 polarized recycling and lateral diffusion and aggregation of CD3 microclusters. In consequence, since the movement of these microclusters is also regulated by a dynein-microtubule dependent mechanism (Hashimoto-Tane et al, 2011), we cannot rule out the possibility that the defect observed in ITAM phosphorylation is also partially caused by a defect in this lateral microcluster movement and fusion due to microtubule instability in MAP4 KD cells.

5.5 PLCγ1 negative control by MAP4

One of the most striking results of this work was the discovery that MAP4 knocking-down resulted in an enhanced expression of T cell activation markers like IL2 and CD69. Unexpectedly, we found that PLCγ1 activity, measured by its phosphorylation at Y783, was enhanced upon TCR activation when MAP4 was reduced. This difference in the levels of activation in comparison with other early activation markers like CD3ζ phosphorylation or LAT Y191 activation suggested us that PLCγ1 could constitute the key protein to explain the increase in T cell activation observed.

PLCγ1 is a protein that catalyzes the transformation of PIP2 into two second messengers that amplify TCR signaling: IP3 and DAG. Its function has been proved essential for T cell activation controlling NFAT or NF-κB activity, IL2 secretion or T cell differentiation, especially in T_{reg} cells (Fu et al, 2010). PLCγ1 activation depends both on its interaction with LAT Y132 phosphorylated residue (Zhang et al, 2000), as well as on its phosphorylation by Itk (Bogin et al, 2007). Since we did not detect any

changes in PLCγ1 total clustering at the IS, which is consistent with the absence of significant changes in LAT Y132 phosphorylation, we wondered what could be affecting to PLCγ1 phosphorylation. Accordingly, PLCγ1 has been proved to be phosphorylated independently of its association with LAT (Braiman et al, 2006). It is thought that SLP-76 interaction with Itk and also with PLCγ1 favors the physical proximity of both proteins allowing its phosphorylation. Then, PLCγ1 would be able to bind to LAT Y132 residue, get close to membrane PIP2 and become fully active (Braiman et al, 2006). Therefore, one possible mechanism is that PLCγ1 enhanced activity is due to an increased phosphorylation by Itk.

Itk regulation is quite complex and involves a phosphorylation by Lck, an autophosphorylation and also some conformational changes (Andreotti et al, 2010). A key event on Itk activation is its interaction with SLP-76. In fact, physical separation of active Itk from SLP-76 drives to the inactivation of Itk, even though being phosphorylated previously (Bogin et al, 2007). Therefore, it would be very interesting to study whether MAP4, or its indirect action over the microtubule network, could be somehow affecting to Itk-SLP76 interaction and therefore to PLCγ1 phosphorylation.

Another possible mechanism to explain PLCγ1 enhanced phosphorylation relies on a reduced activity of its specific phosphatase: CD148. CD148 is a protein tyrosine phosphatase (PTP), which has been seen to dephosphorylate PLCγ1 in T cells, acting as a negative regulator of T cell activation (Baker et al, 2001; Harrod & Justement, 2002). However, the possibility of a reduced activity of this phosphatase in MAP4 knocked-down cells seems less plausible, since CD148 has also been involved in the dephosphorylation of other proteins like ZAP70 or LAT (Harrod & Justement, 2002), whose activity was not enhanced, but even reduced, when MAP4 was blocked. Nevertheless, it would be very interesting to explore this idea, specially taking into account the fact that CD148 microclusters are initially excluded from the IS (Lin & Weiss, 2003). Similarly to other transmembrane phosphatases, like CD45, CD148 microclusters might change its localization during the IS, moving centripetally towards its targets and promoting the termination of T cell activation signal (Varma et al, 2006). Accordingly, the regulation of the position and fusion of the microclusters containing CD148 by the tubulin cytoskeleton and dynein-associated motors (Hashimoto-Tane et

al, 2011), would explain MAP4-dependent enhanced PLCγ1 phosphorylation **(Figure D2)**.

5.6 MAP4 KD enhanced DAG production and effect on NF-KB activity

As we have already described, MAP4 KD cells showed an increase in PLCγ1 activity. This resulted in an enhanced production of DAG, one of the second messengers responsible for TCR signal amplification. DAG production at the IS is mainly regulated by some enzymes called diacylglycerol kinases (DGKs), which catalyze the transformation of DAG into phosphatidic acid (PA), blocking its action. There are ten described DGKs, however, only two predominate in T cells: DGKα and DGKζ (Krishna & Zhong, 2013; Merida et al, 2008). DGKα is rapidly and transiently polarized to the IS (Sanjuan et al, 2003) and it has been proved to accumulate at the pSMAC, where it limits the accumulation of DAG to the cSMAC region (Chauveau et al, 2014). DGKα has been shown to influence MTOC polarization by regulating the spatial accumulation of DAG at the IS (Chauveau et al, 2014). Conversely, DGKζ seems to accumulate at the IS in a more extended distribution and remains attached for longer time. Its function at the IS has been associated with the global control over DAG production, more than with DAG localization, balancing the equilibrium between DAG and PA and acting as a "brake" for T cell activation (Gharbi et al, 2011). Accordingly, although most of the enhanced DAG generation detected in MAP4 KD cells can be explained due to the increased activity of PLCγ1, additionally, it could be also caused by a defect in the proper translocation of DGKs. In this sense, total impairment of MTOC translocation by transfection of HDAC6-GFP or AKAP-450 C-terminal domain resulted in a major DAG production, even when compared with MAP4 KD cells. Since it has been seen that MTOC translocation is highly dependent on DAG production (Quann et al, 2009), maybe the defect in MTOC translocation could act as a feedback loop boosting IS local DAG production and accumulation.

In support of this hypothesis, it has been observed that SNX27, a marker of endosomal compartment, interacts with DGKζ and accumulates at the IS (Rincon et al, 2011). Although SNX27 localization probably does affect to initial DGKζ IS accumulation, it is conceivable that the polarization of SNX27-vesicles towards the IS helps to maintain DGKζ accumulated at that area for longer time. Therefore, MAP4 knocking-down could be affecting to the accumulation of DGKζ at the IS, either due to

its delay in MTOC translocation (and the associated movement of the endosomal compartment) or as a result of a defect in the proper polarization and movement of SNX27-DGKζ bearing vesicles similarly to the one observed for CD3ζ-bearing vesicles. Consequently, it would be interesting to study how MAP4 reduction affects (directly or indirectly) to DGKζ translocation and, therefore, to T cell negative regulation **(Figure D2)**.

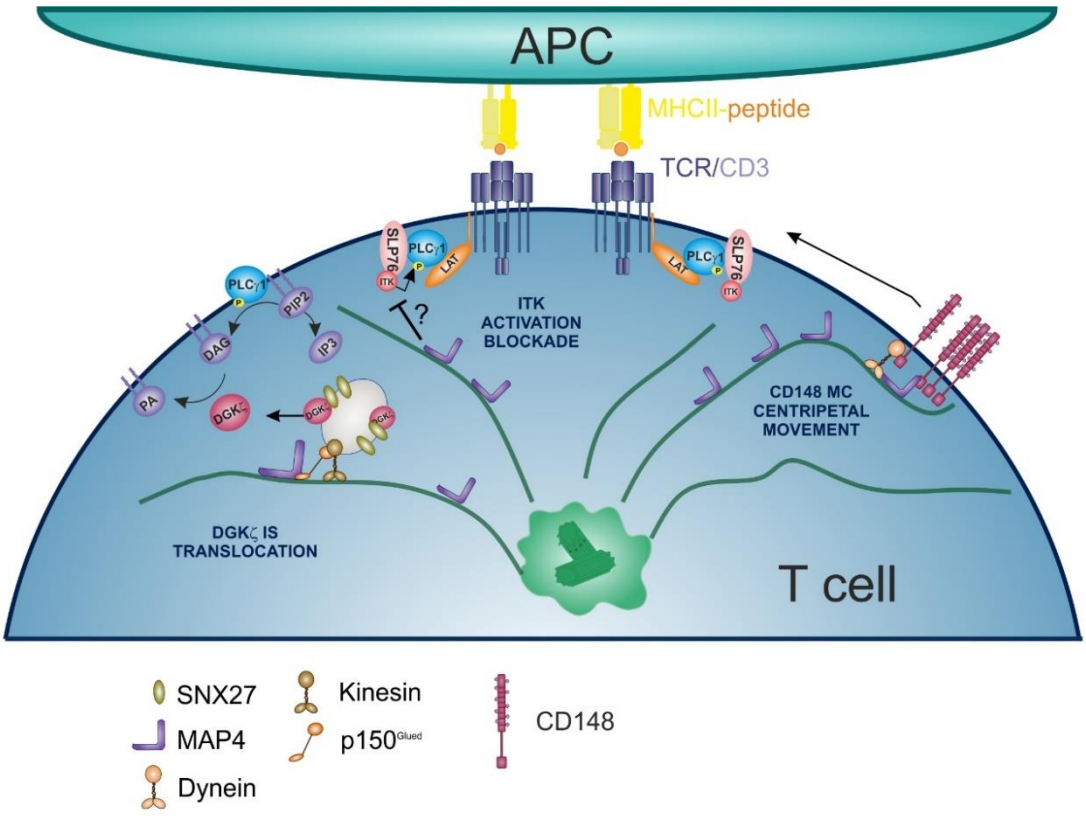

Figure D2. Model for MAP4-mediated control on PLCγ1 activation and DAG production. MAP4 could control PLCγ1 activity through different mechanisms. It could be affecting to the dynamics of CD148 microclusters (MC) along the MTs, favoring its congregation to the cSMAC after activation, and CD148 effect on dephosphorylating PLCγ1. Alternatively, MAP4 could be modulating Itk-SLP76 interaction, dampening Itk activity and therefore its kinase ability over PLCγ1. Finally, DAG production could also be balanced by an enhanced translocation of DGKζ to the contact area, maintaining its lasting accumulation at the IS.

One of the main DAG-downstream effector pathways is the axis consisting of PKCθ and NF-κB activation. Upon TCR engagement, PKCθ activation promotes the phosphorylation of CARMA1 protein leading to the formation of the CBM complex (CARMA1-BCL10-MALT1). This signalosome associates to the membrane due to interactions with the 3-phosphoinositide-dependent protein kinase 1 (PDK1) and CD28 and promotes the activation of IκB kinases (IKKs). IKK kinases phosphorylate IκBα leading to its degradation. Eventually, the degradation of this negative regulator unlocks NF-κB complex (p65-p50 heterodimer) activation and translocation to the nucleus, allowing its transcriptional enhancing activity (Cheng et al, 2011; Paul & Schaefer, 2013).

In our work, we have demonstrated that MAP4 reduction causes an increase on p65 translocation to the nucleus upon TCR activation, as well as an augmented NF-κB transcriptional activity, as detected by the luciferase assay. This supports an enhanced activation of PLCγ1 and the major production of DAG. Nevertheless, no significant differences on PKCθ T538 phosphorylation or on PKCθ IS clustering were detected in MAP4 KD cells.

PKCθ activation mainly depends on two factors: T538 phosphorylation by the germinal center kinase-like kinase (GLK) (dependent on the TCR) (Chuang et al, 2011) and its clustering and localization at the IS (Wang et al, 2012). However, other factors can affect to its degree of activation. For example, different phosphorylation residues, apart from T538, have been identified and their function has been uncovered as modifiers of PKCθ activity or plasma membrane localization (Wang et al, 2012). Additionally, although the initial localization in membrane domains of PKCθ depends on its C1-DAG responsive domain, later stabilization at the pSMAC-cSMAC transition area relies on its interaction with the correceptor CD28 (Huang et al, 2002; Yokosuka et al, 2008). In fact, it has been proved that PKCθ exchange between cytosolic compartment and CD28-plasma membrane domains is very dynamic (Yokosuka et al, 2008). Therefore, although we did not detect any changes in T538 phosphorylation or general clustering in MAP4 KD cells, it would be important to elucidate whether the increase in DAG production is affecting somehow to PKCθ membrane exchange or to any of the other phosphorylation residues that act as modifiers.

In addition, although PKCθ has been the main isoform of PKCs described to function in TCR-mediated NF-κB activation, other isoforms (PKCε and PKCη) display higher affinity for DAG and they are recruited before PKCθ to the contact area (Quann et al, 2011). Therefore, we cannot rule out an effect of one of those other PKCs isoforms on NF-κB complex activation that would bypass PKCθ.

5.7 Calcium regulation: global versus local control

PLCγ generation of IP3 promotes the release of endoplasmic reticulum (ER) stored calcium to the cytosol. This compartment release leads to the activation of STIM1, an ER receptor that physically interacts with calcium activated channels (CRAC) from the plasma membrane called Orai1. Orai1 then favors a flux of calcium entry from the extracellular matrix that increases global calcium levels (Kummerow et al, 2009). Accordingly, apart from DAG increase production, we also assessed calcium levels as marker of PLCγ1 enhanced activity. Surprisingly, no significant changes in global calcium wave were detected in MAP4 KD by FACs analysis.

Alternatively, calcium response was also measured through its activation of NFAT transcription factor. NFAT is a family of transcription factor that controls the secretion of many cytokines. In a basal state NFAT is highly phosphorylated, however, an increase in cytoplasmic calcium promotes the activation of calcineurin protein, leading to NFAT dephosphorylation. Once dephosphorylated, NFAT can translocate to the nucleus and this exerts its transcriptional function (Macian, 2005). In accordance, our results in MAP4 KD cells showed an increase of NFAT transcriptional activity. Since we wanted to use NFAT reporter as an alternative measure for local calcium more than a real reporter of TCR induced NFAT-IL2 activity, we decided to use IL4-promoter NFAT binding sites instead. NFAT activity over IL2 promoter requires additionally the action of AP1 transcription factor to be functional (Macian, 2005); therefore, it would not be exclusively a reporter of calcium local intensity but it would also depend on other factors. In short, the increase on NFAT activity detected could be considered as a very sensitive marker for calcium increase, alternatively to FACs analysis.

A possible explanation for the differences observed in FACs calcium analysis relies on the different regulation of local and global levels of calcium at the IS. While global levels normally increase upon activation, local levels near the contact area remain lower. This effect is due to the mitochondrial repositioning at the IS, where they act as calcium storages, redirecting calcium away from CRAC channels to avoid their inactivation (Quintana et al, 2011). In fact, it has been shown that this mitochondrial repositioning is dependent on tubulin cytoskeleton rotation (Maccari et al, 2016). Therefore, although we did not detect an increase in global calcium levels in MAP4 KD cells, perhaps the defects on tubulin cytoskeleton are causing a mislocalization of mitochondria at the IS. This could result in an increase in local calcium due to increased IP3 production, explaining the results obtained by NFAT-Luciferase marker.

5.8 Concluding remarks

In our work, we have identified a balancing role of MAP4 in T cell activation. On the one hand, it controls positive signals like MTOC timely translocation or polarized movement of CD3ζ-bearing vesicles, which help to sustain TCR phosphorylation and signaling. On the other hand, it negatively regulates the expression of T cell activation markers affecting to its effector function and acting also as a modulator of terminating signals. In short, its role in T cells helps to promote an accurate and fine-tuned response. In fact, considering the importance of IL2 or CD69 genes in T cell function and differentiation (Boyman & Sprent, 2012; Gonzalez-Amaro et al, 2013), it would be, indeed, interesting to know how defects on MAP4 affect to T_h subsets differentiation. Although most of the effects detected in MAP4 KD cells could be explained by its influence over tubulin cytoskeleton the differences obtained in comparison with other microtubule-associated proteins already studied in the context of T cell activation, suggest a differential function of this protein probably due to direct interactions with other components of the IS. Consequently, it can be considered that MAP4 dual role in T cell activation could be very useful to modulate T cell threshold of activation and gene expression pattern, therefore affecting to the coordination and strength of the immune adaptive response.

Conclusions

6. Conclusions

The findings presented herein support the following conclusions:

1) MAP4 decorates T cell microtubules and MTOC during Immune Synapse formation.

2) MAP4 controls T cell microtubule assembly and detyrosinated microtubules stability upon TCR engagement, prompting a timely translocation of the MTOC.

3) CD3ζ-bearing nanovesicles dynamics is affected by MAP4 reduction, leading to a decrease in ITAM phosphorylation and TCR signal sustainment.

4) MAP4 knocking-down promotes an enhanced PLCγ1 activity, which leads to a major production of DAG and an increased NFAT and NF-κB transcriptional activity.

5) MAP4 balances the production of IL2 and expression of T cell early activation marker CD69, therefore regulating T cell effector function.

Conclusiones

7. Conclusiones

Los resultados presentados en este trabajo permiten concluir:

1) MAP4 se localiza en los microtúbulos y en el MTOC de las células T durante la formación de la Sinapsis Inmune.

2) MAP4 controla el ensamblaje de nuevos microtúbulos y la estabilidad de los microtúbulos que contienen tubulina detirosinada en respuesta a la activación del TCR, favoreciendo, en consecuencia, una adecuada y correcta translocación del MTOC.

3) La dinámica de las vesículas de reciclaje de CD3ζ se ve afectada por la reducción de los niveles de MAP4, provocando una disminución en el grado de fosforilación de los ITAMs y en el mantenimiento de la señal dependiente del TCR.

4) La disminución en los niveles de MAP4 en la célula T, promueve una respuesta exacerbada de PLCγ1, que desemboca en una mayor producción de DAG y una actividad transcripcional incrementada de factores como NFAT o NF-κB.

5) MAP4 modula la producción de IL2 y la expresión del marcador de activación T CD69, regulando, por tanto, la función efectora de la célula T.

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Annexes

9. Annexes

9.1 Supplementary information

- **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 MAP4 KD Jurkat 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.

9.2 Publications related with this Thesis work

I. Bustos-Moran E, Blas-Rus N, Martin-Cofreces NB, Sanchez-Madrid F (2017) Microtubule-associated protein-4 controls nanovesicle dynamics and T cell activation. *Journal of cell science* **130:** 1217-1223

II. Blas-Rus N, Bustos-Moran E, Sanchez-Madrid F, Martin-Cofreces NB (2017b) Analysis of Microtubules and Microtubule-Organizing Center at the Immune Synapse. *Methods in molecular biology* **1584:** 31-49

III. Bustos-Moran E, Blas-Rus N, Martin-Cofreces NB, Sanchez-Madrid F (2016) Orchestrating Lymphocyte Polarity in Cognate Immune Cell-Cell Interactions. *International review of cell and molecular biology* **327:** 195-261

9.3 Other publications

I. Blas-Rus N, Bustos-Moran E, Martin-Cofreces NB, Sanchez-Madrid F (2017a) Aurora-A shines on T cell activation through the regulation of Lck. *BioEssays : news and reviews in molecular, cellular and developmental biology* **39**

II. Blas-Rus N, Bustos-Moran E, Perez de Castro I, de Carcer G, Borroto A, Camafeita E, Jorge I, Vazquez J, Alarcon B, Malumbres M, Martin-Cofreces NB, Sanchez-Madrid F (2016) Aurora A drives early signalling and vesicle dynamics during T-cell activation. *Nature communications* **7:** 11389

Annexe I