Galectin-8 Induces Apoptosis in Jurkat T Cells by Phosphatidic Acid-mediated ERK1/2 Activation Supported by Protein Kinase A Down-regulation*

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Galectins have been implicated in T cell homeostasis playing complementary pro-apoptotic roles. Here we show that galectin-8 (Gal-8) is a potent pro-apoptotic agent in Jurkat T cells inducing a complex phospholipase D/phosphatidic acid signaling pathway that has not been reported for any galectin before. Gal-8 increases phosphatidic signaling, which enhances the activity of both ERK1/2 and type 4 phosphodiesterases (PDE4), with a subsequent decrease in basal protein kinase A activity. Strikingly, rolipram inhibition of PDE4 decreases ERK1/2 activity. Thus Gal-8-induced PDE4 activation releases a negative influence of cAMP/protein kinase A on ERK1/2. The resulting strong ERK1/2 activation leads to expression of the death factor Fas ligand and caspase-mediated apoptosis. Several conditions that decrease ERK1/2 activity also decrease apoptosis, such as anti-Fas ligand blocking antibodies. In addition, experiments with freshly isolated human peripheral blood mononuclear cells, previously stimulated with anti-CD3 and anti-CD28, show that Gal-8 is pro-apoptotic on activated T cells, most likely on a subpopulation of them. Anti-Gal-8 autoantibodies from patients with systemic lupus erythematosus block the apoptotic effect of Gal-8. These results implicate Gal-8 as a novel T cell suppressive factor, which can be counterbalanced by function-blocking autoantibodies in autoimmunity.

Glycan-binding proteins of the galectin family have been increasingly studied as regulators of the immune response and potential therapeutic agents for autoimmune disorders (1). To date, 15 galectins have been identified and classified according with the structural organization of their distinctive monomeric or dimeric carbohydrate recognition domain for β-galactosides (2, 3). Galectins are secreted by unconventional mechanisms and once outside the cells bind to and cross-link multiple glycoconjugates both at the cell surface and at the extracellular matrix, modulating processes as diverse as cell adhesion, migration, proliferation, differentiation, and apoptosis (4–10). Several galectins have been involved in T cell homeostasis because of their capability to kill thymocytes, activated T cells, and T cell lines (11–16). Pro-apoptotic galectins might contribute to shape the T cell repertoire in the thymus by negative selection, restrict the immune response by eliminating activated T cells at the periphery (1), and help cancer cells to escape the immune system by eliminating cancer-infiltrating T cells (17). They have also a promising therapeutic potential to eliminate abnormally activated T cells and inflammatory cells (1). Studies on the mostly explored galectins, Gal-1, -3, and -9 (14, 15, 18–20), as well as in Gal-2 (13), suggest immunosuppressive complementary roles inducing different pathways to apoptosis. Galectin-8 (Gal-8) is one of the most widely expressed galectins in human tissues (21, 22) and cancerous cells (23, 24). Depending on the cell context and mode of presentation, either as soluble stimulator or extracellular matrix, Gal-8 can promote cell adhesion, spreading, growth, and apoptosis (6, 7, 9, 10, 22, 25). Its role has been mostly studied in relation to tumor malignancy (23, 24). However, there is some evidence regarding a role for Gal-8 in T cell homeostasis and autoimmune or inflammatory disorders. For instance, the intrathymic expression and pro-apoptotic effect of Gal-8 upon CD4<sup>+</sup>CD8<sup>+</sup> thymocytes suggest a role for Gal-8 in shaping the T cell repertoire (16). Gal-8 could also modulate the inflammatory function of neutrophils (26). Moreover Gal-8-blocking agents have been detected in chronic autoimmune disorders (10, 27, 28). In rheumatoid arthritis, Gal-8 has an anti-inflammatory action, promoting apoptosis of synovial fluid cells, but can be counteracted by a specific rheumatoid

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The abbreviations used are: Gal-8, galectin-8; DAG, diacylglycerol; ERK, extracellular signal regulated kinase; Fas lig Ig; IL-2, interleukin-2; PA, phosphatidic acid; PAP, phosphatic acid phosphohydrolase; PLD, phospholipase D; PKA, protein kinase A; PBMC, peripheral blood mononuclear cells; PDE, phosphodiesterase; SLE, systemic lupus erythematosus; TDG, thiogalactoside; GST, glutathione S-transferase; MEK, mitogen-activated protein kinase/ERK kinase; RT, reverse transcription.
version of CD44 (CD44vRA) (27). In systemic lupus erythematosus (SLE), a prototypic autoimmune disease, we recently described function-blocking autoantibodies against Gal-8 (10, 28). Thus it is important to define the role of Gal-8 and the influence of anti-Gal-8 autoantibodies in immune cells.

In Jurkat T cells, we previously reported that Gal-8 interacts with specific integrins, such as α1β1, α3β1, and α5β1 but not α4β1, and as a matrix protein promotes cell adhesion and asymmetric spreading through activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (10). These early effects occur within 5–30 min. However, ERK1/2 signaling supports long term processes such as T cell survival or death, depending on the moment of the immune response. During T cell activation, ERK1/2 contributes to enhance the expression of interleukin-2 (IL-2) required for T cell clonal expansion (29). It also supports T cell survival against pro-apoptotic Fas ligand (FasL) produced by themselves and by other previously activated T cells (30, 31). Later on, ERK1/2 is required for activation-induced cell death, which controls the extension of the immune response by eliminating recently activated and restimulated T cells (32, 33). In activation-induced cell death, ERK1/2 signaling contributes to enhance the expression of FasL and its receptor Fas/CD95 (32, 33), which constitute a preponderant pro-apoptotic system in T cells (34). Here, we ask whether Gal-8 is able to modulate the intensity of ERK1/2 signaling enough to participate in long term processes involved in T cell homeostasis.

The functional integration of ERK1/2 and PKA signaling (35) deserves special attention. cAMP/PKA signaling plays an immunosuppressive role in T cells (36) and is altered in SLE (37). Phosphodiesterases (PDEs) that degrade cAMP release the immunosuppressive action of cAMP/PKA during T cell activation (38, 39). PKA has been described to control the activity of ERK1/2 either positively or negatively in different cells and processes (35). A little explored integration among ERK1/2 and PKA occurs via phosphatidic acid (PA) and PDE signaling. Several stimuli activate phospholipase D (PLD) that hydrolyzes phosphatidylcholine into PA and choline. Such PLD-generated PA plays roles in signaling interacting with a variety of targeting proteins that bear PA-binding domains (40). In this way PA recruits Raf-1 to the plasma membrane (41). It is also converted by phosphatidic acid phosphohydrolase (PAP) activity into diacylglycerol (DAG), which among other functions, recruits and activates the GTPase Ras (42). Both Ras and Raf-1 are upstream elements of the ERK1/2 activation pathway (43). In addition, PA binds to and activates PDEs of the type 4 subfamily (PDE4s) leading to decreased cAMP levels and PKA down-regulation (44). The regulation and role of PA-mediated control of ERK1/2 and PKA remain relatively unknown in T cell homeostasis, because it is also unknown whether galectins stimulate the PLD/PA pathway.

Here we found that Gal-8 induces apoptosis in Jurkat T cells by triggering cross-talk between PKA and ERK1/2 pathways mediated by PLD-generated PA. Our results for the first time show that a galectin increases the PA levels, down-regulates the cAMP/PKA system by enhancing rolipram-sensitive PDE activity, and induces an ERK1/2-dependent expression of the pro-apoptotic factor FasL. The enhanced PDE activity induced by Gal-8 is required for the activation of ERK1/2 that finally leads to apoptosis. Gal-8 also induces apoptosis in human peripheral blood mononuclear cells (PBMC), especially after activating T cells with anti-CD3/CD28. Therefore, Gal-8 shares with other galectins the property of killing activated T cells contributing to the T cell homeostasis. The pathway involves a particularly integrated signaling context, engaging PLD/PA, cAMP/PKA, and ERK1/2, which so far has not been reported for galectins. The pro-apoptotic function of Gal-8 also seems to be unique in its susceptibility to inhibition by anti-Gal-8 autoantibodies.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Anti-phosphotyrosine ERK1/2 monoclonal antibodies and anti-ERK1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-activated caspase-3 polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-CD3, anti-CD28, and anti-FasL monoclonal antibodies were purchased from BD-Pharmingen (BD Biosciences, San Jose, CA). The ECL system from Amersham Biosciences was used for immunoblot detection. Secondary antibodies: Alexa 488-tagged goat anti-rabbit (Molecular Probes) and HRP-conjugated goat anti-rabbit (Molecular Probes) and HRP-conjugated goat anti-rabbit (Molecular Probes). PD98059, PA, thiodigalactoside (TDG), Hoescht33342, rolipram, propranolol, and Crotonatus atrox snake venom were purchased from Sigma. Primary and secondary alcohols were purchased from Merck. Cell culture reagents were purchased from Invitrogen and Sigma. Tissue culture plastics were purchased from Nalgene Nunc (Thermo Fisher). Recombinant human GST-Gal-8 and Gal-8 released by thrombin cut from this chimera protein were prepared as previously described (10). Wild type PLD-2 and its dominant negative version were kindly provided by Dr. Michael Frohman (Stony Brook University).

**Cell Culture, Gal-8, and Drug Treatments**—Jurkat T cells (ATCC, Manassas, VA) were grown at a density of 1 × 10⁶ cells/ml in RPMI 1640 (Invitrogen) medium containing 10% fetal bovine serum, supplemented with 200 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma). Recombinant GST-Gal-8 was used either as matrix or soluble stimulus and referred to as Gal-8 treatment. GST has no effects on Jurkat cells (10), which also was corroborated here. To avoid blocking interactions of serum glycoproteins, the cells were preincubated for 2 h in serum-free medium before adding the lectin. Rolipram, propranolol, and PD98059 were added 30 min before Gal-8, whereas 1- or 2-butanol was added 5 min before.

**Immunoblotting and Densitometric Analysis for Phosphorylated ERK1/2**—Cell lysates were prepared from 2.5 × 10⁶ cells in the presence of phosphatase inhibitors as described (45). Proteins (25 μg) were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes. Antibodies directed against phosphorylated ERK1/2 or ERK1 (ERK) were used in 1:1000 dilution. The bands in immunoblots were digitalized, and their relative intensities were estimated by National Institutes of Health Image J software. The ratio of phosphorylated ERK versus ERK is represented by numbers below each blot. Each experiment was performed at least three times.
Gal-8 Death Signaling in T Lymphocytes

Transfections with Dominant Negative and Wild Type PLD-2—Transfection experiments for expression of a dominant negative or a wild type PLD-2 were made by electroporating $2 \times 10^5$ Jurkat T cells with $25 \mu$g of the vector in RPMI medium supplemented with 20% fetal bovine serum, using a Bio-Rad-Gene Pulser-II set at 300 mV and 500 microfarad. After 24 h of transfection, the cells were lysed and analyzed by immunoblot for ERK activation. Isolation of PBMC—Ten ml of peripheral blood was diluted with 10 ml of phosphate-buffered saline, layered carefully over 7.5 ml of Histopaque-1077 (Sigma) and centrifuged at $400 \times g$ for 30 min at room temperature. The cells in the opaque interface were collected, diluted twice with 50 ml of phosphate-buffered saline, centrifuged at $250 \times g$ for 10 min, and resuspended in RPMI 1640 medium containing 10% fetal bovine serum.

Phosphatidic Acid Micelles—PA micelles were prepared as previously described (46). Briefly, PA (3-sn-PA) was dissolved in 20 mM imidazole (pH 7.0) containing 1 mM EDTA and 1 mM dithiothreitol. The solution was placed in a bath sonifier (ultrasound) for 15 min twice.

PDE and PKA Assays—PDE activity was measured according to the method of Thompson and Appleman (47). Briefly, the samples were assayed in a reaction mixture of 200 $\mu$l containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 5 mM $\beta$-mercaptoethanol, 0.1 mM $\mu$CAMP, 0.75 mg/ml bovine serum albumin, and 0.1 $\mu$Ci of $^3$H-cAMP for 10 min at 33 °C. The reaction was stopped by adding 200 $\mu$l of 10 mM EDTA in 40 mM Tris-HCl (pH 8.0) followed by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5‘-AMP was hydrolized by incubation of the assay mixture with 50 $\mu$g of C. atrox snake venom for 20 min at 33 °C, and the resulting adenosine was separated by anion exchange chromatography using 1 ml of AG1-X8 resin and quantified by scintillation counting in a LKB Wallac 1217 Rackbeta liquid scintillation counter. PKA activity was assessed with the SignaTECT kit (Promega) according to the manufacturer’s recommendations.

Measurement of PA—Stimulus-induced changes in the levels of PA were assessed as described (46). Jurkat T cells (2 $\times 10^6$ cells/ml) were incubated with [3H]myristic acid (10 $\mu$Ci by dish) in RPMI 0.3% fetal calf serum overnight and then treated with GST-Gal-8 in the presence or absence of 1-butanol for 15 min. Then organic phase extraction was made by adding 300 $\mu$l of cold MetOH:HCl (50:2), 600 $\mu$l of chloroform, and 200 $\mu$l of 1 M NaCl. The organic phase at the bottom of the tube was evaporated under nitrogen flow, and the pellet was resuspended in 60 $\mu$l of chloroform:methanol (90:10). Finally, 20 $\mu$l of the sample was loaded in a TLC plate (Merck), and lipids were separated in a chloroform:acetone:methanol:acetic acid:water (40:15:15:12:7.5) mixture for 2 h at room temperature. PA standard was visualized with iodine crystals. TLC pretreated with ENHANCER™ was developed by autoradiography using Hyperfilm (Amersham Biosciences).

Apoptosis Assessment—Jurkat T cells were either spread on GST-Gal-8 treated coverslips (10) or incubated for 6 h at 37 °C in suspension (2.5 $\times 10^5$ cells) with soluble GST-Gal-8 (0.03 $\mu$M unless indicated) in the absence of fetal calf serum and then were adhered to polylysine-coated coverslips. Apoptosis was assessed by indirect immunofluorescence with anti-activated caspase-3 antibody (1:100) and by Hoescht 33342 nucleus staining to detect condensed or fragmented nuclei. Digital images (1300 $\times$ 1030 pixels) of fluorescence microscopy were acquired on a Zeiss Axiopt microscope with a 63×1.4 oil immersion objective or 40× objective (for quantification) and the 16-bit Zeiss Axiocam camera and transferred to a computer work station running Axiovision imaging software (Zeiss). The percentage of apoptotic cells was estimated from active caspase-3 staining and condensed nuclei. To assess the effect of blocking antibodies, 20 $\mu$g/ml anti-Gal-8 antibodies (10) were pre-incubated with 2 $\mu$g/ml GST-Gal-8 for 30 min at room temperature under continuous shaking, and then the mixture was added to cells, whereas 10 $\mu$g/ml blocking anti-FasL antibodies (48, 49) were added to cells together with soluble GST-Gal-8.

IL-2, Fasl, and β-Actin RT-PCR—Total RNA was purified with TRIzol™ (Invitrogen). The first strand of cDNA was obtained using 0.5 $\mu$g total RNA, oligo(dT) (Invitrogen), and Moloney murine leukemia virus reverse transcriptase (Promega) in a final volume of 10 $\mu$l. For each set of primers, 1 $\mu$l of cDNA was amplified with Taq Polymerase (Promega), and the following primers were used: 5’-GATGACCCAGATCATCTTTGAG-3’ (sense) and 5’-ATCTCTTGCTCGAAGTCCAG-3’ (antisense) for β-actin; 5’-GTCACAAACAGTGCACCTAC-3’ (sense) and 5’-ATGTTGCTGTCTCCAAATCAGC-3’ (antisense) for IL-2; and 5’-TTCAGGCGAACTCCAGCACC-3’ (sense) and 5’-CTTAGGTTCTCATGTAGACC-3’ (antisense) for FasL. The amplification program utilized was 2 min at 94 °C, 2 min at 50 °C, and 2 min at 72 °C using 25 cycles. Preliminary studies with graded numbers of amplification cycles were performed to confirm that the PCR was in the logarithmic phase.

RESULTS

Gal-8 Induces Apoptosis in Jurkat T Cells—Our previous report in Jurkat cells showed that early effects of Gal-8 used as matrix included ERK1/2-dependent cell adhesion and asymmetric spreading within 5–30 min (10). Here we explored long term effects and found that these cells became apoptotic not only when spread on Gal-8 matrix but also when incubated in suspension with soluble Gal-8 for several hours (Fig. 1, A and B). Most of the cells died after 24 h of incubation with Gal-8 (not shown). We noticed that Gal-8 released by proteolysis from the chimera protein produced similar results as GST-Gal-8 (Fig. 1C) but was unstable, rapidly losing activity. Because we previously showed (10) and corroborated here (Fig. 1C) that GST has no significant effects on Jurkat cells, we decided to use GST-Gal-8 in the experiments (referred to as Gal-8, unless indicated).

Jurkat cells spread on a Gal-8 matrix showed clear signs of apoptosis, such as nuclear condensation and caspase-3 activation (Fig. 1A), which we used for quantification (Fig. 1B). In 6 h, ~60% of the attached cells became apoptotic (Fig. 1B), and after 24 h apoptosis reached to 90% (data not shown). The effective concentration of Gal-8 as a matrix cannot be accurately estimated. Therefore, to compare the pro-apoptotic potential of Gal-8 versus that reported for other galectins, we assessed the effect of Gal-8 added in soluble form to Jurkat T cells in suspen-
After incubation, the cells were seeded on coverslips coated with polylysine and analyzed for apoptotic signs as before (Fig. 1A). Similar results obtained by flow cytometry in cells stained with propidium iodide (not shown).

Concentrations as low as 0.03–0.08 μM Gal-8 provoked caspase-3 activation and nuclear condensation in nearly 30% (range 25–40%) of the cells after 6 h of incubation (Fig. 1, A–C). More extreme conditions, such as higher Gal-8 concentrations (0.16 μM), but still in the range used for this lectin family, or 24 h of incubation with Gal-8, provoked apoptosis in more than 90% of the cells. However, to perform quantitative assessments on how drugs and other factors affect the pro-apoptotic potential of Gal-8, we used the less extreme conditions in the following experiments (i.e., Gal-8 inducing 30–40% apoptosis in 6 h).

TDG, which competes with galectin carbohydrate recognition domains, inhibited the Gal-8 apoptotic effect, demonstrating dependence on galectin-sugar interactions (Fig. 1B). As mentioned, GST alone has no effect, and both GST-Gal-8 and isolated Gal-8 gave similar results, justifying the use of GST-Gal-8 (Fig. 1C). In contrast with a previous report (15), we found that Gal-8 (heretofore added as GST-Gal-8) induced DNA fragmentation (Fig. 1D), an unequivocal sign of apoptosis.

In Jurkat cells, we previously reported that Gal-8 interacts with specific integrins, such as α1β1, α3β1, and α5β1 (10). However, blocking antibodies against β1 integrins provoked just 20% inhibition of Gal-8-induced apoptosis (not shown), indicating that other cell surface glycoprotein also participate. Thus we tested the effect of anti-Gal-8 autoantibodies, which we previously showed to inhibit Gal-8 interaction with β1 integrins and the adherence of Jurkat T cells to a Gal-8 matrix (10). Affinity-purified anti-Gal-8 autoantibodies completely blocked the pro-apoptotic potential of Gal-8, assessed on cells in suspension (Fig. 1E). These autoantibodies seem to inhibit the interaction of Gal-8 with cell surface glycoproteins in general, beyond β1 integrins.

Gal-8 Activates ERK1/2 through the PLD/PA Pathway—Apoptosis in T cells has been shown to require ERK1/2 activation (32). Jurkat cells incubated with soluble Gal-8 showed ERK1/2 activation at lectin concentrations as low as 0.008 μM (0.5 μg/ml), reaching a maximal effect at 0.03 μM (2 μg/ml) (Fig. 2A). Inhibition by TDG indicates dependence on galectin interaction with cell surface oligosaccharides (Fig. 2B). ERK1/2 activation occurred within the first 5 min and remained for at least 4 h, although it remained at lower levels (Fig. 2C).

PLD-generated PA has been involved in the regulation of the ERK1/2 pathway in T cells (42). PLD/PA-dependent processes are currently identified by their sensitivity to inhibition by primary alcohols (42). Primary alcohols, but not sec-

FIGURE 1. Gal-8 induces apoptosis in Jurkat T cells. Jurkat T cells maintained for 2 h in serum-free medium were seeded on GST-Gal-8 (referred to as Gal-8)-coated coverslips or treated in suspension with 2 μg/ml of soluble Gal-8 (0.03 μM) for 6 h at 37°C in the absence or presence of TDG and then seeded on lysine-coated coverslips. A, adhered cells assessed for apoptotic signs. Hoechst staining of condensed nuclei and indirect immunofluorescence of activated caspase 3. B, percentage of apoptotic cells (means ± S.E. of triplicates). Gal-8 induces apoptosis both as matrix (60% of the cells) and in suspension (30% of the cells). TDG inhibits the Gal-8 effect indicating dependence on cell surface interaction with glycoconjugates. C, GST-Gal-8 and isolated Gal-8 have similar pro-apoptotic effects, whereas GST has no effect. Therefore, in all other experiments we used GST-Gal-8 and referred to as Gal-8. D, DNA fragmentation in Jurkat T cells incubated in suspension in the absence or presence of 0.03 μM Gal-8 for 12 h. Ethidium bromide staining shows that Gal-8 increases DNA fragmentation. E, anti-Gal-8 autoantibodies abrogate Gal-8-induced apoptosis. The cells were treated with either Gal-8 (0.03 μM) or Gal-8 preincubated with 20 μg/ml of affinity-purified anti-Gal-8 autoantibodies from SLE patients. Apoptosis is expressed as a percentage of the maximal levels achieved under Gal-8 treatment.
ondary alcohols, compete with water in the hydrolysis of phosphatidylcholine by PLD, thus favoring the production of phosphatidyl alcohol rather than PA (50). We found that Gal-8 increased the PA levels within 15 min in a manner completely sensitive to inhibition by 1-butanol (Fig. 3A). Previous incubation with 1-butanol but not 2-butanol also reduced activation of ERK1/2 (Fig. 3B), thus involving PLD-produced PA in this pathway. ERK1/2 activation was also elicited by PA micelles, supporting a direct effect of PA (Fig. 3C). To further assess the involvement of PLD activity, we studied the effect of transiently transfected Jurkat T cells with either wild type PLD2 or dominant negative PLD2 (51). Transfection with wild type PLD2 increased ERK-1/2 activation induced by Gal-8, whereas dominant negative PLD2 had the opposite effect (Fig. 3D). All of these results indicate that Gal-8 induction of the ERK1/2 pathway is secondary to activation of PLD and subsequent generation of signaling PA.

It is well known that PA produced by PLD promotes ERK1/2 activation by recruiting Raf-1 to the plasma membrane and also by PAP-mediated conversion into DAG, which then recruits Ras (42). The contribution of the DAG-dependent pathway can be assessed by inhibiting PAP activity with propranolol, which inhibits PA conversion into DAG, as described in Jurkat T cells (42). We observed that propranolol (150 μM) decreased by ~60% the ERK1/2 activation induced by Gal-8 (Fig. 3E), suggesting that DAG effectively contributes to ERK1/2 activation by the PLD/PA pathway.

**Gal-8 Decreases PKA Activity via PA-dependent Enhancement of PDE Activity**—In addition to recruiting ERK1/2 upstream elements, PA has been described to increase PDE activity, leading to decreased cAMP levels and PKA activity in a rolipram-sensitive manner (44). Rolipram is a specific inhibitor of PDE4 isoforms (44, 52). The cAMP/PKA pathway plays a crucial role in T cells and has been reported to cross-talk with ERK1/2. Thus it is interesting to know whether Gal-8 increases PDE activity and down-regulates the cAMP/PKA pathway.

Jurkat T cells treated with 0.03 μM Gal-8 showed an increased PDE activity, which reached maximum levels in 15 min and remained above basal levels for up to 30 min (Fig. 4A). This effect correlated with a progressive reduction of PKA activity (Fig. 4B). Interestingly, rolipram abrogated not only the increased PDE activity (Fig. 4A) but also the activation of ERK1/2 (Fig. 4C). Therefore, Gal-8 releases a negative regulation of PKA secondarily to stimulating PDE4 activity, which is necessary for strong ERK1/2 activation.
Apoptosis Induced by Gal-8 Involves ERK1/2 and PLD/PA/DAG Pathways—To explore the role of the ERK1/2 pathway in the apoptotic effect of Gal-8, we first tested the MEK inhibitor PD98059. This inhibitor of the kinase that phosphorylates ERK1/2 reduced Gal-8-induced ERK1/2 activation by 60% (Fig. 5A) and abrogated the apoptotic effect of Gal-8. C, preincubation (5 min) with either 1-but or (D) propranolol decreases Gal-8-induced apoptosis. Cells directly incubated with PA micelles (PA) in conditions that activate ERK1/2 (see Fig. 3C) show increased apoptosis (average percentages of three independent experiments ± S.E.). p-ERK1/2, phosphorylated ERK1/2; C, control.

Gal-8 Death Signaling in T Lymphocytes
Gal-8 Death Signaling in T Lymphocytes

apoptosis suggests that other pathways still to be defined might also be involved.

Gal-8 Proves Similar Apoptotic Effects in PBMC—To test whether the pro-apoptotic effect of Gal-8 seen in Jurkat T cells is extensive to peripheral T cells of the organism, we used freshly isolated PBMC. In these cells, Gal-8-induced ERK1/2 activation was sensitive to inhibition by 1-butanol (Fig. 7A), indicating its PLD dependence. It is known that peripheral T cells are resistant to pro-apoptotic stimulus unless they are activated, because during this process the FasL/Fas system becomes competent (58). Incubation with 0.03 μM Gal-8 for 6 h induced apoptosis in 5% of PBMC cells, whereas after activating T cells with anti-CD3 and anti-CD28 for 24 h, apoptosis increased to 13% of the cells (Fig. 7B). Because more than 13% of the T cells were expected to become activated by anti-CD3/CD28 treatment, these results indicate that Gal-8 has pro-apoptotic potential upon subpopulation of activated peripheral T cells.

**DISCUSSION**

Here we show that Gal-8 induces apoptosis in Jurkat T cells, as other galectins do but triggers an ERK1/2-dependent death pathway that requires production of signaling PA by PLD, PKA down-regulation, and expression of FasL that has not been previously reported for any other galectin. Gal-8 also induces apoptosis in freshly isolated human PBMC previously treated for T cell activation. Anti-Gal-8 autoantibodies produced by patients with SLE (10, 28) abrogate the apoptotic effect of Gal-8. The results suggest a model in which Gal-8 triggers a PA-mediated induction of the ERK1/2 pathway strengthened by a concomitant activation of rolipram-sensitive PDEs, which removes a negative control of PKA over ERK1/2. The resulting strong and sustained ERK1/2 activation leads to enhanced expression of FasL, which is known to induce T cell apoptosis (34). Thus Gal-8 is a novel stimulus of FasL expression that could contribute to eliminate activated T cells. This immunosuppressive role can be counterbalanced by function-blocking autoantibodies in autoimmune disorders.

Gal-8, presented either as matrix or soluble stimulus, induced apoptosis in Jurkat T cells involving caspase pathway. Soluble Gal-8 promoted apoptosis at a concentration of 0.03 μM, severalfold lower than those reported for Gal-1 (5–20 μM) and Gal-3 (5 μM), and even for Gal-9 (0.3–1 μM), which so far is considered to be the most potent pro-apoptotic galectin (14, 15, 18). At 0.03 μM, Gal-8 induced ~30% apoptosis within 6 h, higher than the percentage reported for Gal-1 and Gal-3 and close to that of Gal-9, although used at higher concentrations in Jurkat cells (14, 15). A previous study reported that 0.5–2 μM Gal-8 induces phosphatidylserine exposure but not DNA fragmentation in Jurkat T cells, thus raising doubts on its pro-apoptotic potential in T cells (15). We did find clear signs of apoptosis marked by caspase-3 activation and DNA fragmentation. The source of this discrepancy is not clear. It might result from different experimental conditions, including variations in Jurkat T cells and Gal-8 preparations. However, we showed that low concentrations of Gal-8 induce apoptosis also in human PBMC. Therefore, we can conclude that Gal-8 is a potent pro-apoptotic agent in the immune system.

The apoptotic effect of Gal-8 depends on its interactions with cell surface glycoproteins, because it was effectively inhibited by TGD. Lectins can interact with a great variety of cell surface glycoproteins, making it difficult to attribute their effects to a single kind of cell surface molecule. In a previous work, we identified β1 integrins as mediators of Jurkat cell adhesion to a Gal-8 matrix, based on the inhibitory effect of anti-β1 function-blocking antibodies (10). However, β1 blocking antibodies inhibited just 20% of the Gal-8-induced apoptosis (not shown). Therefore, most likely, besides β1 integrins, other cell surface glycoproteins also participate. Gal-8 has been reported to bind αM (CD11b) (26), a β2 integrin that is expressed primarily on NK cells, neutrophils, and monocytes/macrophages (59) and in
Gal-8 induces apoptosis in T lymphocytes

FIGURE 7. Gal-8 induces PA-mediated ERK1/2 and apoptosis in human PBMC and the apoptotic effect increases after T cell activation. PBMC freshly isolated from healthy human donors were incubated with 0.03 μg/ml Gal-8 for 30 min at 37 °C. A, ERK1/2 activation sensitive to inhibition by 1-butanol involves PA produced by PLD. B, apoptosis in activated T cells. Gal-8 induces apoptosis in 5% of freshly isolated PBMC and in 13% of the cells after 48 h of T cell activation with anti-CD3 and anti-CD28 antibodies. p-ERK1/2, phosphorylated ERK1/2.

A small population of CD8^+ lymphocytes (60, 61) and γδ T cells (62, 63).

The mechanism by which Gal-8 induces apoptosis in T cells involves an interesting and still little understood interplay of signaling pathways. Galectins were reported to induce or potentiate ERK1/2 activation in a variety of cells (19, 64–67). However, the contribution of the PLD/PA and PKA pathway has not been previously explored. Here we show that Gal-8 increases the levels of PA and activates ERK1/2 by mechanisms sensitive to inhibition by both 1-butanol and a dominant negative version of PLD2 (51). These results indicate that Gal-8 activates PLD generation of signaling PA, which then promotes activation of the ERK1/2 pathway. It has been described that PA recruits Raf-1 to the plasma membrane (41) and as a substrate of the PAP enzyme is converted into DAG, which in turn recruits Ras to the membrane (42). We used PA micelles to provide direct evidence that PA activates ERK1/2 in Jurkat T cells. An inhibitor of PAP such as propranolol was able to decrease DAG production from PA in Jurkat cells (42) reduced by 50% the levels of ERK1/2 activation in response to Gal-8. Therefore, the ERK1/2 pathway triggered by Gal-8 most likely involves an upstream contribution of both PA and DAG.

In addition, our results showed that full activation of ERK1/2 by PA requires down-regulation of PKA activity. PLD-generated PA has been previously reported to activate PDE4 isoforms, which are specifically sensitive to inhibition by rolipram, leading to decreased cAMP levels and PKA activity (44, 68, 69). The role of this particular pathway remains relatively unknown. It might contribute to decrease the immunosuppressive action of cAMP during T cell activation (44). We showed that Gal-8 increases PDE activity in a manner sensitive to inhibition by rolipram, a characteristic signature of PDE4s isoforms (44, 52). As expected for an increased PDE activity, Gal-8 also decreased basal PKA activity. Therefore, the increment in the levels of PA induced by Gal-8 most likely activates PDE4s isoforms leading to a decreased PKA activity. Strikingly, rolipram not only abrogated the increased PDE activity but also reduced the ERK1/2 activation induced by Gal-8, revealing a negative control of PKA upon ERK1/2. Therefore, PDE4s provide a link between the cAMP/PKA and ERK1/2 pathways through which a negative control of cAMP/PKA on ERK1/2 is released, increasing the intensity and duration of ERK1/2 activation.

Several results indicate that the strong activation of ERK1/2 induced by Gal-8 orchestrates an apoptotic process in Jurkat T cells through an enhancement of FasL expression. PA micelles not only induced ERK1/2 activation but also apoptosis, whereas 1-butanol, propranolol, and MEK inhibitor PD98059, all conditions that inhibited ERK1/2 activation, reduced the percentage of cells that undergo apoptosis by ~50% within 6 h of incubation with Gal-8. T cell apoptosis is predominantly dependent on the function of FasL and its receptor Fas/CD95 (34). FasL is known to mediate T cell apoptosis during the process of activation-induced cell death that terminates the immune response (58, 70). IL-2 was also shown to be involved in T cell apoptosis, presumably contributing to FasL expression (56, 57), and both IL-2 expression and FasL expression depend on ERK1/2 activity (32, 33). We showed that Gal-8 promotes an ERK1/2-dependent expression of both IL-2 and FasL in Jurkat T cells. We also showed that an anti-FasL blocking antibody (49) decreased the apoptotic effect of Gal-8 by 43%. The magnitude of this inhibition correlates with that achieved by blocking the ERK1/2 pathway. Therefore, ERK1/2 activation leading to enhanced expression of the pro-apoptotic factor FasL is at least one of the mechanisms engaged in Gal-8-induced apoptosis of Jurkat cells.

The results with Jurkat T cells are extensive to the activated peripheral T cells. Resting T cells are generally resistant to apoptotic stimuli, but after activation, they express FasL and become susceptible to FasL-induced apoptosis (58, 71). We showed that Gal-8 induces apoptosis in freshly isolated human PBMC, but at low levels (5%). However, after wide stimulation of most peripheral T cells with anti-CD3 and anti-CD28 for 24 h, Gal-8-induced apoptosis increased to 13%. These results suggest that a subpopulation of activated T cells, remaining to
be defined, becomes more sensitive to Gal-8-induced apoptosis. Gal-8 could then exert an immunosuppressive role.

We still do not know the mechanism by which Gal-8 triggers the activation of PLD, leading to increases in PA levels. Given that signaling is not a linear process but is organized in networks of interacting molecules and cross-talks between different pathways, the effect of a particular stimulus would depend on the entire signaling context. It is worthwhile to remark that Gal-8 triggered cross-talk between signaling pathways crucial for a variety of cellular processes, integrating PLD/PA, cAMP/PKA, and ERK1/2 pathways.

Our results provide new clues to understand the role of the cross-talk between PKA and ERK1/2, which has been intensively studied in a number of cellular systems (35). PKA regulates either positively or negatively the activity of ERK, depending on the cell and signaling contexts (35). In cells where PKA exerts a negative control upon ERK1/2, the most accepted mechanism involves a PKA-mediated phosphorylation and inhibition of Raf-1, reducing the activity of downstream ERK1/2 (72, 73). Previous studies reported PKA cross-talk with ERK1/2 mainly in conditions of increased PKA activity (35). Instead, our results indicate that even basal levels of PKA activity exert a blocking action upon ERK1/2 activation, which is modulated by the PLD/PA pathway via PDE4.

PLD generated PA plays important roles in T cells, including T cell receptor-stimulated signaling and proliferation (42, 74–76). Our results indicate that PA-mediated down-regulation of PKA activity is required for full activation of ERK1/2. ERK1/2 and PKA signaling are both finely tuned and integrated in T cells (77, 78). Both orchestrate different responses depending on the intensity and duration of ERK1/2 activity (43, 79) as well as on the PKA cross-talk with other signaling pathways, including ERK1/2 (77, 80). Therefore, by engaging ERK1/2 promoting elements of the PLD/PA and cAMP/PKA pathways, Gal-8 could balance the signaling context toward ERK1/2 and eventually contribute to regulate other T cell outcomes, according to the circumstances of the immune response.

The regulation of PDE/cAMP/PKA system has therapeutic potential in T cells. When T cells become active in immune response, ERK1/2 and PKA are regulated in contraposition. Down-regulation of PKA is achieved by an increased expression or activation of certain PDEs (39, 81–83), including PDE4 isoforms (83), which are major regulators of cAMP levels in T cells (81, 84). Agents that increase the activity of the cAMP/PKA pathway display immunosuppressive effects (36, 85). In particular, PDE4 isoforms have been intensively studied as promising targets to control T cell-mediated diseases (86). The highly specific PDE4 inhibitor rolipram decreases IL-2 production during T cell activation (81, 82). Our demonstration that Gal-8 is an extracellular stimulus that increases the activity of PDE4 in a pathway leading to apoptosis of activated T cells suggests a new kind of intervention on autoimmune disorders. Our results also suggest that clinically used PDE4s inhibitors such as rolipram (52) could exert an unwanted protective action against T cell apoptosis under certain circumstances.

In summary, we show that Gal-8 has pro-apoptotic potential stimulating ERK1/2-mediated Fasl expression, as to play a role in T cell homeostasis. The opposite regulation of ERK1/2 and cAMP/PKA signaling by upstream activation of PLD/PA opens new avenues to explore mechanisms that control PDEs in T cell function and their therapeutic possibilities. The capacity of Gal-8 to engage PLD/PA, cAMP/PKA, and ERK1/2 pathways in an integrative manner can eventually contribute to regulate other T cell outcomes, according to the circumstances of the immune response. It can also play a role in a variety of cellular processes in different kinds of cells, including tumoral cells, in which Gal-8 is widely expressed and can promote immune escape. The function-blocking autoantibodies against Gal-8 described in SLE patients (10, 28) counteract the pro-apoptotic effects of Gal-8. These autoantibodies might control and eventually provoke pathogenic alterations of Gal-8 function in the immune system.

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