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## SAP-MEDIATED INHIBITION OF DIACYLGLYCEROL KINASE ALPHA REGULATES TCR-INDUCED DIACYLGLYCEROL SIGNALING

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### Abstract

Diacylglycerol kinases (DGKs) metabolize diacylglycerol (DAG) to phosphatidic acid (PA). In T lymphocytes, DGK $\alpha$  acts as a negative regulator of TCR signaling by decreasing diacylglycerol levels and inducing anergy. Here, we show that upon co-stimulation of the TCR with CD28 or SLAM, DGK $\alpha$ , but not DGK $\zeta$ , exit from the nucleus and undergoes rapid negative regulation of its enzymatic activity. Inhibition of DGK $\alpha$  is dependent on the expression of SAP, an adaptor protein mutated in X-linked lymphoproliferative disease (XLP), which is essential for SLAM-mediated signaling and contributes to TCR/CD28-induced signaling and T cell activation. Accordingly, over-expression of SAP is sufficient to inhibit DGK $\alpha$ , while SAP mutants unable to bind either phospho-tyrosine residues or SH3 domain are ineffective. Moreover phospholipase C activity and calcium, but not Src-family tyrosine kinases, are also required for negative regulation of DGK $\alpha$ . Finally, inhibition of DGK $\alpha$  in SAP-deficient cells partially rescues defective TCR/CD28 signaling, including Ras and ERK-1/2 activation, PKC $\theta$  membrane recruitment, induction of NF-AT transcriptional activity and IL-2 production. Thus SAP-mediated inhibition of DGK $\alpha$  sustains diacylglycerol signaling, thereby regulating T cell activation and may represent a novel pharmacological strategy for XLP treatment.

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## Introduction

In T lymphocytes, engagement of the TCR by specific antigens, along with stimulation by co-stimulatory receptors such as CD28, leads to T cell activation, cytokine production and differentiation. Moreover, several other receptors influence cell activation by quantitatively or qualitatively modifying immunoreceptor-derived signals. Conversely, stimulation via the TCR alone, while partially activating intracellular signaling pathways, is not sufficient to induce effector functions such as cytokine production and proliferation (1).

SLAM (CD150) is a homotypic transmembrane receptor expressed in T and B lymphocytes, dendritic cells and monocytes (2). Upon engagement, SLAM undergoes a conformational change leading to Fyn-mediated tyrosine phosphorylation and activation of several signaling pathways that modulate TCR-induced responses (2). Fyn recruitment to the activated SLAM is mediated by SAP, an adaptor protein comprising a single SH2 domain and a SH3 domain-binding sequence (3). In humans, SAP loss-of-function mutations cause X-linked lymphoproliferative disease (XLP), an immune disorder characterized by a deregulated immune response to Epstein-Barr virus, susceptibility to lymphoma and defective antibody production (4). Interestingly, SAP-deficient T lymphocytes from either XLP patients or SAP knock-out mice exhibit defective responses to TCR/CD28 co-stimulation *in vitro*: *i*) T cells from XLP patients feature reduced ERK-1/2 and NF- $\kappa$ B activation, decreased IL-2 production and impaired proliferation (5); *ii*) CD4<sup>+</sup> T cells from XLP patients exhibit reduced ICOS expression and IL-10 production (6); *iii*) T cells from SAP knockout mice feature reduced PKC $\theta$  membrane recruitment, Bcl-10 phosphorylation and NF- $\kappa$ B activation, which are associated with defective IL-4 secretion and enhanced INF $\gamma$  production (7).

Antigen-mediated activation of the TCR in the presence of other co-activating molecules triggers a complex signaling network leading to transcriptional activation of specific genes whose expression mediates T cell proliferation and differentiation. Activation of Ras and PKC $\theta$  triggers key signaling pathways, leading, among others, to the activation of NF-AT and NF- $\kappa$ B and contributing to transcription of the IL-2 gene (8, 9). In T cells, activation of Ras and PKC $\theta$  is dependent on the generation of diacylglycerol (DAG) through PLC-mediated hydrolysis of phosphatidylinositol-4,5-bis-phosphate. DAG recruits RasGRP, the Ras-GEF mainly responsible for TCR-induced Ras activation, and PKC $\theta$  to the plasma membrane (10, 11). Notably, engagement of TCR in the absence of co-stimulation results in a weak and transient activation of both Ras and PKC $\theta$ , which drives T cells into anergy, a hypo responsive status characterized by the inability to produce IL-2 and proliferate (12, 13).

DAG generated upon T cell activation is rapidly metabolized by Diacylglycerol kinases (DGKs), a multigenic family of enzymes responsible for phosphorylation of DAG to phosphatidic acid (PA). Consistently with the crucial role of DAG signaling in T cell activation, several pieces of evidence indicate that the DGK $\alpha$  and  $\zeta$  isoforms, which are highly expressed in thymus and T cells, act as negative regulators of TCR signaling and immune cell function (14). Specifically *i*) genetic deletion of DGK $\alpha$  and  $\zeta$  in T cells enhances TCR-induced activation of ERK-1/2 resulting in defective induction of anergy (15, 16); *ii*) DGK $\alpha$  is strongly induced in anergic T cells (13); *iii*) over-expression of either DGK $\alpha$  or DGK $\zeta$  impairs CD3/CD28-induced activation of Ras signaling (17–19); *iv*) pharmacological inhibition of DGKs reverses the inability of anergic cells to produce IL-2 in response to TCR stimulation (13); *v*) DGK $\alpha$  expression is down-regulated within a few hours from T cell activation (19). Collectively, these data support the concept that second messengers signaling is highly dependent on the fine tuning of DAG synthesis and degradation rates. While there is no evidence for regulation of DGK $\zeta$  upon T cell activation,

TCR/CD28 co-stimulation of T cells results in rapid and sustained recruitment of DGK $\alpha$  to the plasma membrane (19), an event mediated by both Lck-dependent phosphorylation of tyrosine 335 and calcium binding to the EF hand domain of DGK $\alpha$  (20, 21).

Based on the role of DGK $\alpha$  as a negative regulator of T cell responses, we investigated the hypothesis that, upon T cell stimulation, DGK $\alpha$  activity might undergo negative regulation. Herein we show indeed that the enzymatic activity of DGK $\alpha$  is inhibited upon co-stimulation of TCR and CD28 through a SAP-mediated mechanism. Moreover, we found that, in SAP-deficient cells, defective TCR/CD28 signaling and T cell activation can be partially rescued by inhibition of DGK $\alpha$ .

## Material and Methods

### Cell Culture

Jurkat A3 cells (LGC) and 293FT (Life Technologies) were cultured respectively in RPMI GlutaMAX medium or DMEM GlutaMAX high glucose (Life Technologies), supplemented with 10% fetal bovine serum (FBS, Life Technologies) and antibiotic-antimycotic solution (Sigma-Aldrich), in humidified atmosphere with 5% CO<sub>2</sub>. Peripheral blood mononuclear cells (PBLs) were isolated by Lymphoprep gradient (Axis-Shield) of ACD (130 mM citric acid, 152 mM sodium citrate and 112 mM glucose)-treated venous blood obtained from healthy volunteers after informed consent. Briefly, blood was layered onto Ficoll–Hypaque separating media, after 20 min centrifugation at 300 RCF, cells were collected, washed and suspended in RPMI GlutaMAX medium supplemented with 10% heat inactivated FBS and antibiotic-antimycotic solution. Monocytes were depleted by plastic adherence at 37 °C for 1 h and the remaining PBLs were maintained for 18 hours in a humidified atmosphere with 5% CO<sub>2</sub> before further stimulation. BI-141 TTS-SAP were a kind gift of A. Veillette (Montreal, Canada).

Jurkat/SAP-shRNA cells were obtained by infection of Jurkat cells with lentiviruses encoding SAP-specific shRNA in pLKO.1-Puro vector (**82712** RNAi Consortium through Sigma-Genosys), sequence:  
CCGGCACAAGGTACTACAGGGATAACTCGAGTTATCCCTGTAGTACCTTGTGTT  
TTTG. Jurkat/control-shRNA were obtained by infection with lentiviruses encoding a shRNA specific for murine DGK $\alpha$ , in pLKO.1-Puro vector (**24825** RNAi Consortium through Sigma-Genosys), sequence:  
CCGGGAGCTAAGTAAGGTGGTATATCTCGAGATATAACCACCTTACTTACTTAGC  
TCTTTTT.

Lentivirus production and Jurkat infection were carried out according to the manufacturer's instructions. Infected Jurkat cells were selected for 14 days in puromycin (1  $\mu$ g/ml) and used as a bulk population in all experiments.

### Reagents

Antibodies used: Pan-Ras (Ab-4, Merck), H-Ras (F235, Cel Signaling), LAT (Santa Cruz), TAC (Abcam), CD3 agonist (OKT3, provided by U. Dianzani, Novara, Italy), CD28 agonist (ANC28.1/5D10, Ancell) except for Fig. 4D where anti-CD28 was from Pharmingen, SLAM agonistic antibody (A12, Biolegend), anti DGK $\zeta$  antibodies (kind gift of M. Topham, Salt Lake City, USA), mixture of DGK $\alpha$  antibodies used for immunoprecipitation (22), DGK $\alpha$  (C-20) and PKC $\theta$  (Santa Cruz) were used for immunofluorescence, ERK1/2 and phospho-ERK1/2 from Cell Signalling for Supplementary material Fig. 2 and from Transduction Laboratories for Fig. 5C, SAP (FL-128, Upstate Biotech),  $\alpha$ -tubulin (Sigma-Aldrich), secondary horseradish peroxidase-conjugated antibodies (PerkinElmer), secondary fluorescein isothiocyanate (FITC)-conjugated antibody (Dako), Alexa Flour 546-phalloidin

(Life Technologies). In all experiments involving stimulation with antibodies, species-matched pre-immune serum (Santa Cruz) was used for controls in equal amount.

Inhibitors used were from Sigma-Aldrich: R59949, DGKs inhibitor; PP2, Src-family inhibitor; U73122, PLC inhibitor; BAPTA-AM, cell permeable Calcium chelator; wortmannin, PI3Ks inhibitor; IPA-3, PAK-specific inhibitor. BAPTA-AM was dissolved in water while others inhibitors were dissolved in DMSO. DMSO was always used in control samples at the same dilution as the inhibitor tested.

### Expression Vectors and Transfections

GFP-SAP-wt, GFP-SAP-R<sub>78A</sub> and GFP-SAP-R<sub>55L</sub> were a kind gift of P. Schwartzberg (NIH, Bethesda, USA). N-terminal yellow fluorescent protein (YFP)-DGK $\alpha$  was obtained by cloning DGK $\alpha$  in pYFP-N-DEST (Life Technologies) using the Gateway kit (Life Technologies) according to manufacturer's instructions. pNFAT-TA-luciferase reporter vector and pRL-TK normalization vector were from Clontech. siRNA and negative control siRNA were from Ambion (Life Technologies): DGK $\alpha$  siRNA (23) sense GGUCAGUGAUGUCCUAAAGTT, antisense CUUUAGGACAUCACUGACCTT

Transient transfections in Fig. 5D and 5E were performed using Lipofectamine2000 reagent (Life Technologies) according to the manufacturer's instructions. Microporation of Jurkat cells for imaging experiments were performed according to manufacturer's instructions with the Microporator MP100 system–Digital Bio technology (Fig. 2 and supplemental Fig. 3B) or Gene pulser II from Biorad (Fig. 5B).

### Cell stimulation, preparation of cell lysates and homogenates, immunoprecipitation, western blotting, DGK assay

Cells ( $3 \times 10^7$ /ml) were resuspended in RPMI and incubated for the indicated time with agonist antibodies or control species-matched pre-immune serum at 37°C. For immunoprecipitation,  $3 \times 10^7$  cells were lysed in 1 ml of lysis buffer A (25mM Hepes (pH 8), 1% NP-40, 10% glycerol, 150mM NaCl, 5mM EDTA, 2mM EGTA, 1mM ZnCl<sub>2</sub>, 50mM ammonium molybdate, 10mM NaF, 1mM sodium orthovanadate and protease inhibitor cocktail from Sigma). An aliquot of cell lysate were retained for western blot analysis and the remaining was immunoprecipitated with a mixture of anti-DGK $\alpha$  antibodies as previously described (24). Whole cell homogenates were prepared by homogenizing  $3 \times 10^7$  cells in 1 ml cold buffer B (buffer A without detergent) by 20 passages in a 23 G syringe. Protein concentration was determined by BCA (Pierce) and equal amounts of proteins were loaded in each lane. SDS–PAGE and western blots were performed as described previously (25). Western blot results were acquired with Versadoc system and quantified using QuantityOne software (Bio-rad).

DGK $\alpha$  activity in cell homogenates (25  $\mu$ l) and anti-DGK $\alpha$  immunoprecipitates was assayed by measuring initial velocities (5 min at 30°C) as previously described (24). Radioactive signals were detected and quantified by GS-250 Molecular Imager and Phosphor Analyst Software (Bio-Rad).

### Immunofluorescence

For immunofluorescence on fixed cells with antibody stimulation, cells were seeded on poly-L-Lysine-coated glass coverslips (Marienfeld) in 24-well plates for 1 hour, and then stimulated with 10  $\mu$ g/ml of agonist antibodies for 1 hour in the presence or absence of the indicated inhibitors. Cells were then fixed with formaldehyde and stained as previously described (26). Confocal images were acquired with Leica confocal microscope TSP2 (objective 63x, numerical aperture 1,32) and analysed with LCS confocal software (Leica).

For the immunological synapse experiments, Raji cells (used as APC) were incubated for 2 h with 10 µg/ml staphylococcal enterotoxin E. Raji cells were washed, mixed with Jurkat control-shRNA or Jurkat SAP-shRNA (1:1) for 15 min and plated on polylysine-coated wells of diagnostic microscope slides (Erie Scientific Company). Cells were allowed to adhere for 15 min and then fixed in methanol at -20°C for 10 min. Samples were then washed for 5 min in PBS and incubated with anti-PKCθ antibody overnight at 4°C. After washing in PBS, samples were incubated for 1 h at room temperature with FITC-labeled anti-goat antibody. Images were taken using an Axio Imager Z1 microscope equipped with a HBO 50-W mercury lamp for epifluorescence and with an AxioCam HR cooled charge-coupled camera (Carl Zeiss).

For live cell imaging experiments, Jurkat A3 cells, Jurkat control-shRNA and Jurkat SAP-shRNA were microporated and serum starved in RPMI + 0.2% BSA + Hepes 50 mM for 2 hours. Cells were seeded on glass-bottom dishes coated with Poly-L-lysine or with the agonistic antibody anti-CD3, anti-CD3+anti-SLAM or anti-CD3+anti-CD28 at the final concentration of 10 µg/ml. Confocal images were acquired at the indicated times with a Zeiss LSM 510 (Carl Zeiss) inverted laser scanning microscope (LSM) using a C-Apochromat X63 water immersion objective lens (Zeiss). LSM image files were processed using the Zeiss "ZEN" LSM image browser software. When comparisons among images were to be made, the photos were taken in identical conditions and equally manipulated using Adobe Photoshop 7.0 software (Adobe Systems).

### Cell fractionation

Cells ( $3 \times 10^7$ /ml) were re-suspended in RPMI and incubated for the indicated time with agonist antibodies or control species-matched pre-immune serum at 37°C. Whole cell homogenates were prepared by homogenizing  $3 \times 10^7$  cells as described above and sonicating the homogenates for 1 minute. Post-nuclear and -mitochondrial fraction (obtained by 10 min centrifugation at 10,000 RCF) was further separated by ultra-centrifugation (30 min at 10,000 RCF). Supernatants (soluble cytoplasmic fraction) and pellet (insoluble membrane fraction) were collected and SDS-PAGE and western blots were performed as described previously (25) using anti-DGKα and anti-LAT antibodies.

### Biochemical Ras activation assays

Recombinant GST-c-Raf-RBD protein was produced in *E. Coli* as described (27). Jurkat cells were serum deprived (2 h in RPMI supplemented with 0.2 % fatty-acid-free/endotoxin-low BSA and 50 mM HEPES pH 7.5). After stimulation, 1 ml of the cell suspension ( $10^7$  cells) was lysed in 1 ml ice cold lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1% NP-40, protease inhibitors) supplemented with 25 µg GST-RBD protein and 100 µM GDP, in order to quench post-lytic GTP-loading and GAP-dependent Ras-bound GTP hydrolysis, respectively. Cell extracts were cleared by centrifugation and GST-RBD/Ras-GTP complexes were collected on Glutathione-sepharose, washed once with lysis buffer and processed for SDS-PAGE analysis.

### Mammalian two hybrid system

A modified Clontech MatchMaker (Becton-Dickinson) mammalian 2/3-hybrid assays was used. Full-length human SAP and its point-mutated variants were cloned into the pM series vectors as GAL4-binding domain fusions.

Either full length DGKα or the N-terminal DGKα fragment were cloned into a pVP vector to direct expression of VP16-activation domain fusion proteins. These were co-transfected into sub confluent HEK293 cells with a GAL4-luciferase reporter plasmid and a pVAX-based

expression plasmid containing full-length human FynT. Luciferase activity was measured after 24h using a commercial kit (Promega).

### NF-AT assay

Jurkat cells ( $4 \times 10^6$ /ml) were co-transfected with pNF-AT-TA-luc and pRL-TK plasmids. After 48 h, cells were stimulated as indicated for 16 hours. Luciferase was assayed with dual Luciferase reporter assay system (Promega) according to the manufacturer's instructions and assessed using Victor3V multilabel counter (Perkin Elmer). NF-AT driven firefly luciferase activity was normalized for the reference renilla luciferase activity to take in account differences in transfection and expression efficiency and all values were expressed as fold increase upon unstimulated controls.

### IL-2 assay

Jurkat cells ( $1 \times 10^5$ ) were plated in 100  $\mu$ l medium supplemented with 10% FBS, and stimulated as indicated for 72 hours. IL-2 released in the media was measured by ELISA (GE Healthcare).

### Statistical Analysis

The data were expressed as means  $\pm$  SE. Statistical analysis was determined by student T test.

## Results

### Negative regulation of DGK $\alpha$ during T cell activation

Since DGK $\alpha$  negatively regulates T cell activation (17, 19), we set out to investigate whether it is regulated in the early phase of lymphocyte activation. To this purpose we assayed the enzymatic activity and sub cellular localization of DGK $\alpha$  upon activation of primary lymphocytes (PBLs) and Jurkat leukemic T cells. DGK $\alpha$  activity was measured in vitro in the presence of exogenous substrates in anti-DGK $\alpha$  immunoprecipitates obtained from either control or stimulated lymphocytes. Following 15 min of co-stimulation of PBLs with agonistic anti-CD3 and anti-CD28 antibodies, the enzymatic activity of DGK $\alpha$  was reduced by about 60% as compared to unstimulated cells (Fig. 1A), without any change in DGK $\alpha$  protein content (Fig. 1A lower right panel). Stimulation of PBLs with anti-CD3 antibody alone did not significantly affect DGK $\alpha$  activity (data not shown). Since activation of SLAM family receptors was reported to enhance TCR signaling (28, 29), we investigated whether SLAM might regulate the enzymatic activity of DGK $\alpha$ . Indeed, 15 min of co-stimulation of PBLs with anti-CD3 and anti-SLAM agonist antibodies resulted in an even stronger inhibition of DGK $\alpha$  activity without affecting DGK $\alpha$  protein content (Fig. 1A). We then measured DGK $\alpha$  activity in anti-DGK $\alpha$  immunoprecipitates from Jurkat leukemia cells following co-stimulation with anti-CD3 and either anti-CD28 or anti-SLAM agonist antibodies. Similar to the data in PBLs, DGK $\alpha$  enzymatic activity was strongly reduced upon 15 min of co-stimulation via the TCR and either SLAM or CD28 and lasted for at least 1 hour, without changes in DGK $\alpha$  protein content (Fig. 1B, 1C). Finally, in order to address the reported ambiguity of how anti-SLAM antibodies may affect SLAM signaling, we used an alternative approach to induce SLAM signaling. We used a chimeric receptor featuring SLAM intracellular domain and the extracellular and transmembrane regions of the human IL-2 receptor  $\alpha$  chain co-expressed with SAP in BI-141 lymphocytes (30). Crosslinking of the chimeric receptor with anti IL-2  $\alpha$ -receptor antibody (TAC) triggers SLAM signaling (30) and it was sufficient to induce a strong decrease of DGK $\alpha$  activity without changes in DGK $\alpha$  protein content (Fig. 1D). This result indicates that signals originating from the intracellular domain of SLAM lead to DGK $\alpha$  inhibition. Taken together, these observations

indicate that upon co-stimulation of the TCR with either CD28 or SLAM, the enzymatic activity of DGK $\alpha$  undergoes a negative regulation, which likely contributes to the accumulation of DAG required for RasGRP-mediated activation of Ras and full T cell activation.

To verify whether this regulation was specific to DGK $\alpha$  we first examined whether anti-CD3 co-stimulation with either anti-CD28 or anti-SLAM antibodies regulated DGK $\zeta$ , which, along with DGK $\alpha$  is highly expressed in T cells. We observed that neither CD3/CD28 nor CD3/SLAM co-stimulation of T cells did affect the enzymatic activity of DGK $\zeta$  in anti-DGK $\zeta$  immunoprecipitates from either control or co-stimulated cells (Supplemental Fig. 1). These observations indicate that TCR activation specifically regulates DGK $\alpha$  enzymatic activity, while not affecting DGK $\zeta$ . In order to verify the contribution of DGK $\alpha$  regulation to the total cellular DGK activity, we measured DGK activity in whole lymphocytes homogenates using exogenous substrates. Following 15 min of TCR/CD28 co-stimulation of either PBLs or Jurkat cells, total DGK activity was not significantly affected, even if the co-stimulation was sufficient to activate ERK-1/2 (Supplemental Fig. 2A and 2B). Conversely, upon 15 min TCR/SLAM co-stimulation, total DGK activity was significantly reduced (Supplemental Fig. 2C and 2D). Given the specific sub-cellular localization of DGK isoforms, these observations suggest that DGK $\alpha$  inhibition does not affect the bulk of DAG metabolism, while selectively promoting DAG accumulation at specific compartments.

As DGK $\alpha$  recruitment from the cytoplasm to the plasma membrane is highly regulated both upon growth factor stimulation of epithelial cells and TCR/CD28-mediated co-stimulation of lymphocytes (20, 24), we assessed DGK $\alpha$  localization following co-stimulation of the TCR with either CD28 or SLAM. Both endogenous DGK $\alpha$  in CD3<sup>+</sup> PBLs and YFP-DGK $\alpha$  transiently expressed in Jurkat cells localize diffusely in the nucleus and in the cytoplasm of unstimulated or TCR-stimulated cells. Upon 1 hour co-stimulation of the TCR with either CD28 or SLAM, DGK $\alpha$  was almost entirely excluded from the nucleus and recruited to the cell periphery in both PBLs and Jurkat cells (Fig. 2A, 2B and Supplemental Fig. 3A and 3B). While inhibition of DGK $\alpha$  enzymatic activity was an early event, starting 5 min following co-stimulation, reaching maximal inhibition at 15 min and lasting up to 1 hour (Fig. 1C), translocation of DGK $\alpha$  became detectable 15 min after co-stimulation, reached its maximum at 30 min and lasted for several hours (Fig. 2B).

In order to distinguish between plasma membrane and cytoplasmic localization, we labeled plasma membrane with either K-Ras-V<sub>12</sub>/A<sub>28</sub> (31) or wheat germ agglutinin (WGA). Upon T cell co-stimulation, DGK $\alpha$  only partially co-localized with K-Ras-V<sub>12</sub>/A<sub>28</sub> (Fig. 2B, 4D) or with WGA (Supplemental Fig. 3B). Accordingly approximately 10% of cytoplasmic DGK $\alpha$  sedimented in the 100,000 RCF fraction of CD3/CD28 co-stimulated Jurkat cells (Fig. 2C). These findings indicate that, upon lymphocytes activation, DGK $\alpha$  undergoes both negative regulation of its enzymatic activity and translocation from the nucleus to the cell periphery, although with different kinetics.

### Regulation of DGK $\alpha$ inhibition and recruitment to the cell periphery

We explored whether translocation to the cell periphery and negative regulation of DGK $\alpha$  were regulated by common signaling pathways. DGK $\alpha$  activity and localization are regulated by Src-mediated tyrosine phosphorylation (21, 24, 25), calcium binding (17, 32), and D-3 phosphoinositides (33). Pharmacological inhibition of PLC by U73122 and calcium chelation by BAPTA-AM blunted DGK $\alpha$  translocation from the nucleus to the cell periphery induced by co-stimulation of TCR with either SLAM or CD28 (Fig. 3A). Interestingly, PP2-mediated inhibition of Src family tyrosine kinases (SFK) inhibited only CD3/SLAM-induced translocation. Conversely, wortmannin did not affect DGK $\alpha$ .

localization, indicating that phosphoinositides 3-kinases are not involved (Fig. 3A). Similarly, pharmacological inhibition of PLC and calcium signaling prevented the negative regulation of DGK $\alpha$  activity induced by TCR/SLAM co-stimulation (Fig. 3B). These data indicate that PLC activity and calcium release mediate both inhibition of DGK $\alpha$  activity and its translocation to the cell periphery. Conversely, inhibition of SFKs impaired specifically negative regulation of DGK $\alpha$  activity and its translocation to the cell periphery induced by TCR/SLAM co-stimulation, but not by TCR/CD28 co stimulation (Fig. 3A, 3C), indicating that SFKs requirement is restricted to SLAM-induced regulation of DGK $\alpha$ . Despite SAP overexpression regulates cdc42, IPA3-mediated inhibition of PAK, a cdc42 effector, does not affect DGK $\alpha$  activity (Fig. 3B).

Upon SLAM engagement, SAP mediates the recruitment of Fyn, thereby promoting tyrosine phosphorylation of SLAM and activation of its downstream signaling (34). Thus, we investigated the role of SAP in negative regulation and membrane recruitment of DGK $\alpha$ . SAP expression was down-regulated in Jurkat cells by lentiviral-mediated stable expression of a SAP-specific shRNA (Fig. 4A and 4B). In SAP-deficient Jurkat cells, but not in control shRNA cells, DGK $\alpha$  activity was not inhibited following stimulation of TCR and SLAM (Fig. 4A), consistently with the essential role of SAP in SLAM-induced signaling. Surprisingly, in SAP-deficient cells, DGK $\alpha$  activity was not inhibited by TCR/CD28 co-stimulation. This observation suggests that SAP is not only required for SLAM signaling, but may also play a more direct role in promoting negative regulation of DGK $\alpha$  enzymatic activity (Fig. 4B). Indeed, over-expression of SAP and myc-DGK $\alpha$  in Jurkat cells resulted in the reduction of DGK $\alpha$  activity by 60% as measured in anti-myc immunoprecipitates, while myc-DGK $\alpha$  protein content was not affected (Fig. 4C). Conversely, in the same assay SAP mutants unable to bind either SH3-domains (SAP-R<sub>78</sub>A) or both tyrosine-phosphorylated proteins and SH3 domains (SAP-R<sub>55</sub>L) (3, 35) failed to inhibit DGK $\alpha$  (Fig. 4C). These findings indicate that SAP over-expression is sufficient to inhibit DGK $\alpha$  through a mechanism which requires SH3-binding ability of SAP.

The sequence surrounding tyrosine 335 of DGK $\alpha$  (SIY<sub>335</sub>PSV) features a high similarity to the SAP-SH<sub>2</sub> binding motif on SLAM (TIY<sub>281</sub>AQV) (36), suggesting that DGK $\alpha$  might bind directly to SAP. However, we could not detect a direct physical association between SAP and DGK $\alpha$  in a mammalian two hybrid assay (Supplemental Fig. 4A) or in co-immunoprecipitation assays using transfected 293T cells (Supplemental Fig. 4B), even when the two proteins were co-expressed with SLAM and Fyn. Altogether these results indicate that SAP does not inhibit DGK $\alpha$  by directly binding to it, but through the SAP-mediated recruitment of a yet unidentified SH3-containing protein.

The role of SAP in DGK $\alpha$  membrane recruitment in Jurkat cells was investigated by shRNA-mediated stable knock down of SAP. SAP silencing selectively impaired the recruitment of DGK $\alpha$  to the cell periphery induced by TCR/SLAM co-stimulation, but not by TCR/CD28 co-stimulation (Fig. 4D). Similar results were obtained upon transient siRNA-mediated down-regulation of SAP in Jurkat cells (data not shown). Thus, following engagement of the TCR and SLAM, SAP is required for DGK $\alpha$  enzymatic inhibition and recruitment to the cell periphery. In contrast, while SAP is required for TCR/CD28-induced enzymatic inhibition of DGK $\alpha$ , it is not essential for translocation of DGK $\alpha$  to the cell periphery. These findings indicate that the localization and enzymatic activity of DGK $\alpha$  are regulated through distinct processes and kinetics, although the mechanisms involved are still partially unknown.

### **Inhibition of DGK $\alpha$ rescues the functional defects caused by SAP deficiency in XLP**

Collectively, these data demonstrate that SAP is essential for regulation of DGK $\alpha$  activity upon T cell activation via the TCR/SLAM or TCR/CD28. We therefore reasoned that,



similarly to SAP-deficient Jurkat cells, T cells of XLP patients lacking functional SAP might be defective in the negative regulation of DGK $\alpha$ , thereby contributing to the defective lymphocyte responses observed in both XLP patients and in SAP null mice. To address this hypothesis, we first characterized the signaling capacity of SAP-deficient Jurkat cells following stimulation via the TCR and CD28. We then assessed whether pharmacological inhibition of DGK $\alpha$  by R59949, or its siRNA-mediated down-regulation, might rescue those aberrant T cell responses.

DAG-dependent recruitment of PKC $\theta$  to the plasma membrane is defective in T cells from SAP null mice, it is potentiated upon SAP over-expression (7, 37) and it is negatively regulated by constitutive activation of DGK $\alpha$  (38). Consistently, in SAP-deficient Jurkat cells, PKC $\theta$  recruitment to the immune synapse with super antigen-loaded APC was impaired (Fig. 5A). Both pharmacological inhibition (Fig. 5A) and siRNA-mediated silencing of DGK $\alpha$  (Fig. 5B) nearly completely rescued the defective translocation of PKC $\theta$  to the immune synapse observed in SAP-deficient Jurkat cells, pointing to a rescue of DAG-mediated signaling.

Upon TCR/CD28 co-stimulation, both T cells from XLP patients and Jurkat cells made SAP-deficient by siRNA-mediated down regulation, exhibit defective ERK-1/2 activation (5, 39), suggesting that DAG-mediated Ras-GTP signaling is impaired. Indeed, upon TCR/CD28 co-stimulation, Jurkat SAP-shRNA cells showed both a decrease in ERK-1/2 phosphorylation and a marked reduction of Ras-GTP loading, as measured by Ras-GTP pull down with GST-RBD (Fig 5C). Pharmacological inhibition of DGK $\alpha$  with R59949 fully restored both ERK-1/2 phosphorylation and Ras-GTP loading (Fig. 5C). These findings confirm that SAP is required for Ras activation in human T cells and provide further support to the hypothesis that negative regulation of DGK $\alpha$  is a critical step in the activation of the Ras pathway downstream of TCR/CD28. Interestingly, R59949 raised basal levels of ERK-1/2 phosphorylation without significantly affecting Ras-GTP loading, suggesting that under these conditions ERK-1/2 phosphorylation may be enhanced through a Ras-independent mechanism, likely through DAG-dependent PKC $\theta$  activation (40).

Activation of PKC $\theta$  and Ras pathways upon TCR/CD28 co-stimulation triggers NF-AT transcriptional activity, which plays a central role in cytokine production (8, 41). Moreover, NF-AT is activated upon SAP over-expression in Jurkat cells (42). Consistently SAP down-regulation in Jurkat cells impaired TCR/CD28-induced stimulation of NF-AT activity, as measured by luciferase reporter system (Fig. 5D, 5E). In SAP-deficient cells, pharmacological inhibition of DGK with 1  $\mu$ M R59949 fully restored TCR/CD28-induced activation of NF-AT without affecting basal NF-AT activity (Fig. 5D), while siRNA-mediated DGK $\alpha$  silencing resulted only in partial rescue (Fig. 5E). These data suggest that either DGK $\alpha$  along with other R59949-sensitive DGKs mediate NF-AT activation downstream from SAP, or that the low quantity of DGK $\alpha$  remaining after RNA interference may still transduce the signaling.

Upon T cell stimulation, activation of Ras, PKC $\theta$  and NF-AT signaling pathways leads to IL-2 production (41, 43, 44), which has been reported to be reduced in lymphocytes from XLP patients (5). Indeed, in Jurkat cells, shRNA-mediated SAP silencing reduced TCR/CD28-induced IL-2 secretion (Fig 5F). Pharmacological inhibition of DGK $\alpha$  by R59949 enhanced TCR/CD28-induced IL-2 production in control cells and fully rescued the defective IL-2 secretion of SAP-deficient Jurkat cells. These findings suggest that SAP-mediated negative regulation of DGK $\alpha$  is a key event in the modulation of T cell activation.

## Discussion

In this paper we demonstrate that, within minutes following co-stimulation of the TCR with either CD28 or SLAM, the enzymatic activity of DGK $\alpha$ , as assayed in immunoprecipitates in the presence of saturating DAG substrate concentration, undergoes a strong negative regulation without protein down-regulation. This finding is surprising, given accumulating evidence that synthesis of PA is increased upon T cell stimulation (15, 16, 45) and that DGK activity is increased in whole cell lysates from *in vivo* activated T cells (19). However, increased PA synthesis through DAG phosphorylation may depend on both positive regulation of one or more DGK isoforms and on increased availability of DAG, whose production by PLC $\gamma$  is increased upon TCR/CD28 co-stimulation (46). A parallel increase of DG and PA levels upon TCR stimulation has been indeed observed (45, 47). Several pieces of evidence suggest that most of the PA generated upon T cell activation derives from phospholipase D2-mediated phospholipids hydrolysis and from DGK $\zeta$ -mediated phosphorylation of DAG, while deletion of DGK $\alpha$  does not significantly affect PA production upon T cell stimulation (15, 16, 45). Nevertheless, recent genetic and biochemical data indicating that DGK $\alpha$  is a negative regulator of DAG-mediated TCR signaling (15, 17) are highly consistent with our finding that enzymatic activity of DGK $\alpha$  is reduced upon TCR co-stimulation with either CD28 or SLAM. This regulation appears to be isoform-specific, as DGK $\zeta$  activity is unaffected by TCR triggering (Supplemental Fig. 1). Interestingly, the previous finding that stimulation of the sole TCR is not sufficient to promote sustained DAG signaling (48) is consistent with our observation that TCR activation in PBLs is not sufficient to inhibit DGK $\alpha$  activity in absence of co-stimulation. Moreover, co-stimulation of TCR/CD28, compared to TCR alone, strongly enhances production of DAG but not of PA (15), suggesting a slowdown in the rate of DAG conversion to PA that is consistent with a negative regulation of DGK activity.

The molecular mechanisms underlying the negative regulation of DGK $\alpha$  have not been elucidated yet. Herein we report that the adaptor function of SAP is required for DGK $\alpha$  inhibition induced by TCR co-stimulation with either SLAM or CD28. SAP is essential for SLAM tyrosine phosphorylation by recruiting the Src-related kinase FynT (3, 34), however, a growing body of evidence indicates that SAP is also involved in T cell responses to antigenic stimulation (2). Indeed SAP binds directly to ITAM sequences of CD3 $\zeta$  subunit (29), while TCR activation promotes the recruitment of SAP and SLAM family receptors to the signalosome (28, 29, 49). Furthermore, genetic deletion of SAP in mice results in the impairment of TCR/CD28-induced DAG-mediated activation of PKC $\theta$  and of downstream signaling events (7). Moreover, TCR/CD28-induced ERK-1/2 activation and IL-2 production, which are both dependent on DAG-mediated activation of RasGRP, are impaired in T cells from SAP-deficient XLP patients (5). Intriguingly, SAP is physically associated to PKC $\theta$  and it has been demonstrated that SAP over-expression, which is sufficient to inhibit DGK $\alpha$ , promotes PKC $\theta$  recruitment to the immune synapse (37). Finally, we and others have shown that, in Jurkat cells, SAP silencing impairs TCR-induced Ras-GTP loading, ERK-1/2 activation, PKC $\theta$  recruitment, NF-AT activation and IL-2 production (5–7). Together, these observations suggest that, upon TCR/CD28 co-stimulation, SAP is required for optimal DAG signaling. The finding that SAP is required for inhibition of DGK $\alpha$  might provide a mechanistic link between SAP and the regulation of DAG signaling. Thus, we propose that, upon stimulation of T cells from either SAP-deficient XLP patients or SAP null mice, DGK $\alpha$  may inappropriately retain a high enzymatic activity, thereby converting DAG to PA and decreasing DAG signaling.

If this hypothesis holds true, we would expect that inhibition or down-regulation of DGK $\alpha$  would rescue, at least partially, the defective signaling of SAP-deficient T cells. Accordingly, we observed that the inhibition of DGK $\alpha$  enzymatic activity in SAP-deficient

Jurkat cells rescued defective DAG-dependent PKC $\theta$  membrane recruitment, Ras-GTP loading, ERK-1/2 and NF-AT activation and IL-2 production. These findings indicate that the excess of DGK $\alpha$  activity contributes to the defective signaling of SAP-deficient cells, and, along with the demonstration that SAP over-expression inhibits DGK $\alpha$ , provide further support to the hypothesis that SAP negatively regulates DGK $\alpha$ . According to these findings, the negative regulation of DGK $\alpha$  activity represents a key event controlling the early phase of T cell activation by contributing to fine tuning of DAG levels required for appropriate signaling.

In here we observed that co-stimulation of the TCR with either SLAM or CD28 induces DGK $\alpha$  exit from the nucleus and accumulation in the cytoplasm with only partial localization at the plasma membrane. This finding appears to contrast previous studies reporting GFP-DGK $\alpha$  localization at the plasma membrane of CD3/CD28 co-stimulated Jurkat cells, however according to the same authors DGK $\alpha$  membrane translocation is rapid and transient and can be visualized in conditions that inhibit its relocalization to the cytoplasm (17, 21). Moreover, DGK $\alpha$  plasma membrane localization was clearly induced by stronger stimuli, such as the activation of ectopically over-expressed muscarinic receptor (20, 21, 50, 51), or antigen challenge in vivo (19). Further support to the hypothesis that enzymatic activity of DGK $\alpha$  regulates DAG level at the plasma membrane of T cells derives both from our finding that uncoupling of DGK $\alpha$  inhibition from TCR stimulation impairs PKC $\theta$  recruitment to the immune synapses (Fig. 5A) and from the observation that pharmacological inhibition of DGK $\alpha$  allows accumulation of DAG at the plasma membrane of T cells, thereby triggering activation of Ras signaling (52).

Interestingly, stimulation with either SLAM or TCR alone did not induce DGK $\alpha$  translocation from the nucleus, indicating that the concerted signaling via both receptors is required. Moreover, the finding that SAP, which is essential for SLAM tyrosine phosphorylation and signaling, is required for translocation induced exclusively by TCR/SLAM, but not by TCR/CD28, suggests that SAP may not directly regulate DGK $\alpha$  sub cellular localization. In addition, the fact that upon TCR/CD28 co-stimulation, SAP is required for inhibition of DGK $\alpha$  activity, but not for its exit from the nucleus, indicates that enzymatic activity and localization of DGK $\alpha$  are regulated independently of each other, as suggested also by the different kinetics of the two processes. Importantly, these findings also indicate that DGK $\alpha$  exit from the nucleus is not required for the inhibition of its enzymatic activity. The massive exit from the nucleus may reflect a potential increase in the availability of DGK $\alpha$  outside the nucleus for control of DAG signaling both at the plasma membrane and at intracellular vesicles. Indeed, several reports indicate a role of DGKs in T cells intracellular trafficking (50, 53, 54). Conversely, DGK $\alpha$  exit from the nucleus may contribute to regulate nuclear pools of DAG and PA acid. Indeed, several DGK isoforms have been reported to localize in the nucleus where they contribute to regulate transcription and cell cycle progression (55).

Previous evidence indicates that, SFK-induced phosphorylation of DGK $\alpha$  on tyrosine 335 mediates its activation and membrane localization upon growth factor stimulation of epithelial and large cell lymphoma cells (24, 56). Moreover in T cells DGK $\alpha$  phosphorylation by LCK on tyrosine 335 mediates CD3/CD28-induced recruitment of DGK $\alpha$  to the plasma membrane (21). Surprisingly, in our study pharmacological inhibition of SFKs did not affect neither TCR/CD28-induced inhibition of DGK $\alpha$  nor its exit from the nucleus, suggesting that both events are independent from SFKs-mediated tyrosine phosphorylation of DGK $\alpha$ . Conversely PP2 completely blocks DGK $\alpha$  inhibition and exit from the nucleus induced by CD3/SLAM, as SLAM signaling is dependent on Fyn tyrosine kinase.

The mechanism by which SAP regulates DGK $\alpha$  still remains to be elucidated. Based on the high similarity between the sequences surrounding Tyr 335 of DGK $\alpha$  and Tyr 281 of SLAM, we investigated the hypothesis that SAP may regulate DGK $\alpha$  by associating with it in a complex. However, we could not detect any direct or indirect physical interaction between the two proteins, even in a reconstituted association assay in a mammalian two-hybrid system. Our data demonstrate that SAP ability to inhibit DGK $\alpha$  requires the interaction with a yet unidentified SH3 domain-containing protein. The finding that inhibition of DGK $\alpha$  is independent of SFKs activity suggests that the SAP interactor required for DGK $\alpha$  inhibition is not Fyn. The previous observation that SAP over-expression activates Cdc42 signaling by interacting with SH3-containing  $\beta$  PIX and independently of Fyn, suggests that DGK $\alpha$  may be regulated by Cdc42-dependent PAK activation. However, the PAK-specific inhibitor IPA-3 did not affect the inhibition of DGK $\alpha$  following TCR/SLAM co-stimulation (Fig. 3B). Finally, upon TCR stimulation of Jurkat cells, SAP silencing results in defective tyrosine phosphorylation of several proteins, including LAT and SLP76 (39). As both LAT and SLP76 regulate PLC $\gamma$  activation (57), it is possible to speculate that SAP regulates DGK $\alpha$  by controlling PLC $\gamma$  activity. Consistently with this possibility, PLC activity and cytosolic free calcium are both required for DGK $\alpha$  inhibition and membrane recruitment. However, lack of SAP in T cells of both SAP null mice and XLP patients does not affect PLC $\gamma$ -mediated intracellular calcium increase (5, 58). Moreover, cell stimulation with a calcium ionophore and phorbol ester failed to inhibit DGK $\alpha$  activity and to recruit it to the plasma membrane, indicating that activation of PLC is necessary but not sufficient to regulate both enzymatic activity and membrane localization (data not shown). Furthermore, the requirement for PLC activity in regulating DGK $\alpha$  suggests that the two enzymes may act as a bi-component unit able to finely modulate the extent and the duration of DAG signaling.

In conclusion our findings suggest that the coordinated, but independent, control of DGK $\alpha$  enzymatic activity and of its localization regulates both its access to DAG and its rate of conversion to PA. Upon T cell stimulation, such coordinated and complementary mechanism of regulation might finely tune the intensity and the duration of DAG-mediated signaling. Indeed, SAP silencing, by uncoupling TCR/CD28 co-stimulation from DGK $\alpha$  inhibition, results in the impairment of TCR/CD28-induced DAG-mediated signaling, providing further evidence that the SAP-mediated negative regulation of DGK $\alpha$  is crucial for the ability of T cells to trigger DAG-mediated responses.

Similarly to cAMP-signaling, which is triggered by G protein coupled receptors (GPCRs) by reciprocal regulation of both adenylylase and phosphodiesterase activities (59), the findings presented here suggest that TCR/CD28 controls DAG-signaling both by means of PLC $\gamma$  activation and DGK $\alpha$  inhibition. Similarly, genetic and biochemical studies in *C. elegans* motoneurons and murine hepatocytes showed that DAG-mediated signaling is controlled by GPCR-dependent reciprocal regulation of both PLC and DGK $\theta$  (60–62).

In summary, our findings demonstrate that SAP-mediated DGK $\alpha$  inhibition is an early event in TCR signaling, which might be required for efficient T cell activation. The impaired regulation of DGK $\alpha$  activity in SAP-deficient lymphocytes may contribute to their defective TCR-induced responses, suggesting that pharmacological inhibition of DGK $\alpha$  could be useful in the treatment of certain manifestations of XLP.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations List

<b>XLP</b>	X-linked lymphoproliferative disease
<b>DAG</b>	diacylglycerol
<b>DGK</b>	diacylglycerol kinase
<b>PA</b>	phosphatidic acid
<b>WCL</b>	whole cell lysate
<b>YFP</b>	yellow fluorescent protein
<b>WGA</b>	wheat germ agglutinin
<b>SFK</b>	Src family kinase
<b>FBS</b>	fetal bovine serum
<b>GPCR</b>	G protein coupled receptor
<b>RCF</b>	relative centrifugal force

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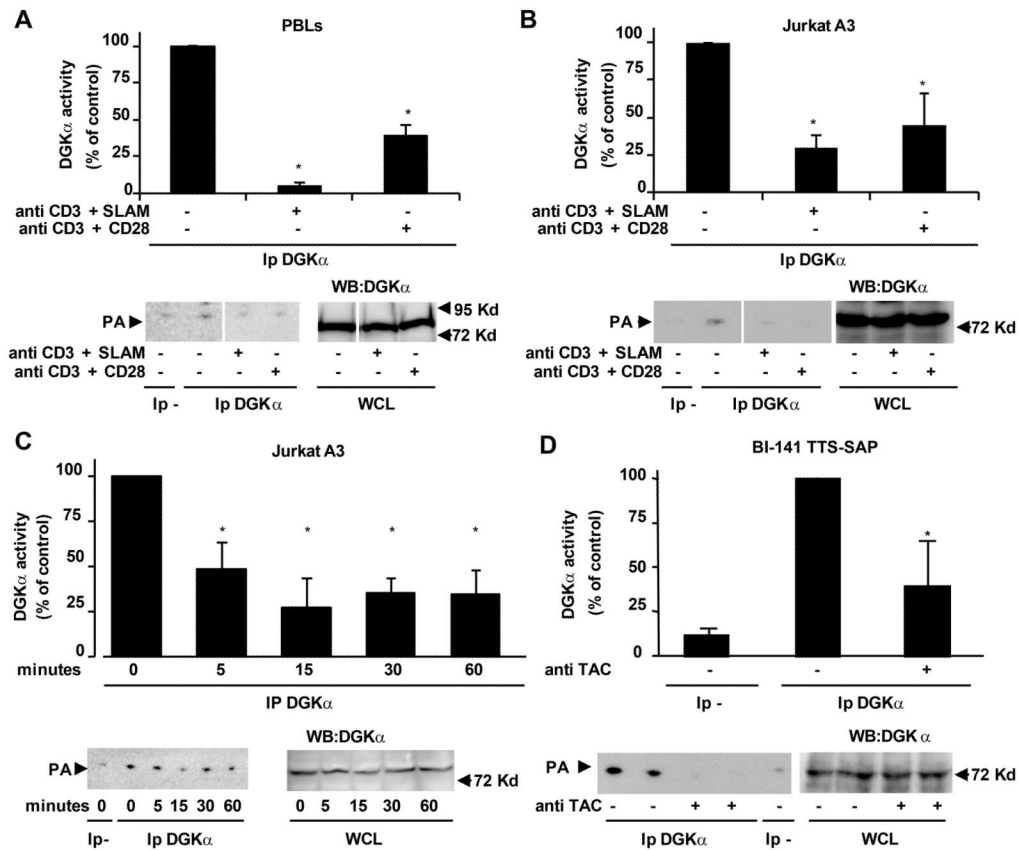
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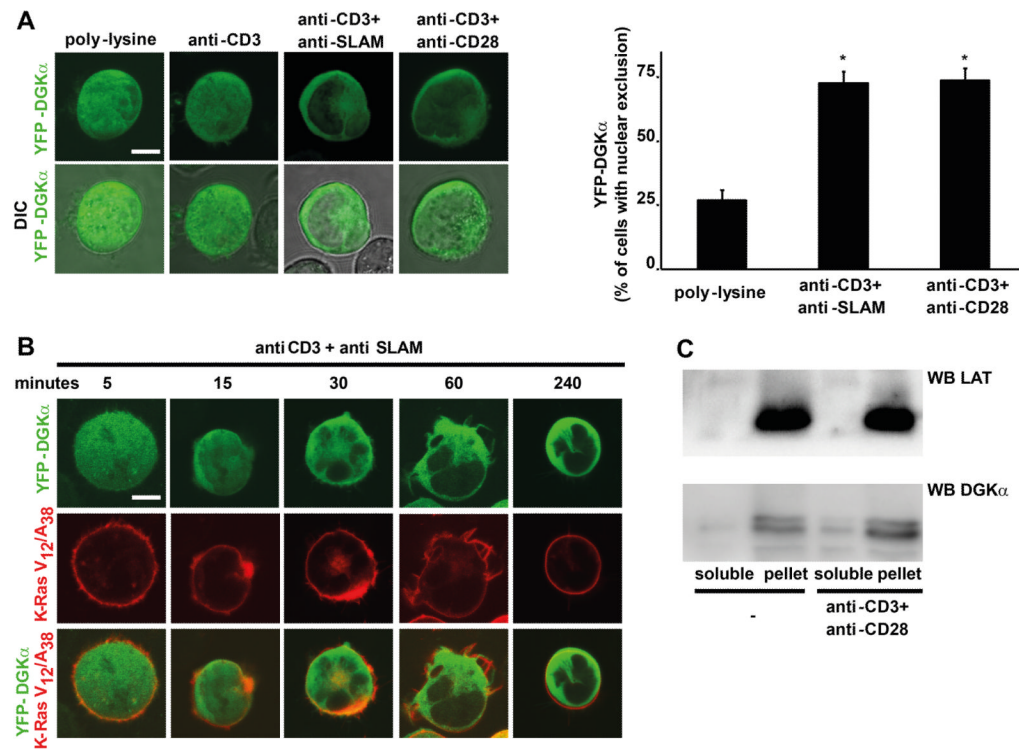


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**Figure 1. DGK $\alpha$  is inhibited upon T cell activation**

PBL (A) and Jurkat A3 cells (B) were stimulated for 15 min with 10  $\mu$ g/ml of the indicated antibodies and lysed. Anti-DGK $\alpha$  immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole cell lysate was analyzed by western blot with anti-DGK $\alpha$  antibody to ensure equal loading. A representative experiment is shown (lower panel) together with a graph showing the mean  $\pm$  SE of four independent experiments shown as percentage of control (upper panel), \*t-test vs. control  $p < 0.05$ . (C) Jurkat A3 cells were stimulated with 10  $\mu$ g/ml anti-CD3 and anti-SLAM antibodies and lysed at the indicated times. Anti-DGK $\alpha$  immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole cell lysate was analyzed by western blot with anti-DGK $\alpha$  antibody to ensure equal loading. A representative experiment is shown (lower panel) together with a graph showing the mean  $\pm$  SE of four independent experiments shown as percentage of control (upper panel), \* t-test vs. control  $p < 0.05$ . (D) BI-141 TTS-SAP cells were stimulated for 15 min with 5 mg/ml anti-TAC and 4 mg/ml anti-IgG and lysed. Anti DGK $\alpha$  immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole cell lysate was analyzed by western blot with anti DGK $\alpha$  antibody to ensure equal loading. A representative experiment is shown (lower panel) together with a graph showing the mean  $\pm$  SE of three independent experiments shown as percentage of control (upper panel), \* t-test vs. control  $p < 0.05$ .

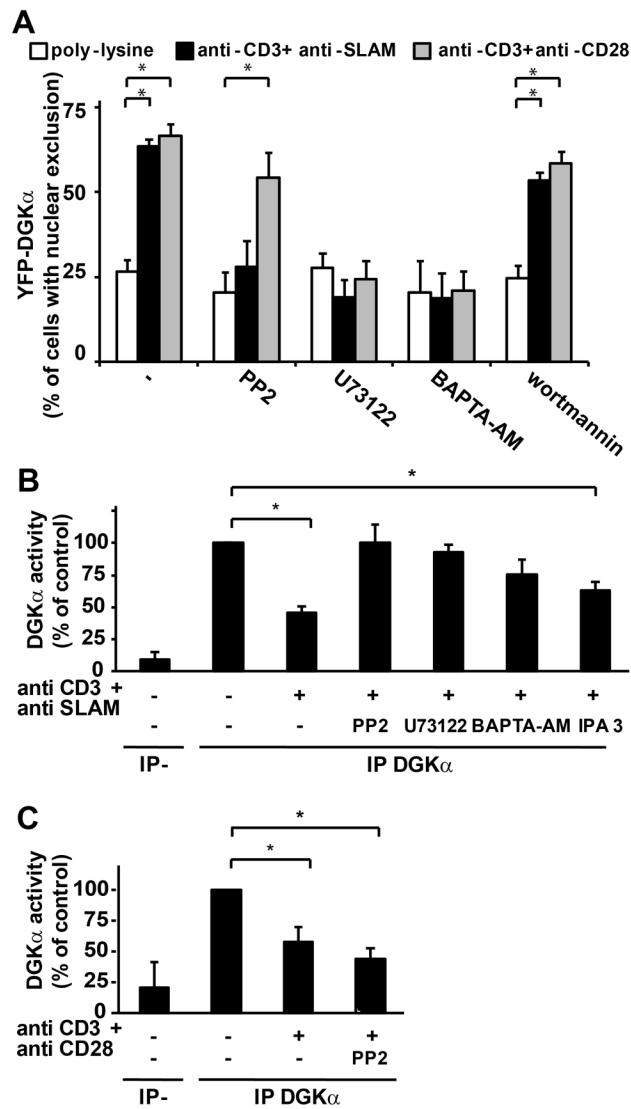


### Figure 2. YFP-DGK $\alpha$ localization upon T cell stimulation

(A) Jurkat A3 cells were transfected with YFP-DGK $\alpha$  (green) and after 24 hours were serum starved for 2 hours and seeded for 1 hour on either poly-L-lysine, anti-CD3, anti-CD3+anti-SLAM or anti-CD3+anti-CD28 (10  $\mu$ g/ml each) coated glass-bottom dishes and microscope images acquired. Representative pictures are shown along with a quantification from three independent experiments, \* t-test vs. control  $p < 0.0005$ . Scale bar = 5  $\mu$ m.

(B) Jurkat A3 cells were transfected with YFP-DGK $\alpha$  (green) and DS-Red-K-Ras V12/A38 (red), and after 72 hours were serum starved for 2 hours and seeded on anti-CD3+anti-SLAM agonistic antibodies (10  $\mu$ g/ml each) coated glass-bottom dishes and images acquired at the indicated times. Representative pictures are shown, scale bar = 5  $\mu$ m.

(C) Jurkat A3 cells were stimulated with anti-CD3 and anti-CD28 agonistic antibodies (10  $\mu$ g/ml each) and homogenized 1 hour later. The post-nuclear and -mitochondrial fraction was separated by centrifugation (100,000 RCF) in a soluble fraction and in a membrane-associated fraction. 1/50 of the soluble fraction and the entire membrane-associated fraction were analyzed by western blotting for DGK $\alpha$  and LAT content.

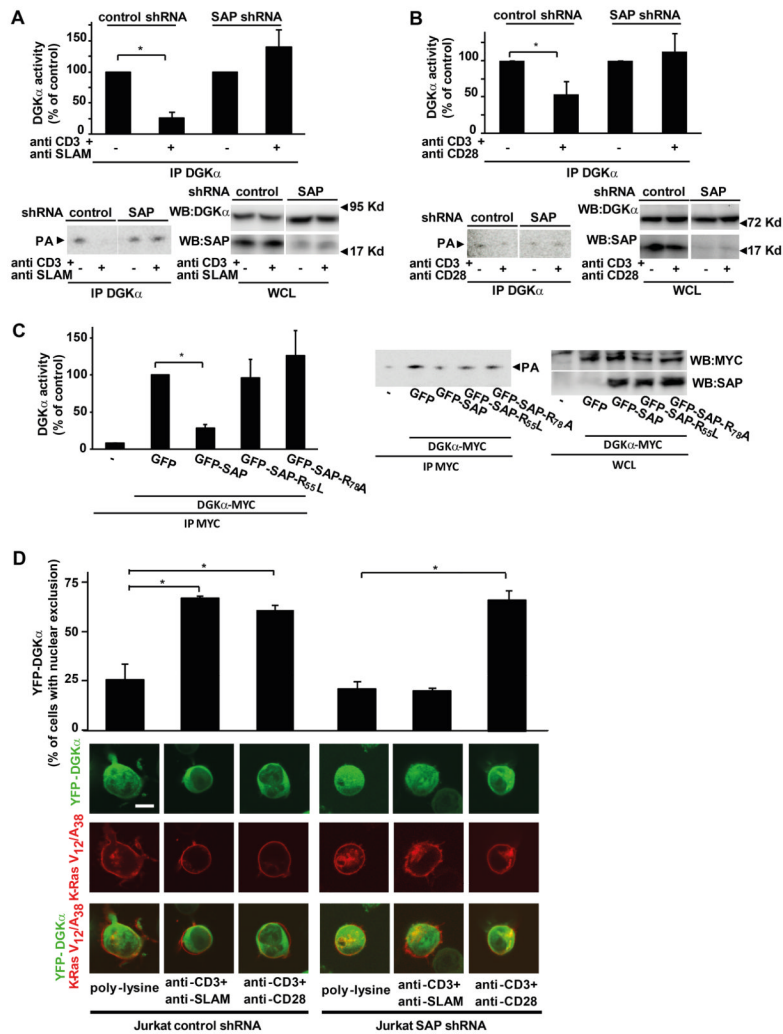


**Figure 3. Phospholipase C and calcium mediate TCR-induced regulation of DGK $\alpha$**

(A) Jurkat A3 cells were transfected with YFP-DGK $\alpha$  and after 24 hours were serum starved for 2 hours, seeded for 1 hour on poly-L-lysine, CD3+SLAM or CD3+CD28 agonistic antibodies (10 $\mu$ g/ml each) coated glass-bottom dishes in the presence or absence of the indicated inhibitors (50 $\mu$ M PP2, 5  $\mu$ M U73122, 10  $\mu$ M BAPTA-AM or 100 nM Wortmannin) and images acquired. The quantification of three independent experiments is shown, \* t-test vs. control p<0.0005.

(B) Jurkat A3 cells were treated with the indicated inhibitors (10  $\mu$ M PP2, 5  $\mu$ M U73122, 10  $\mu$ M BAPTA-AM, 10  $\mu$ M IPA-3) or vehicle for 30 min before stimulation with 10  $\mu$ g/ml anti-CD3 and anti-SLAM antibodies. After 15 min cells were lysed and anti DGK $\alpha$  immunoprecipitates were assayed for DGK enzymatic activity. The graph shows the mean  $\pm$  SE of at least three independent experiments for each inhibitor, \* t-test vs. control p<0.05.

(C) Jurkat A3 cells were treated with 10  $\mu$ M PP2 or vehicle for 30 min before stimulation with 10  $\mu$ g/ml anti-CD3 and anti-CD28 antibodies. After 15 min cells were lysed and anti DGK $\alpha$  immunoprecipitates were assayed for DGK enzymatic activity. The graph shows the mean  $\pm$  SE of at three independent experiments, \* t-test vs. control p<0.05.



#### Figure 4. SAP negatively regulates DGK $\alpha$ activity

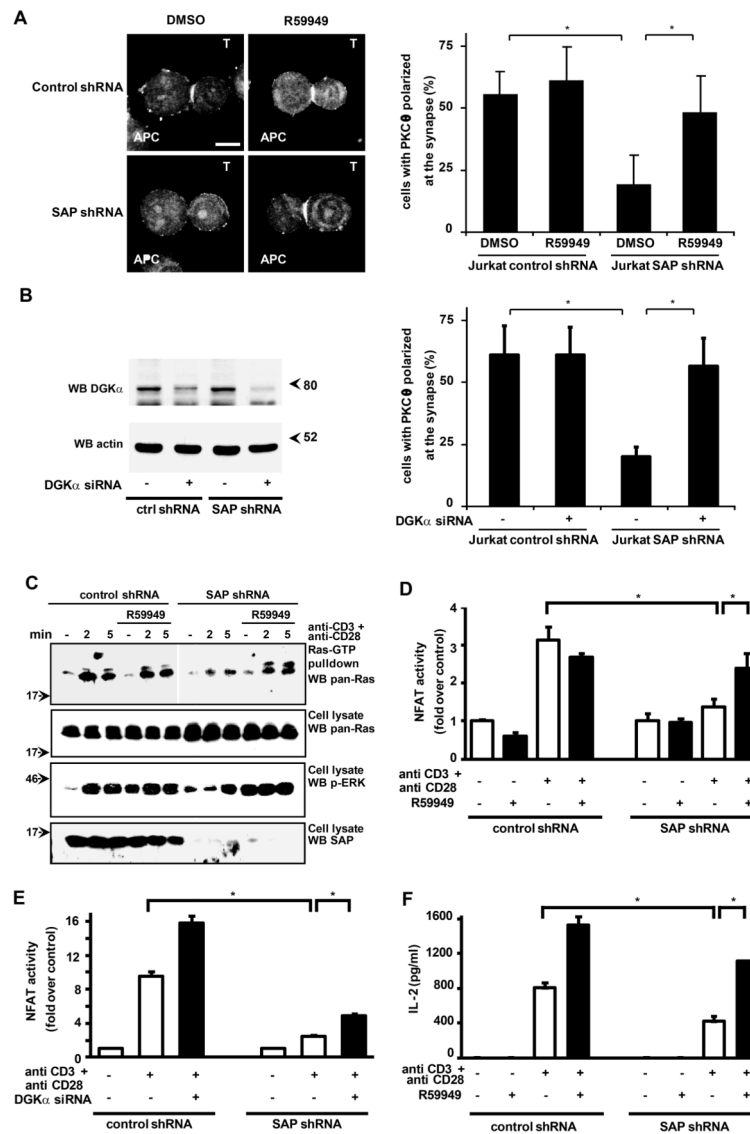
(A) Jurkat control-shRNA or Jurkat SAP-shRNA cells were stimulated for 15 min with 10  $\mu$ g/ml anti-CD3 and anti-SLAM antibodies and lysed. Anti-DGK $\alpha$  immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole cell lysate was analyzed by western blot with anti-DGK $\alpha$  antibody to ensure equal loading and with anti-SAP antibody to verify the down-regulation of SAP expression. A representative experiment is shown together with a graph of the mean  $\pm$  SE of three independent experiments shown as percentage of control, \* t-test vs. control  $p < 0.05$ .

(B) Jurkat control-shRNA or Jurkat SAP-shRNA were stimulated for 15 min with 10  $\mu$ g/ml anti-CD3 and anti-CD28 antibodies and lysed. Anti DGK $\alpha$  immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole cell lysate was analyzed by western blot with anti-DGK $\alpha$  antibody to ensure equal loading and with anti-SAP antibody to verify the down-regulation of SAP expression. A representative experiment is shown together with a graph of the mean  $\pm$  SE of three independent experiments shown as percentage of control, \* t-test vs. control  $p < 0.05$ .

(C) Jurkat A3 cells were transiently co-transfected with myc-DGK $\alpha$  and the indicated GFP-SAP mutants. After 48 hours, cells were lysed and anti-myc immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole cell lysate was assayed by western blot with anti-myc and anti-SAP antibodies to verify transfection efficiency. A

representative experiment is shown along with a graph showing the mean  $\pm$  SE of three independent experiments shown as percentage of control, \* t-test vs. control  $p < 0.05$ .

**(D)** Jurkat control-shRNA and Jurkat SAP-shRNA were transfected with YFP-DGK $\alpha$  (green) and DS-Red-K-Ras V12/A38 (red). After 24 hours, cells were serum starved for 2 hours, seeded for 1 hour on poly-L-lysine, anti-CD3+anti-SLAM or anti-CD3+antiCD28 agonistic antibodies (10  $\mu$ g/ml each) coated glass-bottom dishes and images acquired. Representative pictures are shown together with a quantification from three independent experiments, \* t-test vs. control  $p < 0.05$ . Scale bar = 5  $\mu$ M.



**Figure 5. DGK $\alpha$  inhibition rescues defective TCR-induced diacylglycerol-dependent signaling and IL-2 production of SAP-deficient T lymphocytes**

(A) Jurkat control-shRNA and Jurkat SAP-shRNA cells (T) were pretreated with R59949 (10  $\mu$ M 30 min) incubated with super antigen-loaded Raji cells (APC) for 15 min, fixed and stained for PKC $\theta$ . Representative pictures are shown. Scale bar = 5  $\mu$ m. Cells displaying PKC $\theta$  at the immune synapse were counted. The histogram shows data from 3 independent experiments as mean  $\pm$  SE (\* t-test  $p < 0.05$ ).

(B) Jurkat control-shRNA and Jurkat SAP-shRNA cells (T) were transfected with DGK $\alpha$  specific siRNA or control siRNA. After 72 hours cells were lysed and analyzed by western blot with anti DGK $\alpha$  and anti actin antibodies (left panel). At the same time cells were incubated with super antigen-loaded Raji cells (APC) for 15 min, fixed and stained for PKC $\theta$ . Cells displaying PKC $\theta$  at the synapse were counted (right panel). The histogram shows data from 3 independent experiments as mean  $\pm$  SE (\* t-test  $p < 0.05$ ).

(C) Control shRNA Jurkat or SAP shRNA Jurkat cells were stimulated with 1  $\mu$ g/ml anti-CD3 and 0,1  $\mu$ g/ml anti-CD28 antibodies, in the presence or in absence of 1  $\mu$ M R59949. After 15 min cells were lysed and Ras-GTP separated by pull-down with Raf-RBD and

quantified by western blotting with anti pan-Ras antibody. Total Ras, phospho-ERK-1/2 and SAP content were revealed in whole cell lysates by western blotting.

**(D)** Jurkat control-shRNA and Jurkat SAP-shRNA were transfected with a dual-luciferase NF-AT reporter system. After 48 hours cells were stimulated with 1  $\mu\text{g}/\text{ml}$  anti-CD3 and anti-CD28 antibodies in the presence or absence of R59949 1mM. After 16 hours of stimulation cells were lysed and analyzed for NF-AT-driven luciferase activity. Graph shows the mean  $\pm$  SE of quadruplicates of a representative experiment, \* t-test vs. control  $p < 0.05$ .

**(E)** Jurkat control-shRNA or Jurkat SAP-shRNA were transfected with a siRNA targeting DGK $\alpha$  or a control siRNA and a dual-luciferase NF-AT reporter system. After 48 hours, cells were stimulated with 1  $\mu\text{g}/\text{ml}$  anti-CD3 and anti-CD28 antibodies. After 16 hours of stimulation cells were lysed and analyzed for NF-AT driven luciferase activity. Graph shows the mean  $\pm$  SE of quadruplicates of a representative experiment, \* t-test vs. control  $p < 0.05$ .

**(F)** Jurkat control-shRNA or Jurkat SAP-shRNA were stimulated with 1  $\mu\text{g}/\text{ml}$  anti-CD3 and 0.1  $\mu\text{g}/\text{ml}$  anti-CD28 antibodies. After 72 hours, cells were lysed and the amount of IL-2 released in the medium was measured by ELISA. Graph shows the mean  $\pm$  SE of four replicates of a representative experiment, \* t-test vs. control  $p < 0.05$ .