The Mechanism of CD47-Dependent Killing of T Cells: Heterotrimeric G_i-Dependent Inhibition of Protein Kinase A[1]

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CD47 has been implicated in both positive and negative regulation of T cells as well as in T cell death. To clarify the role of CD47 in T cell function, we have studied the mechanism of T cell death in response to CD47 ligands, including mAb 1F7, thrombospondin-1, and a CD47 agonist peptide derived from it. $CD47^{-/-}$ Jurkat T cells (JINB8) were resistant to killing by all three ligands, indicating the essential role of CD47. Primary human T cells were also killed by CD47 ligands, but only after activation with anti-CD3. CD47-mediated cell death occurred without active caspases, DNA fragmentation, or Bcl-2 degradation. Pretreatment of Jurkat and primary T cells with pertussis toxin (PTX) prevented CD47-mediated death, indicating the involvement of $G_{i\alpha}$. Pretreatment of T cells with 8-bromo cAMP, forskolin, or 3-isobutyl-1-methylxanthine prevented the CD47-mediated apoptosis, and 1F7 dramatically reduced intracellular cAMP levels, an effect reversed with PTX. H89 and protein kinase A (PKA) inhibitor peptide, a specific PKA inhibitor, prevented rescue of T cells by PTX, 8-bromo cAMP, and forskolin, indicating a direct role for one or more PKA substrates. Thus, CD47-mediated killing of activated T cells occurs by a novel pathway involving regulation of cAMP levels by heterotrimeric $G_{i\alpha}$ with subsequent effects mediated by PKA. ***The Journal of Immunology*****, 2003, 170: 3544–3553.**

Activation-dependent cell death is responsible for balancing the number of mature T cells, thus regulating the immune response. Several mechanisms have been found to play roles in T cell killing. Mature T cells respond to activation-dependent apoptosis after ligation of the TCR/CD3 complex (1, 2), upon Fas-Fas ligand interaction (3, 4), and through participation of CTLA-4 (5–7), which is presumed to play an important role in preventing fatal lymphoproliferation by mediating apoptosis. These mechanisms regulate the level of effector T cells in the immune system and thus control the magnitude and duration of the immune response.

In addition to these well-recognized regulators of T cell function, it has become apparent that CD47 (integrin-associated protein) and its ligands, the thrombospondins (TSPs)[3] (8), are able to regulate many facets of activation, differentiation, proliferation, and death of hematopoietic cells including monocyte/macrophages and T and B cells (9). This could represent a spatially defined mechanism for regulating the function of those cells infiltrating specific compartments such as sites of wounding, inflammation, atherosclerosis, or thrombosis, where available levels of TSPs are very high (9–11). CD47 is a 50-kDa glycoprotein of the Ig superfamily that associates with integrins such as $\alpha_v\beta_3$ and serves as a transducer element in activation of cellular signaling pathways mediated via the integrins (9, 12, 13). CD47 stimulation by TSPs can also activate certain integrins such as αIIbβ3 on platelets, leading to enhanced integrin affinity or avidity (14). CD47 has been shown to have a role in migration of polymorphonuclear neutrophils across endothelial and epithelial barriers (15, 16). A CD3-dependent costimulatory function of CD47 has been reported in T cells that is independent of CD28-mediated signaling (16, 17). However, CD47 can apparently play a dual role in the immune response. Recent reports suggest that CD47 causes anergy in immunologically naive neonatal T cells by inhibiting the expression of IL-2 and IL-12R α-chain and induces a state of T cell unresponsiveness characterized by reduced proliferation and cytokine expression (18, 19). Coengagement of TCR and CD47 by immobilized Abs reportedly costimulated and killed T cells (16, 17), whereas soluble CD47 mAbs inhibited an allogeneic mixed leukocyte reaction (20). Negative regulation by CD47 in adaptive immunity is also reflected in selective inhibition of the development of naive T cells into T helper effectors and in inhibition of dendritic cell activation and down-regulation of IL-12 responsiveness (21). Furthermore, TSP1 engagement of CD47 has been shown to inhibit CD3-mediated stimulation of Jurkat and primary human T cells, as reflected in suppression of CD69, egr-1, and phosphatase PAC-1 expression (22). Thus, the role of CD47 and its native ligands in regulation of immunity is apparently widespread, but poorly understood.

Another aspect of CD47 function in the immune response has been revealed by the effects of two anti-CD47 mAbs, Ad22 and 1F7. Cross-linking of the specific epitope on the Ig variable domain of CD47 recognized by Ad22 and 1F7 induces a kind of apoptosis in T cells (23). Mateo et al. (24) also reported that CD47 ligation through TSP or mAb induces cell death in chronic lymphocytic leukemia. The properties of this CD47-mediated cell death are distinct from other modes of apoptosis in the immune system. Phosphatidylserine is rapidly displayed on the cell surface and the mitochondrial transmembrane potential ($\Delta\Psi$m) is compromised. However, caspases are not required and DNA laddering is not seen (23, 24). The mechanism of this CD47-mediated death is not known. In the present study, we investigated the mechanism of this CD47-mediated killing using $CD47^{+/+}$ Jurkat T cells (JE6.1) and the $CD47^{-/-}$ Jurkat T cell line JINB8. We have previously

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[3] Abbreviations used in this paper: TSP, thrombospondin; $\Delta\Psi$m, mitochondrial transmembrane potential; PTX, pertussis toxin; PKA, protein kinase A; IBMX, 3-isobutyl-1-methylxanthine; PKAI, PKA inhibitor peptide; PS, phosphatidylserine; PI, propidium iodide; SIRPα, signal regulatory protein α.

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observed that CD47 is functionally coupled to heterotrimeric G_i (25) in every biological system in which CD47 augments the function of an integrin. In the case of cell spreading, chemotaxis, platelet activation/aggregation, and monocyte and lymphocyte adhesion, the response to CD47 agonists is blocked by incubation of the cells with pertussis toxin (PTX). PTX catalyzes ADP-ribosylation of the α subunit heterotrimeric G proteins of the G_i family (8, 26–29). Thus, we investigated the role of G_i in CD47-mediated apoptosis in Jurkat as well as in primary T cells. We find that CD47-mediated killing of T cells is mediated via heterotrimeric G_i, resulting in a dramatic drop in intracellular cAMP levels. Cell death is prevented by increasing the level of intracellular cyclic AMP, the effect of which depends on active protein kinase A (PKA).

Materials and Methods

Monoclonal Abs and chemicals

Apoptosis inducing anti-CD95 (CH-11, IgM) was purchased from Kamiya Biomedical (Seattle, WA); anti-CD47 mAbs 1F7 (IgG1), 2D3 (IgG1), and B6H12 (IgG1) have been described previously (11, 30, 31). FITC-labeled anti-human Bcl-2, FITC-labeled anti-human CD3, purified anti-human CD3 mAb, and annexin V apoptosis detection kit were purchased from BD PharMingen (San Diego, CA). Cell-permeable C6 ceramide was purchased from Sigma-Aldrich (St. Louis, MO). Mito Tracker red CMX-Ros was purchased from Molecular Probes (Eugene, OR). Forskolin (a direct activator of adenylate cyclases), 8-bromo cAMP, H89 dihydrochloride, 3-isobutyl-1-methylxanthine (IBMX), and PTX were purchased from Calbiochem (San Diego, CA). PKA inhibitor peptide (PKAI) was purchased from Biomol (Plymouth Meeting, PA). The Dneasy Tissue kit was purchased from Qiagen (Valencia, CA). BCA Protein Assay kit was from Pierce (Rockford, IL). Purified mAbs to human CD14, CD19, CD20, CD40, and CD56 were purchased from BD Biosciences (Mountain View, CA). Goat anti-mouse IgG-coated Dynal beads were purchased from Dynal (Oslo, Norway). cAMP enzyme immunoassay kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The preparation of 4N1K peptide (KRFYVVMWKK) from the C-terminal domain of TSP1, the mutant 4NGG peptide (KRFYGGMWKK), and TSP1 were described elsewhere (12).

Cell culture

Both JE6.1 ($CD47^{+/+}$) and JINB8 ($CD47^{-/-}$) have been described previously (32). The Jurkat T cells were cultured in Iscove's medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Human peripheral blood T cells were isolated from PBMCs. In brief, PBMCs were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation. The mononuclear cells were adhered to plastic petri dishes (two times) for removal of adherent cells. A total of 10^7 nonadherent cells were treated with saturating concentrations of anti-human CD19, anti-human CD20, anti-human CD56, and anti-human CD14 plus goat anti-mouse coated Dynal beads for removal of B cells, NK cells, and monocyte/macrophages. The bead-adhered cells were removed with a magnet and the process was repeated three times. The supernatant contained >96% $CD3^+$ T cells as judged by flow cytometry. Cell viability was always >98% as determined by trypan blue dye exclusion.

Transfection of JINB8 Jurkat cells with CD47

JINB8 cells were transfected with full-length human CD47 cDNA cloned in the pcDNA3 vector using electroporation. A total of 1×10^7 cells were resuspended with 15 μg of DNA in 0.5 ml of complete medium (Iscove's supplemented with 10% FBS) in electroporation cuvettes (Bio-Rad, Hercules, CA). Electroporation was done using an Electro Cell Manipulator (Biotrix, San Diego, CA) at 220–260 volts, according to the manufacturer's protocol. After electroporation, the transfected cells were selected in complete medium containing G-418 (1 mg/ml). The expression of CD47 by the transfected cells was tested by FACS analysis with anti-CD47 mAbs. The JINB8 cells with and without transfection with empty vector served as controls.

Cell proliferation assay

A total of 5×10^3 Jurkat T cells were plated in 100 μl of complete medium in 96-well flat-bottom tissue culture plates in the presence or absence of plate-bound or soluble Ab specific to CD3 or CD47 and were incubated for 4 days at 37°C, 5% CO_2. On the fourth day of treatment, cell number was quantified with Cell Titer 96 Aqueous Non-Radioactive Cell proliferation kit (Promega, Madison, WI). This assay depends upon intact functional mitochondria.

Apoptosis induction and detection

A total of 3×10^5 Jurkat T cells were treated with 5 μg/ml 1F7, B6/H12, or 2D3; 100 ng/ml anti-Fas Ab CH-11 (IgM); or 40 μM cell-permeable C6 ceramide for 24 h in 24-well plates (Nunc, Roskilde, Denmark). In some experiments the cells were pretreated with a saturating concentration of caspase 3 inhibitor (DEVD-CHO, 100 μM) or pan-caspase inhibitor (ZVAD-FMK, 100 μM) for a period of 2 h before exposure to 1F7, CH-11, or C6 ceramide. After 24 h the cells were harvested and analyzed for apoptosis by the following methods.

Flow cytometric determination of apoptosis and cell death

Apoptosis was determined by monitoring changes in cell size and externalization of phosphatidylserine (PS) by flow cytometry after exposure to FITC-labeled annexin V according to the manufacturer's instructions. T cells were treated with varying concentrations of mAb vs CD47 or immobilized TSP1-derived peptide (4N1K) in the presence or absence of PTX or cyclic AMP analogs. In some cases, both Jurkat T cells and primary T cells were incubated on plate-bound anti-CD3 with or without treatment with 1F7 or CD47 agonist peptide 4N1K plus PTX. The cells were harvested and stained with FITC-labeled annexin V and propidium iodide (PI) and were analyzed by flow cytometry using the CellQuest software program. A minimum of 10,000 cells was analyzed in each case with duplicate determinations.

Determination of $\Delta\Psi m$

The loss of $\Delta\Psi$m in T cells was studied using flow cytometry. The cells (3×10^5) treated as above were stained with 40 μM CMX-Ros in serum-free medium and were incubated for 45 min at room temperature. The cells were washed and analyzed by FACS analysis.

Quantification of Bcl-2

Intracellular staining for Bcl-2 was done according to the protocol described (33). In brief, cells were treated with 1F7, CH-11, or C6 ceramide at the concentrations indicated, washed in PBS, and resuspended in the same buffer. The cells were fixed with 2% paraformaldehyde for 5 min followed by treatment with octyl-α-glucopyranoside (7 mg/ml) for 5 min for permeabilization. The cells were washed in buffer, stained with FITC-conjugated anti-human Bcl-2 Ab for 45 min, and analyzed by flow cytometry. Isotype-matched Ab was used as a control.

Measurement of cAMP

A total of 3×10^5 Jurkat T cells (Je6.1) pretreated with or without PTX (100 ng/ml) were treated with 1F7 (5 μg/ml) for 24 h. In some experiments purified human peripheral blood T cells pretreated with plate-bound anti-human CD3 were challenged with 1F7 in the presence or absence of PTX. The cells were harvested, and cAMP was extracted and determined using a cAMP enzyme immunoassay kit from Amersham Pharmacia Biotech. The protein concentration was determined with the BCA Protein assay from Pierce according to the manufacturer's protocol. The amount of cAMP is expressed as fmoles/mg of protein.

DNA fragmentation analysis

A total of 3×10^5 Jurkat T cells were either treated with medium alone or treated individually with 1F7 (5 μg/ml), 4N1K (100 μM), CH-11, or C6 ceramide for 24 h. The genomic DNA was prepared from cells using a Dneasy Tissue kit obtained from Qiagen. Approximately 5 μg of DNA was electrophoretically separated on 2% agarose gels containing ethidium bromide (0.5 μg/ml).

Statistical analysis

Statistical analysis of the data was performed using the unpaired *t* test.

Results

Anti-CD47 mAb 1F7 kills only $CD47^{+/+}$ T cells

FACS analysis indicates the complete absence of CD47 on JINB8 Jurkat T cells (Fig. 1). All three mAbs (1F7, B6/H12, and 2D3) used in this study stain $CD47^{+/+}$ Jurkat T cells JE6.1 (Fig. 1, *top*) but do not stain the $CD47^{-/-}$ JINB8 Jurkat T cells (Fig. 1, *bottom*). As previously reported (32), JINB8 cells also express lower

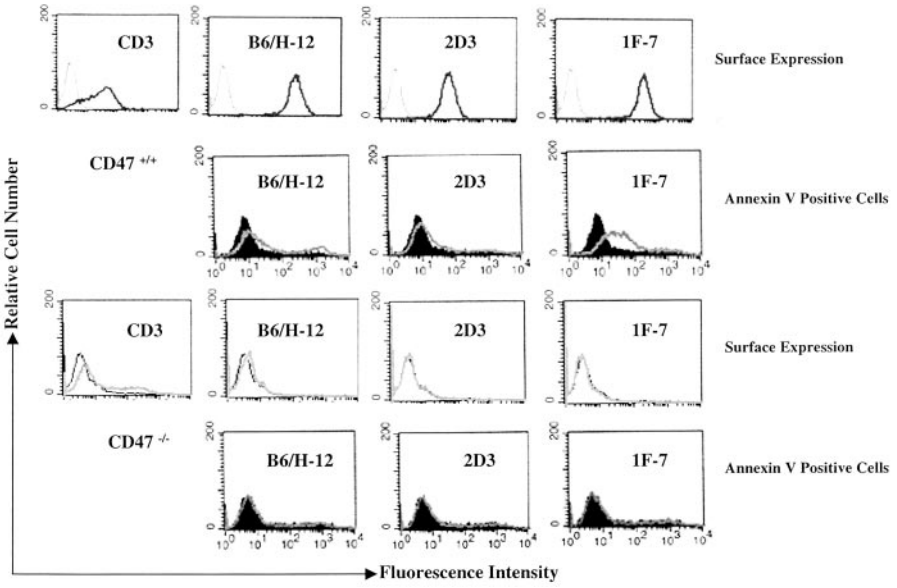

FIGURE 1. JINB8 Jurkat cells are CD47 null. JE6.1 and JINB8 cells were stained with mAbs B6/H-12, 2D3, 1F7, anti-human CD3, and FITC-conjugated goat anti-mouse IgG. The light gray histogram represents the isotype control and the overlaying solid histogram represents the surface expression of the individual Ags. To assess apoptosis, 3×10^5 JE6.1 and JINB8 cells were treated with 5 μg of 2D3, B6/H-12, or 1F7 for 24 h and were stained with FITC-annexin V. The filled black histogram represents the cells alone (control), and the overlaying line histogram represents the annexin V positivity resulting from each mAb treatment. The experiment was repeated three times with identical results.

levels of CD3 than does the parental JE6.1 line (Fig. 1). Incubation of $CD47^{+/+}$ T cells with mAb 1F7 increases annexin V binding in a dose- and time-dependent manner. Significantly increased annexin V binding is seen at concentrations of 1F7 as low as 150 ng/ml and as early as 1 h of treatment (data not shown). The maximum level of apoptosis (62–65%) was observed after 18–24 h of incubation with 5 μg/ml of Ab. Under the microscope, the cells appeared to aggregate and show morphological signs of apoptosis, including granulation and nuclear condensation (data not shown). B6H12, whose epitope overlaps that of 1F7, induced significantly less annexin V binding to JE6.1 cells than did 1F7, whereas mAb 2D3, which binds to a distinct epitope on the CD47 IgV domain, had little effect (Fig. 1). As previously noted for mAb Ad22 (23), the effect of both B6H12 and 1F7 was enhanced when the anti-CD47 mAb was immobilized on the plate along with anti-CD3 (Fig. 2*A*). Anti-Fas CH11 was used as a positive control mAb. Incubation of T cells with plate-bound anti-CD3 along with a CD47-specific Ab markedly enhanced the inhibition of T cell growth (Fig. 2*B*), yielding nearly the same level of inhibition as CH-11 treatment (Fig. 2*B*). The inhibition of cell growth could be related to the induction of apoptosis. To compare the effects of 1F7 with Ad22, which kills cells in a caspase-independent manner (23), we tested the effects of two caspase inhibitors, DEVD-CHO and ZVAD-FMK, on 1F7-induced killing (Table I). Neither inhibitor had any effect on 1F7-induced annexin V positivity. In contrast, ZVAD-FMK virtually blocked the effect of CH11, and to a lesser extent both inhibitors reduced the effect of C6 ceramide (Table I). The effect of the anti-CD47 mAbs on Bcl-2 expression had not been previously investigated. We found that both CH11 and C6 ceramide decreased the Bcl-2 content of Jurkat cells in a caspase-dependent fashion, but that 1F7 had no significant effect on Bcl-2 levels (Table II), once again indicating the caspase-independent nature of CD47-mediated death.

4N1K and TSP1 also induce apoptosis of Jurkat T cells

To determine whether native, i.e., non-Ab, ligands of CD47 also induce the apoptosis of T cells, we tested the effects of TSP1 and 4N1K peptide, both of which act as agonists of CD47 in functions

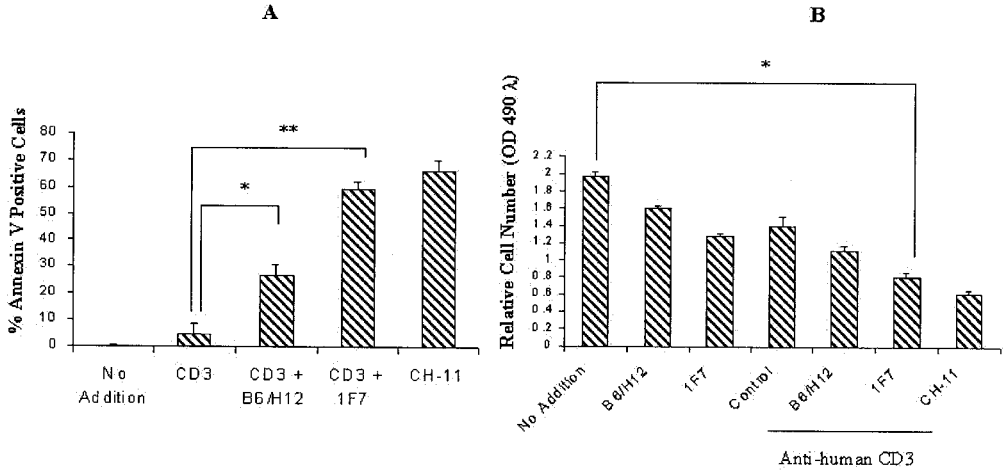

FIGURE 2. Coligation of CD3ε enhances the PS externalization and cell killing induced by mAb 1F7. Jurkat T cells (3×10^5) were cultured on plate-bound anti-CD3 alone (1 μg/ml) or on soluble or immobilized B6/H12, 2D3, or 1F7 in presence or absence of plate-bound anti-CD3 for 24 h. The cells were harvested, stained with FITC-annexin V, and analyzed by flow cytometry (*A*). Cell proliferation was assessed with the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation kit (*B*). The data are presented as mean ± SD of mean fluorescence intensity or OD_{490} of individual treatments with triplicate determinations. The experiments were repeated three times with identical results. *A*, *, $p < 0.01$; **, $p < 0.001$; relative to control cells treated with CD3 alone. *B*, *, $p < 0.0001$ relative to control cells treated with medium alone.

Table I. *Effects of IL-1β converting enzyme and caspase 3 inhibitors on 1F7-induced apoptosis of JE6.1*[a]

Conditions	Annexin V-Positive Cells (%)
Cells alone	9.05 ± 1.25
DMSO control	9.95 ± 0.15
1F7	64.5 ± 3.80
1F7 + DEVD-CHO	64.38 ± 3.95
1F7 + ZVAD-FMK	63.65 ± 0.23
CH-11	88.33 ± 0.56
CH-11 + DEVD-CHO	67.75 ± 0.34
CH-11 + ZVAD-FMK	18.27 ± 0.36
C6 Ceramide	65.42 ± 1.25
C6 Ceramide + DEVD-CHO	54.93 ± 0.44
C6 Ceramide + ZVAD-FMK	46.39 ± 0.40

[a] $CD47^{+/+}$ Jurkat T cells (JE6.1) were cultured in medium alone or were treated with 1F7 (5 μg/ml), CH-11 (100 ng/ml), or C6 ceramide (40 μM) in the presence or absence of 100 μM DEVD-CHO (caspase 3 inhibitor) or ZVAD-FMK (pan-caspase inhibitor). The inhibitors were added 2 h prior to the treatment with 1F7, CH-11, or C6. After 24 h, the cells were stained with FITC-conjugated annexin V and analyzed with flow cytometry. Data are presented as mean ± SD of percentage of annexin V-positive cells with triplicate determinations. The experiment was repeated twice with similar results.

related to integrin regulation and signaling (8–11, 12). Incubation of T cells with TSP1-coated or 4N1K peptide-coated plates induced apoptosis in $CD47^{+/+}$ Jurkat T cells, whereas in $CD47^{-/-}$ T cells TSP1 and 4N1K were no more effective than the negative control peptide 4NGG (Fig. 3*A*). However, neither TSP1 nor 4N1K were as potent as the immobilized 1F7 mAb. At the 100 μM peptide coating concentration, JE6.1 cells were 30–35% annexin V-positive compared with 60–65% with 1F7 (Fig. 3*C*). The combined effect of 1F7 and 4N1K increased apoptosis to 80% under these conditions (data not shown). Longer times of treatment resulted in death of all the cells with all of these treatments (data not shown), indicating that the differences in annexin V positivity that we see at 24 h of treatment are due to kinetic differences. Treatment of JE6.1 cells with 1F7 for a shorter period of time (3 h) resulted in only annexin V-positive cells (Fig. 3*B*), whereas longer incubations led to cell death (PI uptake) as well (Fig. 3*A*). Interestingly, $CD47^{+/+}$ Jurkat T cells are significantly more susceptible to Fas-induced apoptosis compared with the CD47 null Jurkat T cells (Fig. 3*A*), despite having a similar level of Fas expression (data not shown). To determine whether this decreased sensitivity of the JINB8 cells was due to their low level of CD3 expression (Fig. 1), we selected several clones from the JINB8 population, such as JINB8A1, that expressed levels of CD3 comparable to those of the parental JE6.1 Jurkats (Fig. 4*A*). These cells had the same lack of sensitivity to CH11 killing as the CD3-poor JINB8 population (Fig. 4*B*). Clones of JINB8 cells expressing normal levels of CD3 (JINB8-A1) remained resistant to 1F7-induced apoptosis. In contrast, JINB8 cells transfected with human CD47 (JINB8-315 in Fig. 4*A*) are susceptible to 1F7- and CH-11-induced apoptosis (Fig. 4*B*). Thus, it appears that lack of CD47, not CD3, renders the Jurkat cells less sensitive to Fas-mediated apoptosis. This suggests that CD47 plays some role in cell death initiated by the Fas pathway and perhaps other receptor-mediated cell death pathways as well.

Absence of DNA fragmentation in 1F7-induced apoptosis

DNA fragmentation is a common feature of apoptotic cell death in leukocytes (23, 24). Treatment of Jurkat T cells with either 1F7 or 4N1K did not induce genomic DNA fragmentation. In contrast, treatment with CH-11 or C6 ceramide caused characteristic fragmentation of cellular DNA (Fig. 5).

CD47-dependent killing of Jurkat T cells is mediated via G_i

We have reported that the effects of CD47 related to integrin signaling or regulation are mediated by heterotrimeric G_i (8). Thus, we wanted to determine whether this heterotrimeric G protein was also involved in the CD47-mediated killing of T cells. Pretreatment of JE6.1 cells with PTX alone at the highest concentration used (500 ng/ml, 16 h) resulted in no increase in the level of apoptosis (Fig. 6). Furthermore, PTX had no effect on the apoptosis induced by anti-Fas Ab CH-11 (Fig. 6). In contrast, PTX treatment inhibited 1F7-induced death in a dose-dependent manner, resulting in 50% reduction in annexin V-positive cells at a concentration of 500 ng/ml (Fig. 6).

1F7 induces a PTX-sensitive loss of ΔΨm

One of the hallmarks of both apoptotic and programmed necrotic cell death is a loss of potential across the mitochondrial membrane, termed ΔΨm, that drives the synthesis of ATP (34, 35). Thus, we investigated the effect of 1F7 on ΔΨm. 1F7 treatment of JE6.1 cells resulted in a decrease in ΔΨm to nonviable levels in 41% of the cells, compared with 31% of the cells with CH11 or C6 ceramide treatment (data not shown). As seen in Fig. 7, the effect of 1F7, but not of CH11 or C6 ceramide, was markedly reduced by treatment of the cells with PTX. Thus, not only PS display, but also loss of ΔΨm, is sensitive to PTX and is therefore downstream of a G_i protein.

G_i also has a role in CD47-mediated killing of primary T cells

Pettersen et al. (23) have reported that CD3-activated T cells but not naive primary T cells are susceptible to apoptosis by treatment with anti-CD47 mAb Ad22. We also observed that naive primary

Table II. *Effect of caspase inhibitors on expression of Bcl-2*[a]

Conditions	Positive Cells (%)	Mean Fluorescence Intensity
Anti-Bcl-2 control mAb	3.04 ± 0.3	6.04 ± 0.1
No treatment + anti-Bcl-2	78.11 ± 1.25	53.70 ± 3.23
1F7	79.51 ± 0.62	53.14 ± 3.13
1F7 + DEVD-CHO + ZVAD-FMK	73.87 ± 0.01	48 ± 1.0
CH-11	62.36 ± 1.12	31.5 ± 0.5
CH-11 + DEVD-CHO + ZVAD-FMK	77.87 ± 0.76	55.5 ± 2.5
C6 Ceramide	55.87 ± 3.0	23.5 ± 2.5
C6 Ceramide + DEVD-CHO + ZVAD-FMK	85.16 ± 0.25	59 ± 3.0

[a] JE6.1 Jurkat T cells were cultured alone or were treated with 1F7 (5 μg/ml), CH-11 (100 ng/ml), or C6 ceramide (40 μM) in presence or absence of 100 μM each DEVD-CHO and ZVAD-FMK. The inhibitors were added 2 h prior to 1F7, CH-11, or C6 ceramide addition and incubation for 24 h. The cells were harvested, fixed, permeabilized, and stained with FITC-conjugated anti-human Bcl-2 Ab and were analyzed by flow cytometry. An isotype-matched Ab was used as a control. Data are presented as percentage of Bcl-2-positive cells and mean fluorescence intensity. The experiment was repeated two times with similar results.

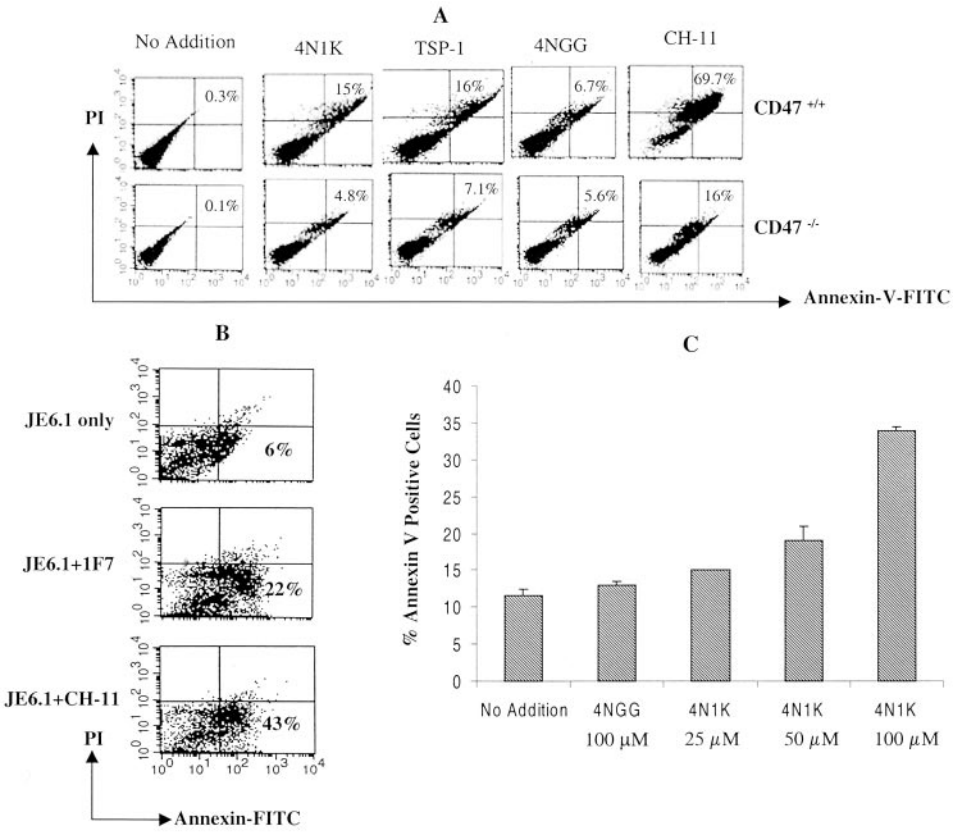

FIGURE 3. TSP1 and its CD47 agonist peptide induce apoptosis. *A*, JE6.1 ($CD47^{+/+}$) and JINB8 ($CD47^{-/-}$) were cultured on plate-bound CD47 agonist peptide 4N1K or mutant peptide 4NGG (100 μM each), TSP1 (5 μg/ml), or CH-11 (100 ng/ml) for 24 h. Apoptosis was determined by FITC-annexin V staining and uptake of PI with flow cytometry. The percentage of annexin V-positive cells is indicated within each histogram. *B*, Jurkat T cells (3×10^5) were cultured for 3 h in presence of medium alone or with immobilized 1F7 or CH-11 and were stained with FITC-annexin V. *C*, JE6.1 cells were incubated for 24 h with increasing concentrations of plate-bound 4N1K, and apoptosis was analyzed by flow cytometry. Data represent the mean ± SD of the percentage of annexin V-positive cells with triplicate determinations. The experiment was repeated three times with identical results.

T cells are not nearly as susceptible to 1F7- or 4N1K-induced apoptosis as are Jurkat T cells. However, exposing primary T cells to immobilized 1F7 along with anti-CD3 resulted in substantial apoptosis. Stimulation with 4N1K also induced apoptosis of CD3-activated T cells, although the effect was modest compared with 1F7 treatment (data not shown). As seen in Fig. 8, the increased apoptosis of CD3-activated T cells due to coimmobilized 1F7 was 80% blocked by pretreatment of T cells with PTX. Thus, the pro-apoptotic function of CD47 is intact in primary human T cells, and heterotrimeric G_i is functionally important as it is in Jurkat T cells.

Elevated cAMP levels inhibit CD47-mediated apoptosis via PKA

Because CD47 signaling is mediated via activation of G_i, the α subunit of G_i could inhibit adenylate cyclase activity, resulting in lower intracellular levels of cAMP (36). In smooth muscle cells and platelets, we found that the CD47 agonist peptide 4N1K, but

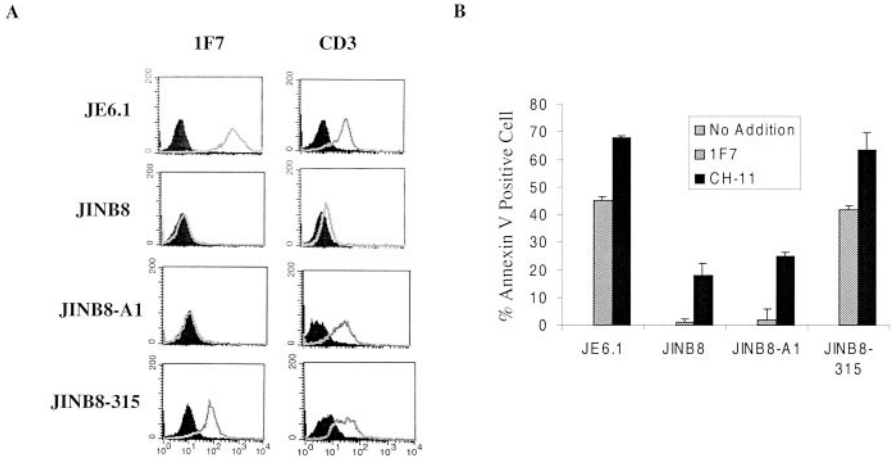

FIGURE 4. Re-expression of CD47 in JINB8 cells (CD47 null) restores susceptibility to Fas-mediated killing. JINB8 cells were transfected with full-length human CD47 (plasmid 315) by electroporation and were selected with G-418 (1 mg/ml). A total of 3×10^5 JE6.1, JINB8, JINB8A1 (a clone of JINB8 cells selected for high CD3 expression), or JINB8-315 (CD47-transfected JINB8) were treated with medium alone or with 1F7 (5 μg/ml) or CH-11 (100 ng/ml) for 24 h. *A*, Surface expression of CD3 and 1F7 in each cell type. The black histogram represents the isotype-matched control, and the light colored histogram represents the expression of the individual Ag. *B*, Percentage of annexin V-positive cells upon treatment with anti-CD47 mAb 1F7 or anti-Fas mAb CH-11. Addition of either mAb resulted in little or no apoptosis. The data are presented as mean ± SD of the percentage of annexin V-positive cells, with triplicate determinations for each condition. The experiment was repeated three times with identical results. $p < 0.001$ between JINB8 vs JE6.1 or JINB8-315 treated with CH-11.

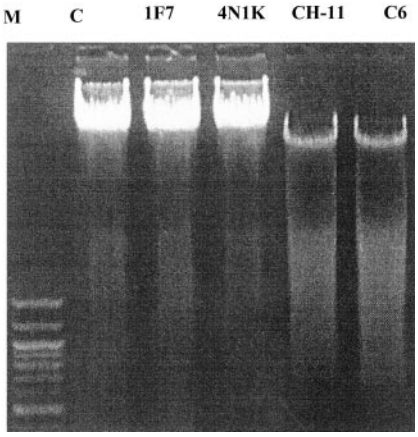

FIGURE 5. Effect of CD47-mediated apoptosis on DNA laddering in Jurkat T cells. A total of 3×10^5 Jurkat T cells were treated in medium alone or with 1F7 (5 μg/ml), 4N1K (100 μM), CH-11 (100 ng/ml), or C6 ceramide (40 μM) for 24 h. The DNA was harvested from cells using a Dneasy Tissue kit obtained from Qiagen. *Lane M* contains markers. See *Materials and Methods* for details.

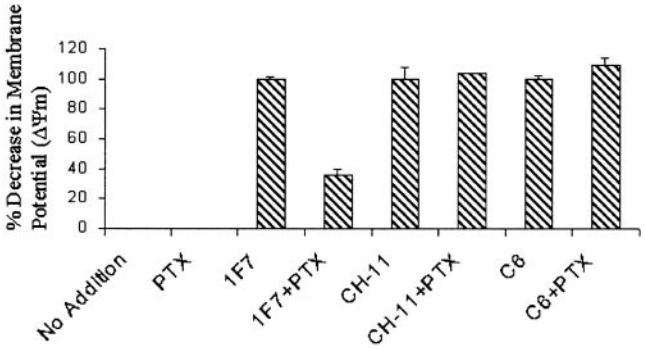

FIGURE 7. 1F7 treatment of Jurkat T cells results in loss of $\Delta\psi$m. JE6.1 cells were treated with medium alone or with PTX (100 ng/ml) for 24 h. The cells were treated with 1F7, CH-11, or C6 ceramide for another 24 h for induction of apoptosis. The cells were harvested, washed with serum-free medium, stained with CMX-Ros (50 nM in PBS for 45 min) followed by two washes in serum-free medium, and analyzed by flow cytometry. The data are presented as the percentage loss of $\Delta\psi$m. The experiment was repeated three times with similar results. A value of $p < 0.0001$ between cells treated with 1F7 vs 1F7 + PTX.

not the control peptide 4NGG, evoked a rapid and substantial (>75%) decrease in cAMP levels (13, 28). Therefore, we determined whether 1F7 caused a decrease of intracellular cAMP levels in T cells. Fig. 9 shows that in both Jurkat JE6.1 (Fig. 9*A*) and normal primary T cells (Fig. 9*B*), 1F7 treatment dramatically reduced intracellular cAMP levels. Furthermore, the decrease was completely blocked by PTX. We then used three means of elevating intracellular cAMP to determine whether reduced cAMP was causal in T cell death. Jurkat T cells were treated with 8-bromo cAMP (a cell-permeable cAMP derivative), forskolin, a direct activator of adenylate cyclase, and IBMX, a cAMP phosphodiesterase inhibitor. As shown in Fig. 10*A*, all three agents strongly inhibited the 1F7-induced apoptosis of Jurkat T cells. Together, 8-bromo cAMP and forskolin resulted in a more pronounced inhibition than either alone. 8-Bromo cAMP and forskolin also each inhibited the 4N1K-induced apoptosis of Jurkat T cells (Fig. 10*B*), and together they reduced apoptosis to the control (no addition) level. These results suggest that G_i signals the CD47-mediated death of T cells and that a large part of the effect is mediated by the decreased levels of intracellular cAMP.

Several reports indicate that cAMP can have positive or negative effects on cell survival depending on the cell type and the death-inducing insult (37–40). Furthermore, some of these cAMP effects depend on the classical pathway of activation of PKA, whereas others appear to be independent of the kinase's activity. Thus, we sought to address this issue in T cells using specific PKA inhibitors. H89 has been used for many years as a PKA inhibitor,

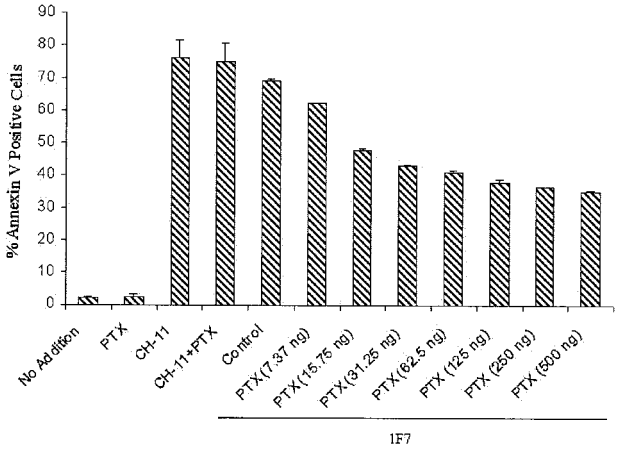

FIGURE 6. CD47-mediated apoptosis requires G_i. JE6.1 cells were treated with medium alone or with different concentrations of PTX for 24 h. The cells were treated with 1F7 (5 μg/ml) for another 24 h to induce apoptosis. Treatment with anti-Fas Ab CH-11 (100 ng/ml) was used as a control. Apoptosis was determined with FITC-conjugated annexin V as assessed by flow cytometry. The data are presented as mean ± SD of the percentage of annexin V-positive cells with triplicate determinations for each condition. The experiment was repeated three times with identical results. A value of $p < 0.005$ between cells treated with 1F7 vs 1F7 + PTX at all concentrations.

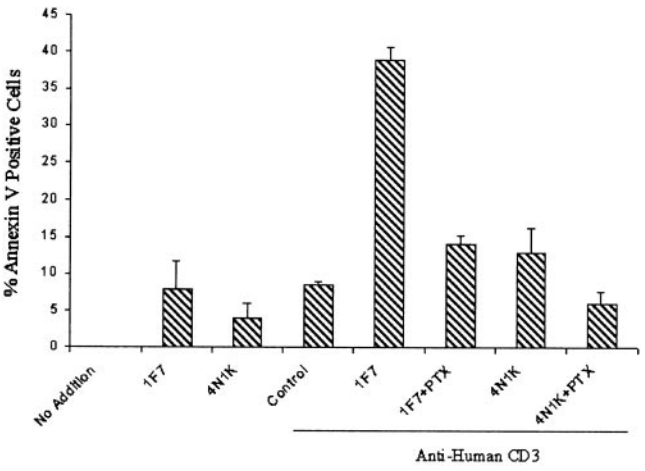

FIGURE 8. CD47-mediated apoptosis of CD3-activated peripheral primary T cells is G_i dependent. Purified peripheral blood T cells (3×10^5) were incubated in medium alone or on plate-bound anti-CD3 (1 μg/ml) in the presence or absence of soluble or plate-bound 1F7 (5 μg/ml) or plate-bound 4N1K (100 μM) for 24 h. In some experiments, the cells were pretreated with PTX (100 ng/ml) before being challenged with 1F7 in the presence of plate-bound anti-CD3 Ab. Apoptosis was determined as above. The data are presented as mean ± SD of percentage of annexin V-positive cells with triplicate determinations. The annexin V positivity of the T cells alone (no addition) was 20 ± 1.5%. The experiment was repeated three times with similar results. A value of $p < 0.005$ between control cells vs control cells with 1F7; $p < 0.0001$ between control cells with 1F7 treatment with or without PTX pretreatment.

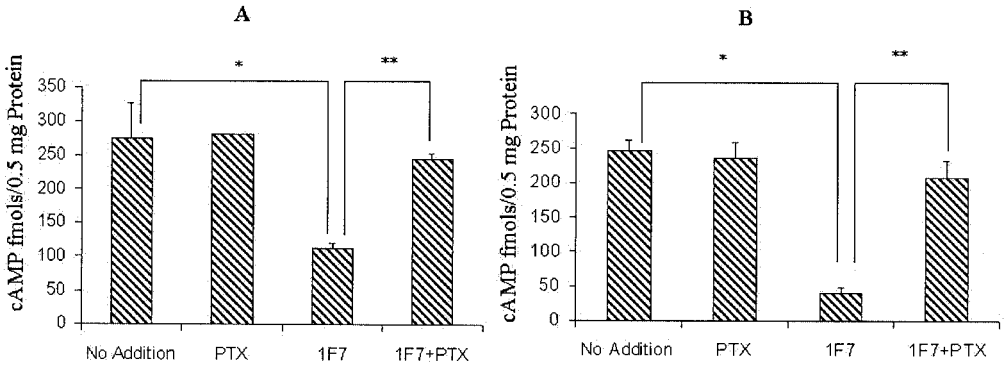

FIGURE 9. 1F7 decreases intracellular cAMP levels and PTX prevents the decrease in cAMP. *A*, 3×10^5 JE6.1 cells were pretreated with or without PTX (100 ng/ml) and then were treated with 1F7 (5 μg/ml) for 24 h. *B*, Purified human peripheral blood T cells pretreated with plate-bound anti-human CD3 were challenged with 1F7 in the presence or absence of PTX. The cells were harvested and cAMP was determined as indicated in *Materials and Methods*. Data presented are fmoles of cAMP/mg protein ± SD of triplicate determinations. The experiment was repeated three times with comparable results. *A*, *, $p < 0.0001$ between bars; **, $p < 0.0005$ between bars. *B*, *, $p < 0.001$; **, $p < 0.0001$.

but it is not completely specific. PKAI provides a means of specifically blocking PKA activity in living cells. As shown in Fig. 11, both H89 and PKAI (1 μg/ml each) reversed the sparing effects of PTX, 8-bromo cAMP, and forskolin. Thus, all of these agents that elevate intracellular cAMP are completely dependent on the activity of PKA to suppress apoptosis. The cellular targets of PKA that lead to opposition of the CD47-generated death signal remain to be determined.

Discussion

CD47, originally called integrin-associated protein (8), has been implicated in many aspects of both innate and adaptive immunity, but the picture as it stands at present is fragmentary and often contradictory in terms of predicting bona fide biological roles for CD47 and its ligands. Furthermore, only some of the effects of the TSP family of CD47 ligands are due to CD47, because TSPs can interact with a plethora of other receptors and extracellular proteins. In fact, Li et al. (41) demonstrated recently that two different sites in TSPs 1 and 2 interact with $\alpha 4\beta 1$ integrin and CD47, resulting in different and opposing effects on T cell behavior. Even in cases in which CD47 has been directly targeted with Abs or peptides, contradictory results have been obtained. In an attempt to find a common denominator that might explain some of these disparate findings, we have investigated the mechanism of CD47 action in several biological contexts and have found, in all cases in which CD47 modulates integrin function, that a PTX-sensitive G protein is involved (8, 12, 13, 25–29). However, in the case of CD47 functions in T cells, it was not clear whether integrins are required or involved or whether CD47 acts via G proteins (8).

In the present study, we first confirmed the observations of Pettersen et al. (23) regarding the ability of certain mAbs, Ad22 and 1F7, to rapidly signal the death of Jurkat T cells independently of caspase activation. Here we have used the CD47 null JINB8 Jurkat cells (32) to prove conclusively that the effects of the mAb are specific and are mediated by CD47. In addition, we show that the peptide agonist of CD47, 4N1K, and the protein from which it is derived, TSP1, also induce apoptosis of CD47 replete Jurkat T cells but not the CD47 null JINB8 cells. We have also found that a combination of a soluble construct containing the IgV domain of signal regulatory protein α (SIRPα), a cell-bound counter receptor for CD47 (8), and a particular anti-SIRPα mAb will also kill CD47 replete Jurkat cells (P. P. Manna, P. A. Oldenborg, A. Zheleznyak, and W. A. Frazier, unpublished observations). Thus, it appears that

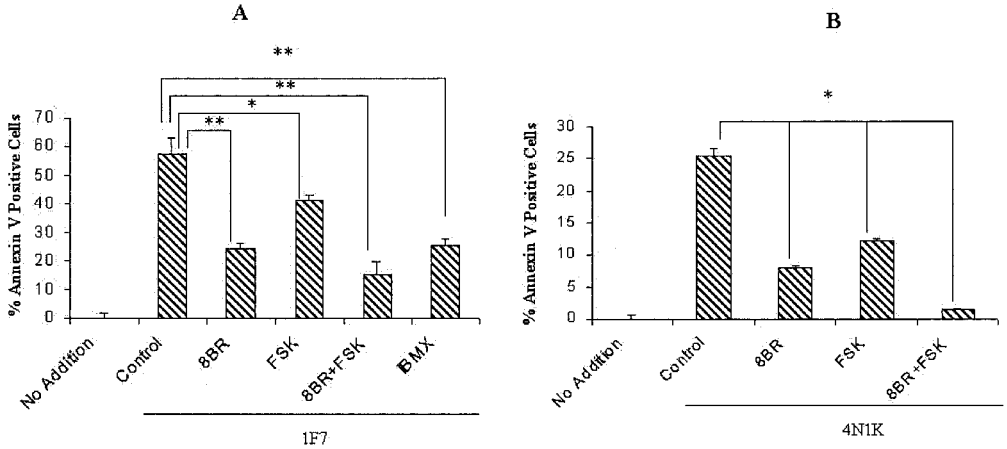

FIGURE 10. Increasing intracellular cAMP levels inhibit CD47-mediated apoptosis in Jurkat T cells. A total of 3×10^5 Jurkat T cells were pretreated with 8-bromo cAMP (100 μM), forskolin (50 μM), both, or IBMX (1 μg/ml) for 1 h before being challenged with 1F7 (*A*) or plate-bound 4N1K (*B*) and were incubated in complete medium for 24 h. The cells were harvested and apoptosis was determined as above. The data are presented as mean ± SD of percentage of annexin V-positive cells with triplicate determinations. The experiment was repeated three times with similar results. *A*, *, $p < 0.02$; **, $p < 0.001$. *B*, *, $p < 0.0001$.

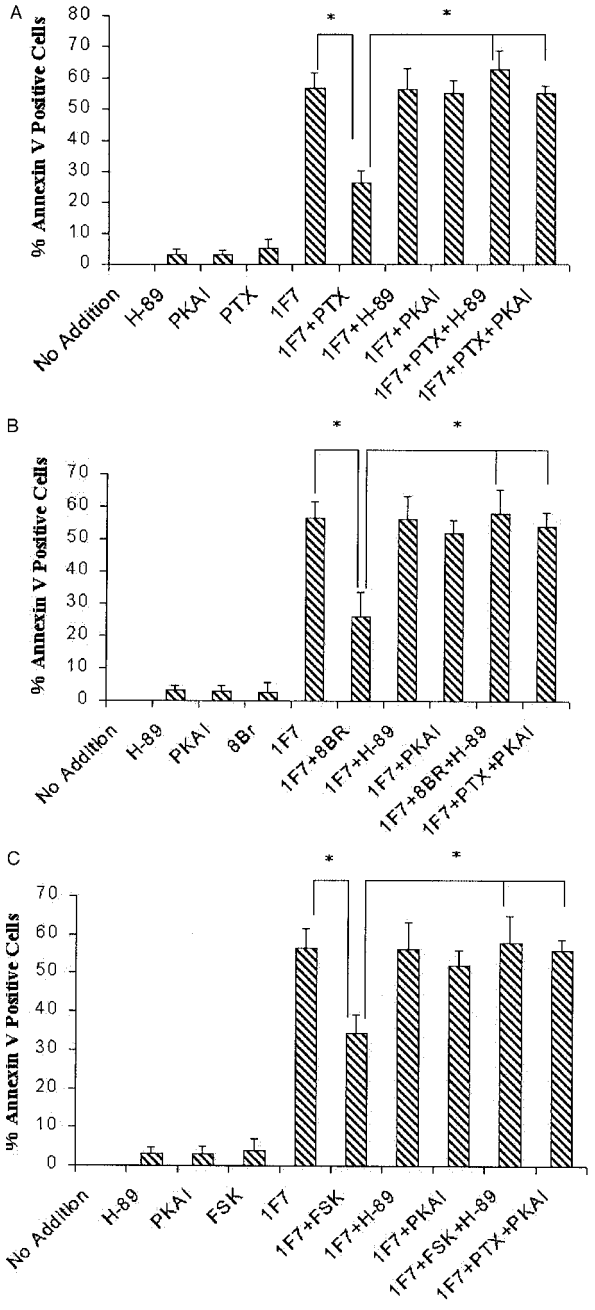

FIGURE 11. Inhibition of PKA suppresses the anti-apoptotic effect of PTX, 8-bromo cAMP, and forskolin. A total of 3×10^5 Jurkat T cells were pretreated with PTX (100 ng/ml) (*A*), 8-bromo cAMP (100 μM) (*B*), or forskolin (50 μM) (*C*) in the presence or absence of H89 or PKAI (1 μg/ml each) before being challenged with 1F7 in complete medium for 24 h. The cells were harvested and apoptosis was determined as above. The data are presented as mean ± SD of percentage of annexin V-positive cells with triplicate determinations. The experiment was repeated three times with similar results. *A*, *, $p < 0.0001$. *B*, *, $p < 0.0003$. *C*, *, $p < 0.005$.

every known ligand of CD47 is capable of initiating a death signal in Jurkat T cells. This leukemic cell line appears to be "activated" by transformation because normal peripheral T cells are insensitive to killing by CD47 ligands unless they are activated by incubation on immobilized anti-CD3. We have also noted that human K562 erythroleukemia cells, also transformed, are very efficiently killed by CD47 ligands (X. Q. Wang, P. P. Manna, J. M. Dimitry, and W. A. Frazier, manuscript in preparation).

The type of cell death induced by CD47 ligation is novel in that it resembles in some ways a classical apoptotic death in which PS accumulates on the external leaflet of the plasma membrane and mitochondria are rendered dysfunctional, as seen in the collapse of their $\Delta\Psi$m (Fig. 8). However, the inhibition of proliferation and attendant cell death proceed without caspase activation, DNA laddering, or Bcl-2 loss. Thus, in some ways this mode of cell death resembles "programmed necrotic death" as seen in other organisms (42), and it shares properties of "death by neglect" as seen in T cells deprived of extracellular survival signals via Ag-MHC-TCR complexes and/or cytokines (43). Death by neglect does not require active caspases (43, 44) and may proceed due to mitochondrial damage per se (43, 45). Although this mode of death has been thought to be a strictly "hands off" process, CD47 and its ligands may actively promote T cell death by neglect in certain contexts where T cells encounter CD47 ligands such as TSPs or SIRPα. The potential for SIRPα to induce death via CD47 raises the interesting possibility that CD47 may promote negative selection due to prolonged or high-affinity APC-T cell interaction (46).

The most novel aspect of this study is the finding that CD47-mediated death requires, in large part, a PTX-sensitive G protein, most likely one or more isoforms of G_i (25). We have been able to find one report of a classical seven-transmembrane G protein-coupled receptor, an α2 adrenoreceptor in *Oryzias latipes*, that couples to G_i and induces apoptosis of melanophores via attenuation of cAMP/PKA action (47). At 24 h of incubation, 1F7-induced PS exposure on Jurkat cells was blocked 50% by PTX treatment (Fig. 5). However, CD3-activated primary human T cells were rescued to an even greater extent by PTX (Fig. 8). This may reflect the different participation of non-PTX-sensitive G proteins in the Jurkat vs the primary T cells. In addition to PS exposure as an endpoint, two-thirds of the drop in $\Delta\Psi$m was prevented by PTX treatment of Jurkats (Fig. 7), suggesting that CD47 activation of G_i is upstream of all branches of this novel death pathway. It is important to note that PTX alone did not kill cells, nor did it affect killing by the Fas pathway (CH11) or by ceramide.

A usual result of G_i activation is a decrease in intracellular cAMP levels. In fact, this has been seen in platelets (25) and smooth muscle cells (28) treated with 4N1K peptide or TSP1. Here we found that 1F7, acting through G_i, dramatically depressed cAMP levels in Jurkat cells and in primary human T cells as well (Fig. 9). Furthermore, restoring cAMP levels in the T cells with 8-bromo cAMP, forskolin, or the phosphodiesterase inhibitor IBMX substantially inhibited CD47-mediated death. The sparing effects of all of these agents, as well as that of PTX, were completely blocked by the specific PKA inhibitor H89 and the highly specific PKAI, suggesting that one or more downstream substrates of PKA strongly opposes the death signal initiated by CD47 activation of G_i.

There are a number of reports in the literature of cAMP inhibiting apoptosis in various cell types. Cyclic AMP analogs and forskolin have been shown to oppose apoptosis initiated by diverse agents in neutrophils, promonocytic leukemia cells, and smooth muscle cells (37–40). However, the mechanisms by which cAMP does this are, in general, not known, although most require active PKA. However, there are reports of cAMP preventing apoptosis by a mechanism independent of both PKA activity and transcription (37). Ad22 kills Jurkats in the presence of inhibitors of transcription and translation (23); thus, a transcriptionally mediated mechanism would appear unlikely at first glance. However, low cAMP would have the effect of reducing cAMP response element-mediated transcription, thus allowing a decay in the level of rapidly turning over mRNA and proteins that might serve a prosurvival function. In fact, a recent report indicates that cAMP response

element-mediated transcriptional activation is involved in cAMP protection of TCR-induced apoptosis (48). Two more direct PKA-dependent mechanisms have been reported that may be relevant here. In one scenario, PKA phosphorylates and inhibits glycogen synthase kinase 3, resulting in reduced apoptosis of neurons via an unknown mechanism (49). In another, PKA directly phosphorylates Bad at a site known to suppress its proapoptotic activity (49, 50). This latter mechanism is interesting in that it directly impacts a mitochondrial mechanism of damage. The potential role of BH3-only proteins in CD47-mediated cell death is under investigation.

Another aspect of CD47 action that cannot be ignored in this context is its role in modulation/augmentation of integrin function. In leukocytes, high intracellular cAMP blocks integrin activation and aggregation (51). This fits with our observation that the CD47 agonist peptide 4N1K caused G_i-dependent activation of αIIbβ3 integrin on platelets but decreases intracellular cAMP levels (13). More recently we have found that 4N1K, TSP1, and 1F7 mAbs kill smooth muscle cells via a G_i-dependent mechanism. The cells are killed by activation of either CD47 by mAbs or of $\alpha_2\beta_1$ integrin by an activating mAb (X. Q. Wang, J. M. Dimitry, P. P. Manna, and W. A. Frazier, manuscript in preparation). It has recently been reported that an unoccupied integrin can signal apoptosis even though a different integrin on the same cell is occupied with its matrix ligand (52). That study used non-marrow-derived cells, and the unoccupied integrins initiated a more classical, caspase-dependent mode of apoptosis than seen in our studies. It may be that in T cells circulating or in culture, unoccupied integrins can signal apoptosis when they become activated by CD3, by a CD47/G_i-dependent mechanism, or perhaps by transformation as might occur in leukemic cells. High cAMP levels and PKA may then suppress this integrin activation, resulting in a decreased apoptotic signal.

In summary, our data show that ligation of CD47 by any of its known ligands rapidly induces $G_{i\alpha}$-dependent but caspase-independent apoptosis in Jurkat T cells and, importantly, in activated primary T cells. Cell killing depends on G_i-mediated reduction of cAMP levels and is effectively blocked by any agent that can maintain intracellular cAMP and, hence, PKA activity. This suggests that CD47 might play an important role in leukocyte homeostasis through clearance of activated cells in the immune system at sites where TSP levels are high. Because the CD47 agonist sequences occur in all five isoforms of TSP (8), it is possible that localized secretion or expression or secretion of any TSP isoform in the proper context could be proapoptotic. Furthermore, if SIRPα can also signal cell death via CD47, as appears likely in preliminary experiments, the interaction of SIRPα on APCs with CD47 on lymphocytes may play a role in negative selection, thus controlling autoimmunity. Therefore, it is proposed that CD47 is a potential therapeutic target in autoimmunity (53), graft-vs-host disease (54), leukemias (24), and certain infectious diseases (55).

Acknowledgments

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