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Abstract: Exosomes are small membrane vesicles that intracellularly accumulate into late or multivesicular endosomes (multivesicular bodies, MVB). Exosomes have a particular lipidic and protein content, reflecting their origin as intraluminal vesicles of late endosomes. The stimulation of several hematopoietic cells induces the fusion of the limiting membrane of the MVB with the plasma membrane, leading to the release of exosomes towards the extracellular environment. In T lymphocytes, stimulation of the T-cell receptor (TCR) induces the fusion of the MVBs with the plasma membrane and exosomes carrying several bio-active proteins are secreted. Among these proteins, the pro-apoptotic protein Fas ligand (FasL) is released as its non-proteolysed form (mFasL), associated to the exosomes. These mFasL-bearing exosomes may trigger the apoptosis of T lymphocytes. Here, we present evidences supporting a role of Diacylglycerol Kinase α

(DGK α), a diacylglycerol (DAG)-consuming enzyme, on the secretion of exosomes carrying mFasL, and the subsequent activation-induced cell death (AICD) on a T cell line and primary T lymphoblasts.

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Editor
Biochimie

6 February 2006

Dear Sir/Madam,

Enclosed you will find our manuscript entitled "**A new role of Diacylglycerol Kinase α on the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes**" by R. Alonso *et al.* that we would like to submit for publication in the Biochimie Special Issue "From lipid analysis to genetic disorders". We review and address here the specific role of Diacylglycerol kinase α (DGK α) on apoptosis, and we show that expression of DGK α decreases FasLigand (FasL)-mediated, apoptosis of T lymphocytes, whereas the inhibition of DGK α enhanced apoptosis. DGK α regulates the release of lethal exosomes/microvesicles bearing FasL. Our results provide insights on a new role of DGK α on the regulation of apoptosis.

We look forward to hearing from you.

Sincerely yours,

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A new role of Diacylglycerol Kinase α on the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes (¹).

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Running Title: Diacylglycerol kinase regulates the secretion of exosomes.

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SUMMARY

Exosomes are small membrane vesicles that intracellularly accumulate into late or multivesicular endosomes (multivesicular bodies, MVB). Exosomes have a particular lipidic and protein content, reflecting their origin as intraluminal vesicles of late endosomes. The stimulation of several hematopoietic cells induces the fusion of the limiting membrane of the MVB with the plasma membrane, leading to the release of exosomes towards the extracellular environment. In T lymphocytes, stimulation of the T-cell receptor (TCR) induces the fusion of the MVBs with the plasma membrane and exosomes carrying several bio-active proteins are secreted. Among these proteins, the pro-apoptotic protein Fas ligand (FasL) is released as its non-proteolysed form (mFasL), associated to the exosomes. These mFasL-bearing exosomes may trigger the apoptosis

of T lymphocytes. Here, we present evidences supporting a role of Diacylglycerol Kinase (DGK), a diacylglycerol (DAG)-consuming enzyme, on the secretion of exosomes carrying mFasL, and the subsequent activation-induced cell death (AICD) on a T cell line and primary T lymphoblasts.

Abbreviations:

7-AAD, 7-Amino-Actinomycin D; Ab, antibody; AICD, Activation-induced cell death; CCh, carbamyl choline (carbachol); CsA, cyclosporin A; DAG, diacylglycerol; DGK, diacylglycerol kinase; DGK_i, DGK inhibitor; DOG, 1,2-dioctanoyl-*sn*-glycerol; FasL, Fas Ligand; HM1R, human muscarinic receptor, type 1; HRP, horseradish peroxidase; mAb, monoclonal antibody; MVBs, multivesicular bodies; PA, phosphatidic acid; PKC, Protein kinase C; PKD Protein Kinase D; PLC, phospholipase C; PS, phosphatidylserine; TCR, T-cell receptor; TGN, trans-Golgi-network; WB, Western blot.

Keywords: Apoptosis/ T lymphocytes/ Fas ligand/Exosomes

1. Introduction

T cell receptor (TCR)¹ stimulation triggers the generation of several second messengers such as calcium and diacylglycerol (DAG) and the subsequent activation of diverse intracellular signalling pathways [1], that may trigger activation-induced cell death (AICD) [2]. AICD is mediated by up-regulation of certain pro-apoptotic genes such as Fas Ligand (FasL) [3]. Interaction of Fas (APO1/CD95) cell surface death receptor with FasL induces apoptosis in T lymphocytes [4]; therefore, AICD plays an important role in maintaining the peripheral immune tolerance and T lymphocyte homeostasis [5] [6].

In the last years, a novel mechanism involved in FasL-mediated apoptosis in Jurkat T cells, T cell lymphoblasts and several tumour cells has been described [7-9]. This mechanism is based on the fact that FasL can be intracellularly stored into small vesicles, termed microvesicles or exosomes, which accumulate inside secretory vesicles with structure of late endosomes/MVBs

[7]. Upon T lymphocyte stimulation, the fusion of MVBs with the plasma membrane results in the release of these exosomes [10,11], which contain pro-apoptotic, non-proteolysed FasL (mFasL) [7,12]. In addition to mFasL, exosomes produced by T lymphocytes have been shown to bear TCR/CD3 complexes, CD8, CD2, MHC class I and cell-adhesion molecules [11,13] at the surface, which may play an important role in the specific delivery of mFasL to the target cells. Secretory vesicular traffic involves the formation of vesicles at the Golgi apparatus, the formation of late endosomes/MVBs and their fusion to the plasma membrane. All these events are regulated by specific lipids and enzymes that metabolize lipids, which supports an essential role of lipid homeostasis in the regulation of protein sorting and Golgi-mediated transport. More specifically, the regulation of the membrane levels of diacylglycerol (DAG) and phosphatidic acid (PA), has been recognized as a crucial factor for protein transport from the Golgi to the plasma membrane [14] [15] [16]. The protein family of diacylglycerol kinases (DGKs) constitutes one of the main regulators of the DAG levels in the cells. DGKs phosphorylate DAG to PA, and downregulate the action and function of this lipid [17]. It has been demonstrated that a type I DGK, DGK α , which is highly expressed in T lymphocytes, transiently translocates to the plasma membrane upon TCR triggering [18]. At this location, DGK α acts as a negative modulator of the DAG levels generated during T cell activation [18] [19,20]. In other studies, it has been shown the translocation of DGK α to the *trans*-Golgi network (TGN) upon receptor stimulation [21]. Moreover, it has been shown that sustained DAG levels at the Golgi facilitate the TGN dynamics [16], and the formation of secretory vesicles [15]. In these studies, the inhibition of DAG production results in the blockade of protein transport from the TGN to the cell surface [16]. All these results suggest that DGK α could be a possible candidate for the negative regulation of DAG levels at Golgi, which in turn would control the dynamics of TGN and the function of secretory vesicles. To investigate this possibility, we analysed the role of DGK α on the secretion of mFasL-bearing exosomes and AICD in T lymphocytes. Here, we show that DGK α expression in T cells inhibits secretion of FasL-bearing exosomes triggered by receptor stimulation, and subsequent AICD is impaired. Conversely, the inhibition of DGK α enhances the secretion of these lethal exosomes carrying mFasL and enhances AICD.

2. Material and Methods

Cell cultures

The human T cell leukemia Jurkat (clone E6.1) was cultured as described [22]. J-HM1-2.2 cells expressing human muscarinic type 1 receptor (HM1R) have been described [23]. Human primary T lymphoblasts were prepared and cultured as described [24]. Human embryonic kidney (HEK) 293 cells were obtained from the ATCC.

Antibodies and reagents

Rabbit polyclonal antibody (Ab) TGN46 was a gift from Dr. Banting (University of Bristol, UK). The anti-DGK Ab was a gift of H. Kanoh (Sapporo Medical University, Japan). The mouse monoclonal antibody anti-CD28 was a gift of Dr. Moretta (Institute Gianina Gaslini, Genove, Italy). The mouse monoclonal antibody anti-CD45 was a gift of Dr. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). The apoptosis-inducer Anti-Fas (clone CH-11) monoclonal Ab (mAb) was obtained from MBL. Anti-Fas (CD95) DX2 mAb, anti-Fas Ligand (CD95L) NOK-1 mAb, anti-CD3 (UCHT1) and Annexin V-PE were obtained from BD. Rabbit polyclonal anti-Fas Ligand (CD95L) Q-20 for WB was from Santa Cruz Biotechnology. Anti-CD63 (clone NKI-C-3) was from Oncogene. Anti-GFP polyclonal Ab (Living Colors) was from Clontech. Rabbit polyclonal against Giantin (cis and medial Golgi marker, PRB-114C) was from Covance. All horseradish peroxidase-coupled second-step Abs were from Dako. Actinomycin D was obtained from SIGMA. DGK inhibitor II (R59949) was purchased from Calbiochem. Secondary, Cy5-conjugated Abs were from Jackson ImmunoResearch Inc.

Expression vectors and transfection assays

The plasmids pEF-GFP, pEF-GFP-DGK have been described [18]. For transfection experiments, cells were transiently transfected with 20-30 µg of the plasmids as described [22]. Human FasL cDNA was subcloned from pBXhFL-1 plasmid into pEF4/Myc-HisB (Invitrogen). Sub-confluent HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen, Life Technologies).

Assessment of apoptosis

Phosphatidylserine (PS) exposure in the outer leaflet of the cytoplasmic membrane was evaluated by staining the cells with Annexin V-PE, and late apoptosis was measured by cell permeability to 7-Amino-Actinomycin D (7-AAD). GFP-DGK⁺ and GFP-DGK⁻ cells were gated by green fluorescence, and Annexin-V-PE binding and 7-AAD permeability in both populations were analysed by flow cytometry.

Western blot analysis

Cells and exosomes were lysed for 10 min in RIPA supplemented with protease inhibitors. Cellular and exosomes proteins were separated by SDS-PAGE under reducing conditions and transferred to HybondTM ECLTM membranes (Amersham Pharmacia). For CD63 detection, proteins were separated under non-reducing conditions as described [25]. After the incubation with the appropriate primary antibody, the blots were developed with peroxidase-conjugated secondary antibodies using enhanced chemiluminescence (ECL) reagents and following standard protocols.

Cell fractionation

J-HM1-2.2 cells were disrupted in 2 ml of lysis buffer [26] and the lysate was fractionated on a discontinuous Percoll (Amersham Pharmacia) gradient as described [26]. Fractions were analysed for the presence of CD63, FasL, DGK⁻, PKD1 and Lamp-1 proteins by WB. Density was calibrated by using density marker beads (Amersham Pharmacia).

Immunofluorescence analysis

J-HM1-2.2 cells were stained as described [22] with primary Abs (anti CD63, anti TGN46, anti-Giantin) and appropriate secondary, Cy5-conjugated Abs. Confocal microscopy was performed using a Bio-Rad Radiance 2100 scan head mounted on a Nikon ECLIPSE TE 2000U microscope and using 60x NA 1.4 Plan Apo objective. Single cells were observed and 0.4 μ m serial optical sections were collected (iris diameter 1.2 mm). No labelling was observed when using the secondary Abs alone. Digital images and co-localization analysis were performed with LaserSharp2000 software (Bio-Rad).

3. Results

3.1 T lymphocytes secrete exosomes carrying FasL upon cell activation and DGK α regulates this process.

In human T lymphocytes, the stimulation of TCR by PHA or anti-TCR induces the secretion/release to the extracellular medium of intact, non-proteolized FasL protein (mFasL) associated with exosomes [7,12]. These mFasL-bearing exosomes have been shown to induce paracrine apoptosis when assayed on target cells bearing Fas death receptor [27] [7] [8]. However, the molecular mechanisms involved in exosome secretion remain undefined. Previous results have shown that DAG levels at Golgi play a crucial role in TGN dynamics [16], the formation of secretory vesicles and the protein transport from the Golgi to the plasma membrane [15]. DAG phosphorylation to PA is mediated by DGK activity, and J-HM1-2.2 cells express the type I DGK, DGK [18]. Therefore, we studied the role of DGK , which controls DAG levels, on secretory vesicle formation and/or dynamics and the subsequent secretion of exosomes.

JHM1-2.2 Jurkat cells expressing both the human muscarinic receptor (HM1R) and TCR have been used as a model to study the signals during T lymphocyte activation [28] [23] and AICD [29]. In these cells, HM1R stimulation with the agonist Carbachol (CCh), which mimics in part TCR triggering, induces FasL expression and AICD mediated by Fas/FasL interaction [29]. First, we analysed whether CCh stimulation could induce the secretion of exosomes in these cells. Using standard protocols [12], we purified the fraction containing exosomes by ultracentrifugation of the culture supernatants from J-HM1-2.2 cells stimulated 10 h with CCh. We analysed the presence of two late endosomal markers associated with exosomes: CD63 and lamp-1 [30] by WB. As shown in Fig. 1A, CCh increased the amount of protein CD63 and lamp-1 in the pellet containing exosomes, which reflects an increase in the number of released exosomes [11] [7]. We also analysed the presence of two transmembrane proteins, CD45 and CD28, that are highly expressed on the cell surface of T lymphocytes but are absent in exosomes [11]. Both plasma membrane markers were not present in the fraction containing the exosomes, which excludes that these fractions may contain plasma membrane blebs or apoptotic bodies (Fig 1A, lower panels).

To address the role of DGK on the secretion of these exosomes we used, as a first approach, the type I DGK specific inhibitor R59949 [31]. It is noteworthy that out of the three

type I DGK isozymes (δ , ϵ and ζ) inhibited by R59949, DGK δ is the only one present in T lymphocytes and Jurkat cells [17]. Therefore, this inhibitor constitutes a valuable tool to analyse the contribution of DGK δ to the secretion of exosomes. Pre-incubation with the DGK inhibitor R59949 increased the amount of CD63 and hence exosomes [7,11] recovered in the supernatants from JHM1-2.2 CCh-stimulated cells (Fig. 1B), and primary human T blasts stimulated with anti-CD3 (Fig. 1C), whereas the plasma membrane marker CD45 was not found in these fractions.

In activated cytotoxic T lymphocytes (CTLs) CD8⁺ clones, pre-formed FasL has been observed intracellularly located at the limiting membrane of pre-lysosomal multivesicular structures called secretory lysosomes [26]. Upon activation of CTLs, these secretory lysosomes undergo fusion with the plasma membrane and re-localisation of FasL to the cell surface occurs at the immunological synapse [26]. Once at the cell surface, mFasL is rapidly processed by cell matrix metalloproteinases, which shed the extracellular domain of the protein producing an inactive soluble FasL (sFasL) [32], leading to down-regulation of its proapoptotic activity. The presence of FasL in the exosomes secreted by JHM1-2.2 (Fig. 1D) and human T blasts (Fig. 1C) was analysed by WB using the Q-20 polyclonal Ab. This Ab is raised against a N-terminal epitope of FasL protein, therefore recognizes the full-length, non-processed form of FasL (40 kDa), and also the processed (N-terminal), 16 kDa membrane form. As shown in the Fig. 1D, CCh induced an increase in the FasL protein recovered in the secreted exosomes, and the DGK inhibitor R59949 also increased the CCh effect on the secretion of FasL protein. Interestingly, these exosomes only contained the full-length, non-processed form of FasL (mFasL) (Fig. 1D). The last results show that inhibition of DGK increases the secretion of exosomes containing intact mFasL. Consistent with these results, the expression of DGK δ should inhibit the secretion of these exosomes. To analyse the consequences of DGK δ expression, we made use of a GFP-tagged DGK δ construction (GFP-DGK δ) (Fig. 2A, B) [18]. We analysed the ability of CCh to induce the release of exosomes in cells expressing GFP-DGK δ , in comparison with GFP-DGK δ ⁻ cells. As shown in Fig. 2C, the expression of DGK δ decreased the amount of CD63 and hence of exosomes secreted upon CCh stimulation. Concomitantly, the amount of full-length mFasL associated to these exosomes decreased [33].

3.2 Modulation of DGK α pathway affects activation-induced apoptosis via the release of lethal exosomes.

The observed effect of DGK α on the secretion of mFasL-bearing exosomes suggests that DGK α could regulate the AICD induced by these exosomes in T lymphocytes. In J-HM1-2.2 cells, HM1R stimulation with CCh induces early up-regulation of FasL expression and subsequent Fas/FasL mediated AICD [29]. To analyse the contribution of DGK α to AICD in JHM1-2.2, first we used the DGK inhibitor R59949. Pre-treatment with the DGK inhibitor increased both CCh-induced and PHA-induced apoptosis, whereas anti-Fas (Fig. 3A) or Actinomycin D (not shown), which evoke death pathways that do not involve DAG metabolism, were refractory to DGK α inhibition. In addition, the blockade of Fas/FasL interaction by blocking anti-Fas DX2 or anti-FasL (NOK-1) mAbs (Fig. 3A) reverted the positive effect of the DGK inhibitor on cell death. Thus, inhibition of DGK α enhances FasL-dependent AICD. If the effect of R59949 inhibitor increasing AICD were exerted via inhibition of DGK α , then the expression of DGK α should decrease CCh-induced AICD. To analyse the consequences of DGK α expression, we made use of the GFP-tagged DGK α construction (GFP-DGK α) [18]. Apoptosis induced by CCh after 15h (measured as % of Annexin V⁺ cells) and 20h (measured as % of 7-AAD-permeable cells, not shown) was analysed in GFP-DGK α ⁺ and GFP-DGK α ⁻ cells (Fig. 3B). The expression of DGK α decreases early AICD (from 46 $\pm$ 2 % to 27 $\pm$ 3 % of Annexin V⁺ cells, after 15h of CCh treatment). When late AICD was measured after 20 h, the inhibition of AICD achieved by DGK α expression was even higher (not shown). The inhibitory effect of DGK α was specific for CCh-induced AICD, since no effect of DGK α on apoptosis induced by Actinomycin D or direct Fas triggering was observed (Fig. 3B).

All these data suggest that the effect of DGK inhibitor increasing CCh-induced apoptosis could be due to an increase in the release of exosomes bearing non-processed FasL. To test this hypothesis, we analysed the ability of the exosomes obtained from culture supernatants from JHM1-2.2 and human T lymphoblasts to induce apoptosis on target cells. To exclude the possibility of any effect of CCh on target cells, we used Jurkat E6.1 cells as target cells since they do not express HM1R and CCh doesn't induce apoptosis in these cells. When Jurkat E6.1 target

cells were challenged with the fraction of exosomes secreted by JHM1-2.2 cells, this fraction induced FasL-dependent apoptosis (Fig. 3C), which was enhanced by pre-treatment of the effector JHM1-2.2 cells with the DGK inhibitor. In addition, the culture supernatants that were depleted of exosomes by ultracentrifugation were unable to trigger apoptosis of the target cells (not shown). The challenge of JE6.1 target cells with the exosomes secreted from CD4⁺ T lymphoblasts induced FasL-dependent apoptosis (which was inhibited by anti-Fas DX2 and anti-FasL NOK-1 blocking Abs, Fig 3D). As observed in JHM1-2.2 cells (Fig. 3C), pre-treatment of effector T blasts with the DGK inhibitor enhanced FasL-induced cell death.

3.3 DGK α is associated to TGN and late endosomes/MVBs, and is found into secreted exosomes.

In T lymphocytes, FasL is directly sorted from the TGN to secretory lysosomes/MVBs [10]. DAG at the Golgi membranes is required for the formation of secretory vesicles [15,34] and for protein transport from the TGN [16]. Therefore, it may be expected that DGK, by affecting the levels of DAG at the Golgi, could regulate the generation of vesicles from TGN and subsequent FasL transport to the MVBs. The location of DGK at Golgi membranes could constitute the molecular basis underlying this phenomenon. Thus, we studied the subcellular location of DGK by immunofluorescence analysis and confocal microscopy. When we expressed GFP-DGK in JHM1-2.2 cells, we found it throughout the cytoplasm with an accumulated area in a perinuclear region of the cell (Fig.4A). These cells were stained with Abs against Giantin (Cis and medial Golgi marker) and TGN46 (TGN marker). We observed partial colocalisation of GFP-DGK with TGN46 (Fig. 4A) in a perinuclear region and no colocalisation with Giantin (Fig.4B). In addition, we could not observe any colocalisation when cells overexpressing GFP were stained for TGN46, although GFP was thoroughly distributed in the cell, which supports the partial colocalisation of GFP-DGK was specific (not shown). Next, we studied the subcellular location of DGK by subcellular fractionation on a Percoll density gradient, approach that has previously taken to characterise mFasL in secretory vesicles from CTLs [26]. The distribution of FasL, DGK, the late endosome/MVB markers CD63 (Fig. 4C) and lamp-1 (not shown) was analysed by WB in the percoll density fractions obtained. As shown

in Fig. 4 C, high amounts of FasL and DGK were found in CD63⁺ fractions (fractions 5, 9, 12-13). The association of Protein Kinase D1 (PKD1) to the TGN is implicated in the stimulation of vesicle budding from TGN [16], therefore we analysed the presence of PKD in these fractions. As shown in Fig. 4C, the PKD⁺ fractions (no. 9, 12 and 13) contained the highest amounts of DGK, supporting that these fractions may corresponded to the TGN/emerging vesicle compartment. Therefore, the biochemical distribution of DGK and the location of GFP-DGK by immunofluorescence microscopy indicate that DGK is associated with TGN and late endosomal compartments in JHM1-2.2 cells.

The immunofluorescence analysis of the intracellular distribution of CD63 protein in JHM1-2.2 cells stimulated with CCh for 6 h, showed a vesicular pattern for this protein (Fig. 5A). When cells were treated with DGK inhibitor R59949 we observed an increase in the formation of these CD63⁺ vesicular structures upon stimulation with CCh. A recent advance has been the identification into exosomes of lipids and specific proteins that are part of the machinery involved in late endosomes/MVBs biogenesis [35]. The presence of DGK in CD63⁺ late endosomes/MVBs and the increase in the formation of these structures by DGK inhibitor, could suggest that DGK may also be involved during the formation/dynamics of these structures. When we performed WB analysis in lysates from the exosomes secreted by JHM1-2.2 cells (Fig. 5B and C), both endogenous DGK and overexpressed GFP-DGK were found into secreted exosomes, and the amount of DGK associated to the exosomes was increased by the DGK inhibitor. Therefore, the subcellular location of DGK observed and the presence of DGK in the secreted exosomes is compatible with the idea of DGK being a possible regulator of the generation of MVBs and the subsequent secretion of exosomes [33].

3. Closing comments

The inhibition of DGK with R59949 increases the secretion of lethal exosomes bearing mFas Ligand and subsequent apoptosis. On the contrary, the over-activation of the DGK pathway inhibits exosome secretion and subsequent apoptosis. DGK was found associated with the *trans* Golgi Network and late endosomal compartments, but also was found in the secreted exosomes. Our results support the hypothesis that DGK effect on apoptosis occurs via the regulation of the

release of lethal exosomes by the exocytic pathway [33]. The fact that PKD1 was found associated to DGK at the TGN suggests that a possible regulatory step during exocytic traffic where DGK may act involves the generation of export vesicles at the TGN (Scheme 1). Another possibility, which does not exclude the former one, implies a role of DGK during the maturation/biogenesis of MVBs (Scheme 1). Experiments are in progress in order to establish these important points, and to study the function of the secretion of lethal exosomes carrying mFasL during some physiopathological processes.

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Figure legends

Fig. 1. HM1R and TCR stimulation induces the secretion of exosomes bearing FasL, and inhibition of DGK increases the secretion of exosomes. A) Secretion of exosomes was induced by treatment of J-HM1-2.2 cells with CCh (50 and 500 μ M, 10h). Exosomes were isolated as described in Material and Methods. WB of protein extracts from these exosomes was developed with an anti-CD63, anti-lamp-1, anti-CD45, anti-CD28. B) JHM1-2.2 cells pre-incubated in the presence or the absence of R59949 (DGK_i, 10 μ M), were treated with CCh for 10h to induce the secretion of exosomes. CD63 and CD45 protein in these microvesicles was analysed as in A). C) T lymphoblasts were treated with anti-CD3 Ab (10 h, 5 μ g/ml), pre-incubated or not with DGKi (10 μ M). The exosomes were analysed by WB developed with anti-CD63 and anti-FasL specific Ab. As a reference, protein extracts from T blasts were run in parallel in the left-side lane. D) Same as B), but FasL protein was analysed by WB using the anti-FasL Ab Q-20. In the right panels, protein

extracts from HEK293 cells transfected with a plasmid coding for FasL (pEF4BFasL) or a control vector (pEF4B) were used as controls.

Fig. 2. Expression of DGK α decreases the secretion of FasL-bearing exosomes. J-HM1-2.2 cells were transfected with either an empty vector (pEF4B) or an expression vector for DGK (pEFGFP-DGK) and 48 h later were analysed for GFP-DGK expression. A) WB analysis developed with an anti-GFP to visualise the GFP-tagged DGK protein. The protein bands below 62 kDa are non-specifically recognised by anti-GFP Ab, since they also appear in pEF4B empty vector-transfected cells. B) Flow-cytometry expression profile for the GFP-DGK chimera. The marker represents the percentage of GFP-DGK⁺ cells. C) J-HM1-2.2 cells transfected with pEFGFP-DGK (20 μ g) were stimulated with CCh (500 μ M) for 10 h to induce exosomes secretion, and this secretion was compared to that of control vector (pEF4B)-transfected cells. WB of protein extracts from these microvesicles was developed by using anti-CD63 and FasL (Q-20) specific Abs.

Fig. 3. DGK inhibition enhances FasL-dependent, AICD and expression of DGK α inhibits AICD. A) J-HM1-2.2 cells were pre-incubated with blocking Anti Fas (DX2) or Anti-FasL (NOK-1) Abs, and stimulated for 15 hours with CCh (500 and 50 μ M), anti-Fas mAb CH-11 (40 ng/ml) or Actinomycin D (0.1 μ g/ml) in the presence or the absence of R59949 (DGK_i, 10 μ M). Apoptosis was determined by analysing Annexin-V binding as indicated in Material and Methods. B) J-HM1-2.2 cells were transfected with either an empty vector (pEF4B) or an expression vector for DGK (pEFGFP-DGK) and 48 h later were stimulated with CCh (50 and 500 μ M, 15h) or anti-Fas (CH-11 Ab, 40 ng/ml, 15h). Early apoptosis was assessed in GFP-DGK⁺ and GFP-DGK⁻ cells by AnnexinV-PE binding. C) Target JE6.1 cells were challenged with the exosomes obtained from J-HM1-2.2 effector cells that were stimulated with CCh (500 μ M, 10h) in the presence or absence of DGKi (10 μ M). In parallel cultures, JE6.1 target cells were pre-incubated 30 min with the blocking anti-Fas antibody (DX2) before the treatment with the exosomes. After 24 hours of treatment, apoptosis was assessed by analysing Annexin-V binding. D) Same as in C, but exosomes were obtained from effector T lymphoblasts that were unstimulated (control) or stimulated with PHA or

with anti-CD3 Ab in the presence or absence of DGKi. The data presented are representative of 5 different experiments.

Fig. 4. Subcellular location of DGK α . J-HM1-2.2 cells expressing GFP-DGK were stained with an Ab against TGN46 (A) or Giantin (B), and a Cy5-conjugated second Ab. Cells were imaged by confocal microscopy and representative, single optical sections (0.4 μ m thick) are shown. The right-hand panels show the merge images, with coincident labelling appearing yellow. C) Sub-cellular fractionation of J-HM1-2.2 cells was performed as indicated in Material and Methods; fractions from a percoll density gradient were analysed for CD63, DGK, PKD1 and FasL by WB. In the left side lane, a non-fractionated cellular lysate was run as a reference.

Fig. 5. DGK inhibition potentiates the formation of CD63⁺ vesicular structures upon CCh stimulation. GFP-DGK α and DGK α were detected in secreted exosomes. A) JHM1-2.2 cells pre-incubated in the presence or the absence of R59949 (DGKi, 10 μ M), were treated with CCh for 6 h to induce formation of MVBs. Cells were stained with an Ab against late endosomes marker CD63 and a Cy5-conjugated second Ab. Cells were imaged by confocal microscopy and Z-projection of optical sections were shown. B) JHM1-2.2 cells pre-incubated in the presence or the absence of R59949 (DGKi, 10 μ M), were treated with CCh for 10h to induce the secretion of exosomes. WB of protein extracts from these exosomes was developed with an anti-DGK specific Ab. C) J-HM1-2.2 cells were transfected with either an empty vector (pEF4B) or an expression vector for DGK (pEFGFP-DGK) and stimulated with CCh (500 μ M) for 10 h to induce exosomes secretion. WB of protein extracts from these secreted exosomes was developed by using anti-DGK specific Ab.

Scheme 1: Hypothetical model of DGK α function during the generation and dynamics of MVB containing lethal exosomes

Secretory vesicular traffic sequentially involves the formation of vesicles at the *trans* Golgi network (TGN), the formation of late endosomes and multivesicular bodies (MVBs), and the fusion of the MVBs with the plasma membrane. In T lymphocytes, Fas ligand (FasL) is stored into small vesicles inside the MVBs, termed exosomes. These exosomes are formed via invagination /inward budding of the limiting membrane of the late endosomes. Stimulation of T lymphocytes induces the fusion

of the MVBs with the plasma membrane, and as consequence intact mFasL is released associated with the exosomes. Diacylglycerol (DAG) at the Golgi membranes is required for the *trans* Golgi network (TGN) dynamics and the formation of secretory vesicles [16] [34]. The interaction of DAG with the C1a domain on Protein Kinase D1 (PKD1) regulates the recruitment of PKD from the cytosol to the TGN [36]. At this location, PKD1 is implicated in stimulating vesicle budding from TGN and the formation of emerging vesicles [16]. Reduction of the cellular levels of DAG inhibits PKD recruitment and blocks vesicular transport from the TGN [16]. Our results support that DGK was found associated to sub-cellular structures corresponding to the TGN, or associated to CD63⁺ subcellular structures (a marker for late endosomes/multivesicular bodies) [33] and PKD1 (which can be found at the TGN, see [16]), and DGK was also detected into the secreted exosomes (this paper). Thus, DGK is a candidate to modulate the PKD1 effect on vesicle budding from TGN, via the downregulation of DAG levels in the TGN, and the subsequent generation of emerging vesicles mediated by PKD1. In addition, we show that inhibition of DGK increases the formation of vesicular structures/MVBs, and the secretion of FasL-bearing exosomes in T lymphocytes. The observation of DGK in the secreted exosomes and MVBs [33], supports a possible role for DGK in controlling the inward budding process responsible for MVB biogenesis.

Fig. 1 Alonso et al.

A **B** **C**

Fig. 2 Alonso et al.

Fig. 3 Alonso et al.

Fig 4 Alonso et al

C

Fig. 4C Alonso et al.

A

CCh - R59949

CCh + R59949

JHM1-2.2 CD63

B- DGK_i+ DGK_i

Mw (kDa)

83 -

Exosomes

CJHM1-2.2 GFP-DGK α

Mw (kDa)

175 -

83 -

Exosomes

GFP-DGK α
DGK α

Scheme 1 Alonso et al.