Diacylglycerol Kinase α Regulates the Secretion of Lethal Exosomes Bearing Fas Ligand during Activation-induced Cell Death of T Lymphocytes*§

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Roberto Alonso§, M. Carmen Rodríguez‡, Jose Pindado¶, Ernesto Merino¶, Isabel Mérida¶, and Manuel Izquierdo‡**

From the §Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas-Universidad de Valladolid, Facultad de Medicina, Ramón y Cajal, 7, 47005 Valladolid and Instituto de Investigaciones Biomédicas "Alberto Sols" Facultad de Medicina, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Arturo Dupuyer, 4, 28029 Madrid and the ¶Department of Immunology and Oncology, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas, Campus de Cantoblanco, 28049 Madrid, Spain

Fas ligand (FasL) mediates both apoptotic and inflammatory responses in the immune system. FasL function critically depends on the different forms of FasL; soluble Fas ligand lacking the transmembrane and cytoplasmic domains is a poor mediator of apoptosis, whereas full-length, membrane-associated FasL (mFasL) is pro-apoptotic. mFasL can be released from T lymphocytes, via the secretion of mFasL-bearing exosomes. mFasL in exosomes retains its activity in triggering Fas-dependent apoptosis, providing an alternative mechanism of cell death that does not necessarily imply cell-to-cell contact. Diacylglycerol kinase α (DGKα), a diacylglycerol (DAG)-consuming enzyme, is involved in the attenuation of DAG-derived responses initiated at the plasma membrane that lead to T lymphocyte activation. Here we studied the role of DGKα on activation-induced cell death on a T cell line and primary T lymphoblasts. The inhibition of DGKα increases the secretion of lethal exosomes bearing mFas ligand and subsequent apoptosis. On the contrary, the overactivation of the DGKα pathway inhibits exosome secretion and subsequent apoptosis. DGKα was found associated with the trans-Golgi network and late endosomal compartments. Our results support the hypothesis that the DGKα effect on apoptosis occurs via the regulation of the release of lethal exosomes by the exocytic pathway, and point out that the spatial orchestration of the different pools of DAG (plasma membrane and Golgi membranes) by DGKα is crucial for the control of cell activation and also for the regulation of the secretion of lethal exosomes, which in turn controls cell death.

T cell receptor (TCR) stimulation triggers the generation of several second messengers such as calcium and diacylglycerol (DAG) (1). DAG may induce Ras activation and subsequent ERK cascade stimulation (2). Calcium and DAG/Ras pathways control T cell activation, which involves up-regulation of hundreds of genes (3), and activation-induced cell death (AICD), which is mediated by up-regulation of proapoptotic genes such as Fas ligand (FasL) (4). AICD is one of the main mechanisms involved in maintaining the peripheral immune tolerance and T lymphocyte homeostasis. Once a T cell clone has been activated and expanded in response to an antigen, it can be eliminated through AICD to prevent autoimmunity and lymphoproliferative disorders (5).

The fact that the same signaling pathways, calcium and DAG/Ras, contribute to both activation and apoptosis raises the question how these responses are controlled by TCR. This issue may be addressed considering that a strict balance between positive and negative regulators acting on each pathway must determine both the magnitude and the quality of the final response. Supporting this hypothesis, the existence of TCR mutants, which are apoptosis-defective but exhibit normal activation, revealed that a subtle control exerted on the Ras/ERK pathway may constitute the molecular basis to explain how these responses are regulated by TCR (6, 7). The evidence showing that the Ras/ERK pathway is required for activation signals leading to positive selection of thymocytes but is irrelevant for AICD of thymocytes (8) supports the view that these antithetical responses to TCR stimulation are distinguishable.

Diacylglycerol kinases (DGKs) constitute one of the negative regulators controlling the DAG/Ras/ERK pathway (9, 10). The lipid second messenger DAG is phosphorylated to phosphatidic acid (PA) by DGKs (11). Therefore, the clearance of DAG by DGKs is thought to be crucial to down-regulate abnormally prolonged, receptor-triggered DAG/Ras signaling and subsequent T lymphocyte activation (9, 10). It has been shown that a type I DGK, DGKα, which is highly expressed in T lymphocytes, transiently translocates to the plasma membrane upon...
TCR triggering (12, 13). At this location DGKα acts as a negative modulator of the DAG levels generated during T cell activation (12). Moreover, constitutively active forms of DGKα located at the plasma membrane attenuate T lymphocyte activation (12), and the inhibition of DGKα induces prolonged activation signals (12) via sustained signaling through RasGRP (Ras guanyl nucleotide exchange factor) (13, 14). Therefore, the regulation of DAG accumulation in the plasma membrane appears critical for the correct propagation of DAG/Ras-derived signals leading to activation (10), and this occurs through the precise, spatial, and temporal control by DGKα activity (12, 13). These facts, together with the evidence showing that the DAG/Ras/ERK pathway may act as a divergence point in the signals leading to activation and AICD, prompted us to hypothesize such a subtle control by DGKα may act also on the DAG-derived signals leading to cell death. To investigate this, we analyzed the role of DGKα on AICD of T lymphocytes. Our results provide insights on a new role of DGKα on the regulation of AICD.

EXPERIMENTAL PROCEDURES

Cell Cultures—The human T cell leukemia Jurkat (clone E6.1) was cultured as described previously (15). J-HM1-2.2 cells expressing the human muscarinic type 1 receptor (HM1R) have been described (16). Human primary T lymphoblasts were prepared and cultured as described previously (17). HEK293 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-DGKα Ab was a gift from H. Kanoh (Sapporo Medical University, Japan). The mAb anti-CD28 was a gift from Dr. Moretta (Institute Gianina Gaslini, Genova, Italy). The anti-CD45 mAbs RP2/21 and RP2/18 were a gift from Dr. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). The apoptosis inducer anti-Fas (clone CH-11) monoclonal Ab (mAb) was obtained from Medical and Biological Laboratories Co., Ltd., Nagoya, Japan. DGK inhibitor II (R59949) and GM6001 were obtained from Sigma. Z-Val-Ala-DL-Asp-fluoromethyl ketone (Z-VAD-fmk) was from Bachem AG.

Expression Vectors and Transfection Assays—The plasmids pEF-GFP, pEF-GFP-DGKα, pEF-GFP-(A1–192)-DGKα, and pEF-Myr-DGKα-DGKα have been described previously (12). Human FasL cDNA was subcloned from pBShFl-1 plasmid into pEF4/Mye-HisB (Invitrogen). For transfection experiments, cells were transiently transfected with 20–30 μg of the plasmids as described previously (12). Subconfluent HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen).

Assessment of Apoptosis—Phosphatidylserine (PS) exposure in the outer leaflet of the cytoplasmic membrane was evaluated by staining the cells with annexin V-FITC, and late apoptosis was measured by cell permeability to 7-amino-actinomycin D (7-AAD). GFP-DGKα-deficient cell lines were transfected with a GFP-DGKα reporter plasmid (pEF-GFP-DGKα). GFP-DGKα+ cells were gated by green fluorescence, and annexin V-FITC binding and 7-AAD permeability in both populations were analyzed by flow cytometry.

Dual Luciferase Reporter Assays—J-HM1-2.2 cells were cotransfected with an inducible vector containing the luciferase reporter gene, a Renilla luciferase vector pRL-TK (Promega), and either (i) pEF-Bos (pEF) control vector or (ii) pEF-GFP-DGKα. As inducible reporter genes, we used pLuc-486FasL (18) and CD69 promoter-based reporter pAM1.4-Luc (19). 48 h post-transfection, cells were stimulated as described, and the dual luciferase assay was performed according to the manufacturer’s instructions (Promega).

Intracellular Fas Ligand Detection—Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, preincubated in 5% bovine serum albumin, and incubated for 1 h with mouse anti-human Fas ligand (NOK-1) in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% Triton X-100. Cells were washed three times with the same buffer, incubated for 1 h with goat F(ab')2 anti-mouse CH-11 (Jackson ImmunoResearch Inc.), and analyzed by flow cytometry.

Isolation of Microvesicles and Functional Assays—The isolation and purification of exosomes/microvesicles from culture supernatants of T cells have been described previously (20–22). By using these standard protocols, culture supernatants of 20 × 106 cells were centrifuged at low speed in sequential steps and then clarified to eliminate cells and cell debris/apoptotic bodies. Then the microvesicles were spun down by ultracentrifugation (100,000 × g for 12 h), as described previously (21), and resuspended in 50 μl of RIPA lysis buffer. We usually loaded 25 μl of this lysate per lane for WB analysis (see below). We commonly obtained between 50 and 150 μg of protein in the 100,000 × g pellet from 20 × 106 CCh-stimulated cells. For functional assays, target Jurkat JEB.1 cells were challenged with the microvesicle fraction from the culture supernatants, and after incubation, the extent of apoptosis was determined by annexin V-PE/7-AAD staining. To analyze the involvement of FasL in the toxicity of these supernatants, the bioassays were performed in the presence (500 ng/ml) or the absence of anti-human FasL mAb (NOK-1) or anti-human Fas mAb (DI2).

Western Blot Analysis—Cells and microvesicles were lysed for 10 min in RIPA supplemented with protease inhibitors. Cellular and microvesicle proteins were separated by SDS-PAGE under reducing conditions and transferred to Hybond ECL membranes (Amersham Biosciences). For CD63 detection, proteins were separated under non-reducing conditions as described previously (23). After the incubation with the appropriate primary antibody, the blots were developed with peroxidase-conjugated secondary antibodies by using enhanced chemiluminescence (ECL) reagents and by following standard protocols.

Concentration of DGK Activity—DGK activity in J-HM1-2.2 cells were disrupted in 2 ml of lysis buffer (24), and the lysate was sonicated on a discontinuous Percol gradient (Amersham Biosciences) as described previously (24). Fractions were analyzed for the presence of DGK (1,2-dioctanoyl-sn-glycerol) (12), by using DOG-PA as standard.

Inhibition of T Cell Subpopulations—T lymphoblasts cultured for 6 days with IL-2 were incubated for 15 min at 4 °C with anti-human CD4 or anti-human CD8 mAbs (Pharmingen). After being washed twice with culture medium, cells were incubated with rat anti-mouse IgG1 magnetic microbeads (Miltenyi Biotec) to isolate CD4+ and CD8+ subpopulations and were cultured in the presence of IL-2 (20 ng/ml).

Immunofluorescence Analysis—J-HM1-2.2 cells were stained as described previously (15) with primary Abs (anti-CD3, anti-TCR, and anti-Giantin) and appropriate secondary Cy3-conjugated Abs (Jackson ImmunoResearch) or Cy5-conjugated Abs were from Jackson ImmunoResearch. Z-VAD-fmk was from Bachem AG.

Expression Vectors and Transfection Assays—The plasmids pEF-GFP, pEF-GFP-DGKα, pEF-GFP-(A1–192)-DGKα, and pEF-Myr-DGKα-DGKα have been described previously (12). Human FasL cDNA was subcloned from pBShFl-1 plasmid into pEF4/Mye-HisB (Invitrogen). For transfection experiments, cells were transiently transfected with 20–30 μg of the plasmids as described previously (12). Subconfluent HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen).

RESULTS

Inhibition of DGK Increases FasL-dependent, Activation-induced Cell Death—J-HM1-2.2 Jurkat cells expressing HM1R and TCR have been used to study the signals during T lymphocyte activation (16) and AICD (15, 25). In these cells, HM1R stimulation with the agonist CCh induces early up-regulation of FasL expression (25). Blocking Abs against FasL or FasL (NOK-1) mAbs inhibited CCh-induced apoptosis. Therefore, binding of FasL to its receptor was necessary for CCh to induce Fas/FasL-dependent apoptosis.

Stimulation with CCh induces phospholipase Cβ-mediated hydrolysis of inositol phospholipids, which leads to DAG elevation and subsequent phoshatidic acid (PA) production (12, 16). DAG phosphorylation to PA is mediated by DGK activity, and...
J-HM1-2.2 cells express the type I DGK, DGKα (12). Thus, pretreatment with the type I DGK-specific inhibitor R59949 (27) inhibits CCh-induced PA production (12). It is noteworthy that out of the three type I DGK isozymes (α, β, and γ) inhibited by R59949, DGKα is the only one present in T lymphocytes and Jurkat cells (11). Therefore, this inhibitor constitutes a valuable tool to analyze the contribution of DGKα to AICD. Pretreatment with the DGK inhibitor increased CCh-induced apoptosis, whereas anti-Fas (Fig. 1B) or actinomycin D (not shown), which evoke death pathways that do not involve DAG metabolism (28), were refractory to DGKα inhibition. In addition, the blockade of Fas/FasL interaction by blocking Abs DX2 (Fig. 1B) and NOK-1 (not shown) reverted the positive effect of the DGK inhibitor on cell death. The DGK inhibitor also enhanced PHA-induced AICD (data not shown), a process that has been shown to depend on Fas/FasL interaction (21). Thus, inhibition of DGKα enhances FasL-dependent AICD.

Expression of DGKα Inhibits AICD—HM1R stimulation induces transient translocation of DGKα from the cytosol to the plasma membrane. At this location, DGKα attenuates DAG-derived activation responses (12, 13). If the effect of the R59949 inhibitor increasing AICD were exerted via inhibition of DGKα, then the overexpression of DGKα should decrease CCh-induced AICD. To analyze the consequences of DGKα expression, we made use of a GFP-tagged DGKα construction (GFP-DGKα) (Fig. 2, A–C) whose translocation to the plasma membrane is similar to that corresponding to the endogenous enzyme (12). CCh induction of early apoptosis (% of annexin V+ cells) and late apoptosis (% of 7-AAD-permeable cells) was analyzed in GFP-DGKα+ and GFP-DGKα− cells (Fig. 2, D and E). Fig. 2, D and E, compares the apoptosis induced by CCh after 15 and 20 h (early and late apoptosis, respectively), in the two populations. As shown in Fig. 2D, the expression of DGKα decreases early AICD (from 46 ± 2% to 27 ± 3% of annexin V+ cells, after 15 h of CCh treatment). When late AICD was measured after 20 h, the inhibition of AICD achieved by DGKα expression was higher (Fig. 2E). The degree of inhibition was dependent on the expression level of DGKα as shown in Fig. 2F; those cells expressing higher levels of DGKα underwent less apoptosis.

The inhibitory effect of DGKα was specific for CCh-induced AICD, because no effect of DGKα on apoptosis induced by actinomycin D (data not shown) or direct Fas triggering (Fig. 2D) was observed. DGKα Does Not Affect the Transcriptional Regulation of FasL and the Intracellular Levels of FasL—CCh stimulation in JHM1-2.2 cells triggers Ras/mitogen-activated protein kinase activation (29), which is involved in the transcriptional control of the FasL gene (30), and it has been shown that DGKα attenuates the Ras/mitogen-activated protein kinase pathway (12–14). These observations may suggest that the effect of DGKα on CCh-induced apoptosis could be due to the inhibition of FasL gene expression. To test this, we analyzed the effect of DGKα expression on FasL promoter regulation in reporter gene assays. Cells were cotransfected with a reporter for the FasL gene and either an expression vector for DGKα (pEFGFP-DGKα) or a control vector (pEF). As shown in Fig. 3, the reporter driven by the FasL promoter was activated (4–5-fold induction) by CCh, and the overexpression of DGKα did not
inhibit FasL reporter induction (Fig. 3, A and B). However, DGKα partially inhibited the activation of the CD69 reporter (Fig. 3C), an activation marker that is regulated by the effect of DGKα on the DAG/Ras pathway (12). In addition, treatment with the DGK inhibitor R59949 did not increase the FasL luciferase reporter activity of CCh-stimulated cells (Fig. 3D).

These results support that the inhibition by DGKα of FasL-dependent apoptosis was not exerted on the transcriptional regulation of FasL. Consequently, post-transcriptional regulation of FasL may underlie the DGKα effect on AICD. It is remarkable that plasma membrane mFasL is quickly processed by cell matrix metalloproteinases to produce a C-terminal inactive soluble FasL (24 kDa) and an N-terminal membrane form (16 kDa) (31), leading to down-regulation of its proapoptotic activity. By using NOK-1, a mAb that recognizes the extracellular, C-terminal epitope on FasL, we analyzed by flow cytometry the presence of the unprocessed form of FasL (mFasL) at the cell surface upon stimulation. We were unable to detect any plasma membrane, full-length mFasL, even in the presence of a metalloproteinase inhibitor, GM6001, which inhibited FasL processing as shown in supplemental Fig. 3, A and B; therefore, we analyzed intracellular FasL. It has been shown that Jurkat cells express pre-formed intracellular FasL protein, and stimulation through TCR and HM1R up-regulates FasL mRNA (maximal induction between 3 and 5 h of stimulation) and protein (21, 25). To analyze whether DGKα might regulate translational events involved in intracellular FasL expression, we measured the levels of FasL protein after CCh stimulation for 6 and 8 h in permeabilized GFP-DGKα− and GFP-DGKα+ cells. As shown in supplemental Fig. 3C, both cell populations expressed comparable levels of pre-formed FasL. CCh induces up-regulation of FasL protein; no significant differences on the levels of FasL were found between DGKα− and DGKα+ cells, independently of the expression levels of the DGKα (data not shown). In addition, the DGK inhibitor R59949 neither affected the levels of pre-formed FasL nor the induction of intracellular FasL protein of CCh as detected by WB analysis (supplemental Fig. 3D).

Inhibition of DGK Increases the Secretion of Microvesicles Bearing FasL—The previous results showed that DGKα neither interferes with the transcription of FasL gene (4–6 h) nor with FasL protein synthesis (6–8 h); thus, DGKα could interfere with FasL function at a post-translational level. The fact that we cannot detect full-length mFasL at the cell surface upon stimulation prompted us to analyze another mechanism involved in FasL-mediated apoptosis, which implies the secretion of exosomes/microvesicles bearing mFasL (21, 22, 32). These exosomes intracellularly accumulate in late or multivesicular endosomes (multivesicular bodies, MVBs) (33), and in T cells FasL is stored inside these MVBs (24, 34, 35). Stimulation of T lymphocytes induces the fusion of the MVBs with the plasma membrane (20, 35), and as consequence bioactive mFasL is released as an intact, nonproteolized protein associated with the exosomes (21, 35). PHA-stimulated Jurkat cells release FasL-bearing exosomes following a biphasic pattern with a first translational independent peak after 1 h of stimulation (release of pre-formed FasL) and a second peak at about 7 h, corresponding to de novo mRNA and protein synthesis of FasL (21). Previous results have shown that sustained DAG levels at Golgi facilitate the trans-Golgi network (TGN) dynamics (36) and the formation of secretory vesicles (37). Therefore, we hypothesized that DGKα, by decreasing DAG levels, may inhibit the formation and/or dynamics of MVBs and subsequent cell death. In human T cells the stimulation of TCR by PHA or anti-TCR induces the secretion of exosomes, which contain the tetraspanin protein CD63, and they originate from endocytic compartments (20, 35). We analyzed first whether CCh could induce the secretion of microvesicles and the effect of R59949 on this secretion. By using the protocols described previously, we purified the microvesicle fraction by ultracentrifugation from the culture supernatants from J-HM1-2.2 cells stimulated for 10 h with CCh. We analyzed the presence of the two late endosomal markers CD63 (microvesicle-specific protein) and Lamp-1 (38) by WB. As shown in Fig. 4A, CCh increased the amount of protein CD63 and Lamp-1 in the pellet containing microvesicles. These microvesicles also contain FasL (Fig. 4A,
lower panel). We also analyzed the presence of two transmembrane proteins, CD45 and CD28, that are highly expressed on the cell surface but absent in exosomes (20). Both plasma membrane markers were not present in the fraction containing the microvesicles, which excludes that these fractions may contain plasma membrane blebs or apoptotic bodies (Fig. 4 lower panel). Moreover, preincubation with the DGK inhibitor R59949 increased the amount of CD63 and hence the microvesicles (20, 35) recovered in the supernatants from CCh-stimulated cells (Fig. 4B), whereas the plasma membrane marker CD45 was not found in these fractions. The presence of FasL in these microvesicles was analyzed 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. D and E, densitometry corresponding to the WB in B and C, respectively. F, target JE6.1 cells were challenged with the microvesicle fraction obtained from J-HM1-2.2 effector cells that were stimulated with CCh (500 μM, 10 h) in the presence or absence of DGKi (10 μM) or CsA (200 ng/ml). In parallel cultures, JE6.1 target cells were preincubated 30 min with the blocking anti-Fas antibody (DX2) before the treatment with the microvesicles. After 24 h of treatment, apoptosis was assessed by analyzing annexin V binding. See also supplemental Fig. 4. The inset shows the WB developed with anti-CD63 of the microvesicle fraction obtained from JHM1-2.2 cells pretreated with CsA and stimulated with CCh for 10 h. G, JHM1-2.2 cells were stimulated with CCh (500 μM, 10 h) plus R59949 (10 μM) or ActD (0.5 μg/ml) for 10 h. Apoptosis was determined by annexin V binding. Microscopy transmission images were taken in parallel to show the induction of plasma membrane blebbing/cell debris. Lower left panel, effect of Z-VAD-fmk pretreatment (100 μM) and ActD on microvesicle secretion in JHM1-2.2 effector cells. Lower right panel, microvesicle fractions from JHM1-2.2 effector cells that were pretreated with Z-VAD-fmk and stimulated with CCh (500 μM, 10 h), or stimulated with ActD (0.5 μg/ml, 10 h), were washed twice by ultracentrifugation (100,000 × g, 15 h) and then added to JE6.1 target cells. Cell death of target cells was analyzed by annexin V staining after 24 h of culture. The data presented are representative of at least four different experiments. Cont, control.
Q-20 polyclonal Ab. This Ab is raised against an N-terminal epitope of FasL protein and therefore recognizes the full-length FasL (40 kDa) and also the processed (N-terminal) 16-kDa membrane form. As shown in the Fig. 4C, CCh induced an increase in the FasL protein recovered in the microvesicles, and the DGK inhibitor R59949 increased the CCh effect on the secretion of CD63 and FasL protein (see densitometry in Fig. 4, D and E). Most interestingly, the microvesicles only contained the full-length FasL, in contrast to the processed FasL protein (16 kDa), which was detected in HEK293 cells expressing FasL (Fig. 4C, right panel). After 10 h of stimulation with CCh, we were unable to detect cell death (Fig. 4G), which rules out that these microvesicle fractions may contain apoptotic bodies or intracellular membrane structures released from dying cells. The intact mFasL recovered in the microvesicles obtained from the cell culture supernatants has a slightly higher molecular weight when compared with the intact intracellular FasL (32, 39) (see also Figs. 4A and 7B), suggesting differences in the glycosylation stage of mFasL in the microvesicles (32). It is remarkable that this high molecular weight form of mFasL was the only one we found in the microvesicle fraction, which also supports that this fraction did not include intracellular membrane structures.

These data suggest that the effect of the DGK inhibitor increasing CCh-induced apoptosis could be due to an increase in the release of microvesicles bearing nonprocessed FasL. Next, we confirmed that the increase in AICD observed after DGK inhibition was because of the enhanced release of microvesicles containing bioactive FasL. To this end, we analyzed the ability of the microvesicle fraction obtained from culture supernatants to induce apoptosis. To exclude the possibility of any effect of CCh on target cells, we used Jurkat E6.1 cells as target cells because they do not express HM1R, and CCh does not induce apoptosis in these cells. When Jurkat E6.1 target cells were challenged with the microvesicle fraction from the cell culture supernatants, these microvesicles induced Fasl-dependent apoptosis (Fig. 4F and supplemental Fig. 4), which was enhanced by pretreatment of the effector with the DGK inhibitor. In addition, the culture supernatants that were depleted of microvesicles by ultracentrifugation were unable to trigger apoptosis of the target cells (not shown). The blockade of de novo FasL gene expression by cyclosporin A (CsA) (25, 40) in JHM1-2.2 effector cells inhibited the ability of the culture supernatants from these cells to induce apoptosis (Fig. 4F), but the release of microvesicles containing CD63 was not affected (Fig. 4F, inset), which supports that the presence of newly synthesized mFasL in the secreted microvesicles is necessary to induce apoptosis.

To confirm that the microvesicle fraction does not derive from dying cells, we analyzed the secretion of the microvesicle in an apoptosis-free environment, treating JHM1-2.2 effector cells with the general caspase inhibitor Z-VAL-fmk. As shown in Fig. 4G, pretreatment with Z-VAL neither affected the secretion of CD63+ microvesicles (lower left panel) nor the ability of the microvesicle fraction to induce apoptosis of JEM6.1 target cells (lower right panel). In addition, actinomycin D, which induced apoptosis of JHM1-2.2 effector cells (40 ± 8% of annexin V+ cells) and concomitantly produced cell debris (Fig. 4G), did not induce the secretion of microvesicles. When JEM6.1 target cells were challenged with the microvesicle fraction of culture supernatants from JHM1-2.2 effector cells treated with ActD (Fig. 4G, lower panel), we were unable to detect cell death.

**Expression of DGKα Decreases the Secretion of Microvesicles and Exogenous DAG Induces the Release of Microvesicles**—The last results show that inhibition of DGK increases the secretion of microvesicles containing FasL. Consistent with these results, if the inhibitory effect of DGKα on AICD was exerted at the level of microvesicle secretion, then the expression of DGKα should inhibit secretion. To address this point, we analyzed the ability of CCh to induce the release of microvesicles in cells expressing GFP-DGKα, in comparison with GFP-DGKα- cells. As shown in Fig. 5, A and B, the expression of DGKα decreased the amount of CD63 and hence of microvesicles which were secreted upon CCh stimulation. Concomitantly, the amount of FasL associated to these microvesicles decreased.

To support further the requirement for DAG in microvesicle secretion, we used a cell-permeant analog of DAG, 1,2-di-octanoyl-sn-glycerol (DOG). DOG undergoes partitioning across the plasma membrane and through the intracellular membranes. As shown in the Fig. 5C, treatment of cells with DOG was sufficient to induce the secretion of microvesicles containing CD63, Lamp-1, and FasL.

![Figure 5: Expression of DGKα decreases the secretion of microvesicles, and exogenous DAG induces the release of microvesicles.](http://www.jbc.org/)

**A**. J-HM1-2.2 cells transfected with pEFGFP-DGKα (20 μg) were stimulated with CCh (500 μM) for 10 h to induce microvesicle secretion, and this secretion was compared with that of control vector (pEF4B)-transfected cells. The percentage of GFP-DGKα-cells was 67%. WB of protein extracts from these microvesicles was developed by using anti-CD63, Lamp-1, and FasL-specific Abs. B, densitometry corresponding to the WB in A. C, microvesicles secreted from cells treated with DOG (100 and 25 μg/ml) for 10 h. Cont, control.
Inhibition of DGK Increases Both the Secretion of Exosomes Containing FasL and AICD in Primary T Lymphoblasts—Next, we extended some of the results obtained in the cell line model to primary cultures of T lymphoblasts. As shown in Fig. 6B (inset), T lymphoblasts cultured in the presence of IL-2 express DGKα. AICD induced by anti-CD3 stimulation was partially inhibited by the prevention of the Fas/FasL interaction with blocking anti-Fas (DX2) or anti-FasL (NOK-1) Abs (Fig. 6A). In addition, the blockade of de novo FasL synthesis by CsA (40) (Fig. 6A, inset) also partially inhibited AICD. These results suggest that other mechanisms apart from FasL could contribute to AICD (i.e. Apo2L, tumor necrosis factor-α) (21, 41). The DGK inhibitor R59947 weakly enhanced early apoptosis (Fig. 6B) and late apoptosis (Fig. 6C) induced by anti-CD3 (from 38 ± 4 to 50 ± 3% of annexin V+ cells) or PHA (1 μg/ml, from 48 ± 4 to 68 ± 3% of annexin V+ cells) in T lymphoblasts. The polyclonal activation stimuli we used may trigger the cytotoxicity induced by the cytotoxic CD8+ T lymphocytes (CTLs) contained in this heterogeneous population. This cytotoxicity can be mediated by either Fas relocation to the cell surface of CTLs or by the secretion of perforin/granzymes (34) and could contribute to the observed AICD. To rule out any of these possibilities and to exclude the contribution of other death mechanisms acting on each cell subpopulation (41), we analyzed AICD on sorted CD4+ and CD8+ subpopulations. Pretreatment with the DGK inhibitor increased AICD in CD4+ lymphoblasts (Fig. 6D), and the blockade of Fas/FasL interaction with DX2 or NOK-1 Abs, or the inhibition of de novo FasL gene expression with CsA, reverted the effect of the DGK inhibitor on AICD (Fig. 6D). Next, we analyzed in both J-HM1-2.2 and CD4+ lymphoblasts the following: 1) the amount of FasL into the released microparticles produced upon TCR stimulation, and 2) the ability of the microparticles fraction from unstimulated or stimulated CD4+ lymphoblasts to induce apoptosis. As shown in Fig. 7, the DGK inhibitor increased the secretion of microvesicles induced by anti-CD3 in both J-HM1-2.2 cells and CD4+ lymphoblasts (A and B, respectively), whereas the plasma membrane marker CD45 was absent in these fractions. Concomitantly, the amount of FasL secreted in the microvesicles from both cell types was increased (Fig. 7, A and B, lower panels). In addition, when CD4+ lymphoblasts were activated with PHA, we observed as well an increase in the secretion of microvesicles and associated FasL (Fig. 7B). The challenge of JE6.1 target cells with the microvesicles derived from CD4+ lymphoblasts induced FasL-dependent apoptosis (which was inhibited by anti-Fas blocking Ab DX2 or by pretreatment of effector T lymphoblasts with CsA) (Fig. 7C). As observed in JHM1-2.2 cells (Fig. 4F), pretreatment of the effector T lymphoblasts with the DGK inhibitor enhanced cell death.

Expression of Constitutively Active Forms of DGKα Does Not Inhibit AICD—DGKα expression partially inhibited the activation of a CD69 reporter gene (Fig. 3C), and constitutively active forms of DGKα located at the plasma membrane act as negative modulators of the DAG generated during T cell activation (12). Therefore, these constitutively active mutants constitute useful tools to analyze the role of the DAG produced at the plasma membrane on the process of vesicle secretion. There are several stages of membrane rearrangement involved in secretory vesicular traffic that may be regulated by DGKα as follows: the fission of vesicles from Golgi donor membranes, the formation of MVBs, and their fusion with plasma membrane. The structural properties of lipids contribute to these rearrangements; cone-shaped lipids such as PA mark sites of membrane fusion by remodeling membrane curvature and fluidity (37). Therefore, it is conceivable that the DGKα effect on microparticle secretion could be exerted at the level of the fusion of MVBs with the plasma membrane. If this possibility were correct, constitutively active forms of DGKα, GFP-(Δ1–192)-DGKα and Myr-GFP-DGKα, which consume DAG and produce PA elevation at the plasma membrane (12–14), should increase microvesicle-mediated AICD. However, GFP-(Δ1–192)-DGKα and Myr-GFP-DGKα, which are expressed at the plasma membrane, did not affect CCh-induced apoptosis (Fig. 8). This result together with the fact that the expression of a catalytically inactive mutant (kinase-dead) of DGKα (GFP-DGKα-Kd), which is located at the plasma membrane upon CCh stimulation (12) and inhibits endogenous DGKα at this location (13, 14), did not affect CCh-induced apoptosis (Fig. 8), suggest that the control point where DGKα acts should be different from the fusion of MVBs with the plasma membrane.

DGKα Is Associated with TGN/MVBs—FasL is directly sorted from the TGN to secretory lysosomes/MVBs in T lymphocytes (34). DAG at the Golgi membranes is required for the generation of secretory vesicles (37) and for protein transport from the TGN. Therefore, it may be expected that DGKα, by affecting the levels of DAG at the Golgi, could regulate the generation of vesicles from TGN and subsequent FasL transport to MVBs. Thus, we studied the subcellular localization of DGKα. We performed subcellular fractionation on Percoll density gradients. This approach has been used previously to isolate pre-formed mFasL in secretory lysosomes from CTLs (24). As a marker of MVBs and microvesicles, we analyzed the distribution of two late endosomal proteins, Lamp-1 and CD63. Lamp-1 is mostly present on the MVBs limiting membrane, whereas CD63 is abundant within internal membranes (microvesicles) (38). WB analysis of Golgi p58K protein was also included as a Golgi marker. As shown in Fig. 9, CD63+ fractions from nonstimulated cells included pre-existing FasL and also DGKα. The higher density fraction (fraction 25, density >1.12) of the gradient had the expected density of MVBs (38); accordingly, this fraction was CD63+ and Lamp-1+ and also included DGKα and FasL. Most interestingly, high amounts of DGKα and FasL were also found in the low density Percoll fractions (5, 8–15) that corresponded to Golgi (Golgi p58K+, CD63+, and Lamp-1+) (38). The association of protein kinase D (PKD) to the TGN is involved in the stimulation of vesicle budding from TGN, and the reduction of cellular levels of DAG inhibited the recruitment of PKD and blocked vesicle budding and protein transport form TGN to the cell surface (38). Therefore, we analyzed the presence of PKD in these fractions. As shown in Fig. 9, the PKD+ fractions (fractions 9, 12, and 13) contained the highest amounts of DGKα and FasL, supporting that these fractions corresponded to the TGN/emerging vesicle compartment. The presence of DGKα in these fractions was confirmed by measuring DOG phosphorylation to the corresponding PA by using an in vitro kinase assay (supplemental Fig. 9A). A good correlation was found between the levels of DGKα protein detected by WB and the ability to phosphorylate DOG of these fractions (Fig. 9). It is noteworthy that FasL was also found in Golgi p58K+, Lamp-1+, and CD63+ fractions (fractions 17–19), whereas DGKα was mainly found in the TGN (fractions 5 and 8–15) and also in MVBs (fraction 25). This suggests that the sorting of DGKα to the TGN and subsequently to MVBs occurs after FasL entry into the secretory pathway.

In addition, we analyzed the distribution of DGKα by immunofluorescence analysis and confocal microscopy with cellular markers. When we expressed GFP-DGKα, we found it throughout the cytoplasm with an accumulated area in a perinuclear region of the cell (supplemental Fig. 9B). J-HM1-2.2 cells transfected with GFP-DGKα were stained with Abs against Giantin...
Inhibition of DGK increases Fas/FasL-dependent AICD in primary T lymphoblasts. T lymphoblasts were preincubated for 30 min with DX2, NOK-1 Abs, and CsA (200 ng/ml) (A) or with R59949 (DGKi, 10 μM) (B and C), and subsequently stimulated with PHA (0.3 and 1 μg/ml) or anti-CD3 Ab bound to plastic (5 μg/ml) for 24 h. After this period, apoptosis was determined as described under “Experimental
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FIG. 7. Inhibition of DGK increases the secretion of lethal microvesicles bearing FasL. Secretion of microvesicles was induced by treatment of J-HM1-2.2 cells (A) or T lymphoblasts (B) with anti-CD3 Ab (10 h, 5 µg/ml) or PHA (50 µg/ml, 5-min pulse and cultured 10 h) (21), preincubated or not with DGKi (10 µM). The microvesicles were analyzed by WB and developed with anti-CD63, anti-CD45, and anti-FasL. As a reference, protein extracts from J-HM1-2.2 or T lymphoblasts were run in parallel in the absence of anti-FasL. As a reference, protein extracts from J-HM1-2.2 or T lymphoblasts were analyzed by WB and developed with anti-CD63, anti-CD45, and anti-FasL (21). It is noteworthy that DGKα expression did not affect the overall organization of the Golgi apparatus when comparing the staining for Giantin and TGN46 with that corresponding to DGKα cells (not shown). In addition, we could not observe any colocalization when cells overexpressing GFP were stained for CD63 and TGN46, although GFP was thoroughly distributed in the cell, which supports the partial colocalization of GFP-DGKα was specific.

The biochemical distribution of DGKα and the location of GFP-DGKα by immunofluorescence indicated that DGKα is associated with TGN and late endosomal compartments.

DISCUSSION

Exosomes are small membrane vesicles formed by inward budding of the limiting membrane of late endosomes into their lumen (33). The exosomes accumulate in secretory vesicles/multivesicular bodies (MVBs); the stimulation of hematopoietic cells induces the fusion of the limiting membrane of the MVBs with the plasma membrane (33) and the secretion of exosomes. Among hematopoietic cells, CTLs kill Fas+ target cells by exposing pre-formed FasL on the plasma membrane (mFasL) and releasing soluble proteins (perforin and granzymes) stored in secretory vesicles at the immunological synapse (24, 42). Because activated T lymphocytes, but also a variety of cell types, express functional Fas receptor and therefore are sensitive to mFasL, a strict regulation of FasL expression at the surface of the effector cell is essential to avoid nonspecific killing. To fulfill this restriction, pre-formed mFasL has been observed to be intracellularly located in activated CTL CD8+ clones at the limiting membrane of secretory lysosomes or cytoplasmic lytic granules with MVB structure (24). Upon activation of CTLs, MVBs undergo fusion with the plasma membrane, and re-localization of FasL to the cell surface occurs (24). Once at the cell surface, mFasL is rapidly processed by cell matrix metalloproteinases, which shed the extracellular domain of the protein producing inactive soluble FasL (31).

Apart from this pathway for the delivery of mFasL to the cell surface, another mechanism involved in the secretion of bioactive mFasL exists in T lymphocytes. This mechanism is based on the fact that mFasL can be sorted from the limiting membrane of the late endosomes to the internal membranes (microvesicles) via inward budding (33). As one consequence, mFasL is stored in the microvesicles contained in the MVBs (35). Upon T lymphocyte activation, the fusion of MVBs with the plasma membrane results in the release of exosomes (20, 34) containing mFasL correctly oriented (C terminus facing toward extracellular medium) (21, 35). Cell surface mFasL is proteolytically cleaved by matrix metalloproteinases to generate its soluble form, leading to down-regulation of its apoptotic activity (31). One unique feature of this secreted mFasL is in comparison to the relocated mFasL at the cell surface of CTLs is conferred by the fact that cell matrix metalloproteinases do not have access to the mFasL on microvesicles; therefore, mFasL would remain bio-active (this paper and see Ref. 21). It is remarkable that high order multimerization of the Fas death receptor has been shown to be a crucial event in apoptosis induction (28). Microvesicle-bound mFasL would guarantee cytotoxic potential, because it retains its multimerization ability and cross-linking efficiency and fully preserves the target range of the mFasL expressed on the cell surface (43). Therefore, mFasL on microvesicles provides evident advantages for apoptosis induction over cell surface mFasL and the secretion of the soluble protein, given the higher efficiency in triggering cell death of mFasL when compared with soluble FasL, and the fact that processing of mFasL to its soluble form leads to down-regulation of the apoptotic activity (31).

Procedures.” The inset in the A shows the WB developed with anti-FasL Ab corresponding to protein extracts from T lymphoblasts that were preincubated with CsA (200 ng/ml), and subsequently stimulated for 6 h with PMA plus ionomycin (50 ng/ml and 0.5 µg/ml) or anti-CD3 Ab bound to plastic (5 µg/ml). The inset in B shows the WB developed with anti-DGKi corresponding to the protein extracts from T lymphoblasts cultured with IL-2. D and E, same as A and B, but analyzing the apoptosis of sorted CD4+ and CD8+ subpopulations. Cont, control.
The specificity of the released lethal exosomes for the target is one important issue that must be satisfied in order to hypothesize a role for the exosomes in T lymphoid homeostasis. A comparable requirement is satisfied by the lytic granules from CTLs to avoid the killing of bystander cells because of the following: 1) degranulation is controlled by TCR cross-linking (clonal activation); 2) release of secretory lysosomes is polarized, and mFasL is delivered exclusively to the cell surface at the synapse between CTL and the target (34). However, during AICD of T lymphocytes, it is conceivable that lethal exosomes may function beyond the local area controlled by cells expressing mFasL on the cell surface; therefore, their specificity toward the target cells could be given by the fact that only previously TCR-activated cells are mFasL-sensitive (44). In addition, exosomes produced by T lymphocytes have been shown to bear TCR/CD3 complexes, CD8, CD2, major histocompatibility complex class I, and adhesion molecules (20, 45) at the surface, which may play an important role in the specific delivery of mFasL to the target cells, avoiding the damage of bystander cells. Moreover, exosomes are enriched in combinations of ligands, costimulatory molecules, and accessory proteins that are not present on the cell surface (46) and may simultaneously engage different cell-surface receptors that in turn could control their diffusion. For example, oligomer-forming tetraspanin proteins, such as CD63, play an important role in antigen presentation, T lymphocyte activation, and cell motility and adhesion, facilitating multiple protein interactions (33). Such protein networks provided by exosomes might facilitate novel intermolecular interactions with target cells (46). Thus, secreted microvesicles containing TCR/CD3, adhesion, and pro-apoptotic molecules may contribute to cell death during antigen-driven, T lymphocyte AICD, and thus to immune regulation.

Here we show that expression of DGKα decreases FasL-mediated AICD of T lymphocytes, whereas the inhibition of DGKα enhanced AICD. DGKα regulation of AICD does not affect the transcription of the FasL gene. Instead, DGKα regulates the release of exosomes bearing mFasL.

Secretory vesicular traffic involves the formation of vesicles at the TGN, the formation of MVBs, and their fusion with the plasma membrane. These processes are exquisitely tuned by enzymes that modify lipids such as DAG and PA (37), and the efficiency of post-TGN secretory vesicle formation critically depends on the pool of DAG in the Golgi (36). Thus, inhibition of DAG production results in the blockade of protein transport from the TGN to the cell surface (36). These results support a highly conserved role for DAG in maintaining the formation of secretory vesicles from the TGN. A possible negative regulator of DAG levels in TGN is DGKα. Supporting this, DGKα was found associated with the TGN in CHO-K1 cells (47) and for the first time in T lymphocytes (this paper). The distribution of DGKα in density gradients, and the analysis by confocal microscopy, reveals that a fraction of DGKα was associated with CD63⁺ subcellular structures (Fig. 9), and also partially colo-
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Fig. 9. DGK\( \alpha \) is associated with subcellular fractions containing CD63 and FasL. Cellular fractionation of J-HM1-2.2 cells was performed as indicated under "Experimental Procedures"; fractions from a Percoll density gradient were analyzed for CD63, DGK\( \alpha \), Golgi p58k, FasL, Lamp-1 and PKD by WB. In the left side lanes, microvesicles induced with CCh and a cellular lysate were run as a reference. See also supplemental Fig. 9.

The expression of a catalytically inactive form (kinase-dead) of DGK\( \alpha \), located at the plasma membrane after stimulation (12), does not increase CCh-induced apoptosis (Fig. 8). This mutant was only found at the plasma membrane after stimulation (12) and therefore acts as a dominant interfering mutant of endogenous DGK\( \alpha \) at the plasma membrane (14). This fact hinders the use of this mutant to inhibit DGK\( \alpha \) function at subcellular locations different from the plasma membrane. However, the fact that all these plasma membrane-located mutants do not affect CCh-induced apoptosis supports the hypothesis that DGK\( \alpha \) effect on secretory vesicle traffic must be exerted at a different regulatory step than the fusion of MVBs with the plasma membrane. Most interestingly, DGK\( \alpha \) was associated with subcellular structures corresponding to TGN but was not found in cis-Golgi fractions (Fig. 9). In addition, CCh stimulation increased the amount of DGK\( \alpha \) at TGN and MVBs, where secretory vesicles are produced. The observations of DGK\( \alpha \) in the secreted microvesicles and MVBs (Fig. 9) support a possible role for DGK\( \alpha \) in controlling the inward budding process responsible for MVB biogenesis. All together, these results suggest that DGK\( \alpha \) regulates secretion of secretory vesicles at the TGN and/or during MVB biogenesis.

PKD provides a platform on which a DAG-regulated event may control secretory vesicle budding. PKD is a DAG-binding protein, and this lipid regulates the recruitment of PKD to the Golgi, which is mediated via interaction of DAG with the C1a domain on PKD (36). Prevention of the dephosphorylation of PA into DAG inhibited recruitment of PKD to the Golgi and protein transport from TGN (36). This supports a role of DAG, but not PA, in maintaining the formation of secretory vesicles from the TGN (36, 48). Once at Golgi, PKD is involved in stimulating vesicle budding from TGN; the level of vesicle budding is proportional to PKD activity, which supports a central role of PKD in TGN budding (48). The downstream effectors of PKD and their role on secretory vesicle budding are in large part unknown; however, DGK\( \alpha \) is a candidate to modulate the DAG/PKD effect on vesicle budding from TGN (48). By supporting this hypothesis, we have found PKD in CD63\( ^{\pm} \), DGK\( \alpha \), and FasL\( ^{\pm} \) Percoll fractions from untreated cells, and CCh stimulation induced an increase in the levels of DGK\( \alpha \) and PKD in these TGN fractions.

Remarkably, although relocation of DGK\( \alpha \) to the plasma membrane is involved in the attenuation of receptor-controlled DAG, and the subsequent down-regulation of activation responses (12–14), location of the enzyme in a different subcellular compartment controls a different biological effect. These facts point out that the spatial orchestration by DGK\( \alpha \) of the different pools of DAG is crucial for the control of activation and also for the regulation of the secretion of lethal exosomes, which in turn controls cell death. Very little is known about the mechanisms regulating late endosome dynamics, microvesicle formation, and secretion. Our results provide new insights into the mechanisms involved in secretory vesicle secretion and function. In addition, we provide data indicating that DGK\( \alpha \) is a key player in the negative regulation of the signals triggered by receptor stimulation which are critical, apart from the control of activation, in the AICD of T lymphocytes and hence important for the regulation of the immune system.

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2 R. Alonso, unpublished data.
Diacylglycerol Kinase α Regulates the Secretion of Lethal Exosomes Bearing Fas Ligand during Activation-induced Cell Death of T Lymphocytes
Roberto Alonso, M. Carmen Rodríguez, Jose Pindado, Ernesto Merino, Isabel Mérida and Manuel Izquierdo

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