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CD47 Augments Fas/CD95-mediated Apoptosis*

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Fas (CD95) mediates apoptosis of many cell types, but the susceptibility of cells to killing by Fas ligand and anti-Fas antibodies is highly variable. Jurkat T cells lacking CD47 (integrin-associated protein) are relatively resistant to Fas-mediated death but are efficiently killed by Fas ligand or anti-Fas IgM (CH11) upon expression of CD47. Lack of CD47 impairs events downstream of Fas activation including caspase activation, poly(ADP-ribose) polymerase cleavage, cytochrome *c* release from mitochondria, loss of mitochondrial membrane potential, and DNA cleavage. Neither CD47 signaling nor raft association of CD47 is required to enable Fas apoptosis. CH11 induces association of Fas and CD47. Primary T cells from CD47-null mice are also protected from Fas-mediated killing relative to wild type T cells. Thus CD47 associates with Fas upon its activation and augments Fas-mediated apoptosis.

Fas (CD95) is expressed on many cell types and mediates apoptosis during development in response to stress and in normal functioning of the immune system. Fas-mediated apoptosis is regulated by modulation of expression levels of Fas and Fas ligand (1) as well as downstream regulators of the death pathway (2, 3). Thus, the susceptibility of cells to killing by Fas ligand and anti-Fas antibodies is highly variable, particularly in leukocytes such as T cells, where activation and developmental status play a role in regulating Fas-mediated killing (4, 5). Expression of Fas *per se* does not automatically lead to cell death, because other proteins play important roles in regulating Fas-mediated killing. There are several well characterized cases in which expression or modification of intracellular proteins inhibits or promotes apoptotic events downstream of Fas (3, 6). In contrast, few examples exist in which Fas activity is modulated in either a positive or negative fashion by membrane proteins expressed in *cis* (7).

CD47 and its ligands, thrombospondins (TSPs)[1] and signal inhibitory regulatory protein α (SIRPα), have important roles in leukocyte migration (8, 9), T cell homeostasis (8, 10), recognition of "self" (11, 12), and autoimmunity (13). CD47 consists of an extracellular IgV domain followed by five transmembrane segments and a cytoplasmic tail (8). CD47 can augment integrin function through signaling via heterotrimeric G_i (14) with which it associates in cholesterol-rich rafts (15, 16). The penta-spanning transmembrane region of CD47 is required for G protein signaling. CD47 can also form complexes with integrins in *cis* that augment the avidity (clustering) of the integrin for its ligand without the apparent need for activation of intracellular signaling pathways or sequestration in rafts (16). This mode of integrin activation appears to require only the extracellular IgV domain of CD47.

We (17, 18) and others (19–21) have reported that ligation of CD47 by certain mAbs and by TSP1 and a peptide, 4N1K, derived from TSP1 (17, 18), can induce a novel, caspase-independent, but partially G_i-dependent form of apoptosis characterized by rapid mitochondrial dysfunction and annexin V display. In the course of these experiments we observed that a Jurkat T cell line deficient in CD47 expression (JinB8) was much less sensitive than wild-type Jurkat cells to killing by the anti-Fas IgM mAb CH11 (17). Here we have investigated the potential role of CD47 in Fas-mediated apoptosis and report that CD47 augments Fas-dependent apoptosis not only in Jurkat T cells but in normal mouse T cells as well. The activation of Fas causes it to associate with the extracellular IgV domain of CD47, leading to enhanced activation of downstream, caspase-dependent death pathways.

MATERIALS AND METHODS

Cell Lines and Reagents—JE6.1 ($CD47^{+/+}$), JinB8 ($CD47^{-/-}$) and JinB8–315 ($CD47^{+/+}$) have been described earlier (17). β1 integrin-deficient Jurkat T cells were provided by Dr. Yoji Shimizu (University of Minnesota Medical School, Minneapolis). All Jurkat cell lines were cultured in Iscove's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), and G418 (1 mg/ml) as needed. JINB8 cells transfected with the extracellular domain of CD47 linked to GPI (pIAP148) and CD7 (pIAP323) were provided by Dr. Eric Brown (University of California, San Francisco). Anti-human CD95 antibody CH-11 (IgM) was obtained from Coulter Immunotech. Anti-CD47 monoclonal antibodies 1F7 (IgG_1), 2D3 (IgG_1), and B6H12 (IgG_1) have been described previously (15, 17, 22). Annexin V apoptosis detection kit, phycoerythrin-labeled anti-active caspase-3, mouse anti-human PARP antibody, mouse anti-human caspase-7, anti-mouse FAS antibody Jo-2, and DX-2 were from Pharmingen, San Diego, CA. C_6-ceramide was purchased from Sigma-Aldrich. MitoTracker® red CMXRos was purchased from Molecular Probes (Eugene, OR). The DNeasy® tissue kit was purchased from Qiagen (Valencia, CA). Anti-human cytochrome *c* antibodies SC-7159 and SC-13561 and Rabbit anti-Fas antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Z-VAD-fmk was from BIOMOL (Butler Pike, PA). Polyclonal

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[1] The abbreviations used are: TSP, thrombospondin; mAb, monoclonal antibody; FASL, Fas ligand; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PARP, poly(ADP-ribose) polymerase; GPI, glycosylphosphatidylinositol; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; FACS, fluorescence-activated cell sorter; PCI, pancaspase inhibitor; WT, wild type.

anti-human caspase-3 antibody was obtained from Cell Signaling (Beverly, MA).

Clonogenic Survival Assay—The clonogenic assay was done according to the method described by Johnstone *et al.* (23) with some modification. Cells (10 μl from a stock of 1×10^5 cells/ml) treated with various apoptotic stimuli were plated in triplicate on soft agar. Cells were diluted in 5 ml of RPMI containing 10% (v/v) fetal calf serum, the supplements listed above, and 0.3% noble agar (Difco, Detroit, MI) and plated in 60-mm dishes. Once set, the dishes were overlaid with 2.5 ml of medium and incubated at 37 °C. In some wells, cells were preincubated for 2 h with 100 μmol of the pancaspase inhibitor Z-VAD-fmk as indicated in Fig. 1. After 12 days, the total number of colonies/plate was counted. Data were calculated as mean ± S.D. of triplicate determination and are representative of three individual experiments with similar results.

Apoptosis Initiated by L Cells Expressing Fas Ligand—Fas ligand (FASL)-transfected and mock-transfected L cells (10^4) were cultured overnight in 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin/streptomycin, and β-mercaptoethanol. The L cells attached firmly to the plate. Any unbound cells were removed by repeated washing. JE6.1, JINB8, and JINB8–315 cells were cultured on the L cells for 8–10 h. The loose cells were collected after gentle shaking and analyzed for annexin V staining and staining with W6/32 to confirm their identity as human Jurkat cells.

Isolation of Primary T Cells from Wild Type and CD47-deficient Mice—Splenic T cells were isolated from both wild type and CD47-deficient mice (back-crossed more than 16 generations to The Jackson Laboratory, Bar Harbor, ME, C57Bl/6 strain). In brief, single cell suspensions were made from spleens with collagenase digestion in phosphate-buffered saline. The red blood cells were lysed by lysing buffer. The mononuclear cells were adhered to a Petri dish for 2–4 h. The nonadherent cells (10^7) were collected and treated with saturating concentrations of anti-mouse CD14, CD16, and CD19 plus immunomagnetic Dynal beads to remove monocytes, NK cells, and B cells, respectively. The bead-adhered cells were removed with a magnet, and the process was repeated three times. The supernatant contained more than 98% $CD3^+$ T cells as judged by flow cytometry. Cell viability was always >98% as determined by trypan blue dye exclusion.

Apoptosis Induction and Detection—3×10^5 Jurkat T cells were treated with 5 μg/ml 1F7, B6/H12, or 2D3, 100 ng/ml of anti-Fas antibody CH-11 (IgM), or 40 μM cell-permeable C_6-ceramide for 6–8 h in 24-well plates (Nunc). In some experiments the cells were pretreated with Z-VAD-fmk, 100 μM for a period of 2 h, before exposure to 1F7 or CH-11. After 6 h the cells were harvested and analyzed for apoptosis (17). Apoptosis was determined by monitoring changes in cell size and externalization of phosphatidylserine by flow cytometry after exposure to FITC-labeled annexin V according to the manufacturer's instructions. The cells were harvested, stained with FITC-labeled annexin V and propidium iodide, and analyzed by flow cytometry using the CellQuest software program. A minimum of 10,000 cells was analyzed in each case with triplicate determinations.

Determination of Mitochondrial Transmembrane Potential ($\Delta\Psi m$)—The loss of mitochondrial membrane potential in T cells was studied using flow cytometry as described (17). The cells (3×10^5) treated as described above were stained with 40 μM CMXRos in serum-free medium and incubated for 45 min at room temperature. The cells were washed and analyzed by FACS analysis.

DNA Fragmentation Analysis—3×10^5 Jurkat T cells were treated as above for 24 h. Genomic DNA was prepared using a DNeasy® tissue kit (Qiagen). Approximately 5 μg of DNA was separated electrophoretically on 2% agarose gels containing ethidium bromide (0.5 μg/ml).

Western Blot Analysis of Apoptotic Events—1×10^6 cells were treated as indicated for 6 h in 24-well plates. The cells were harvested, sonicated for 10 s, resuspended in solubilization buffer (1% Nonidet P-40, 1 mM EDTA, 50 mM Tris-HCl, 165 mM NaCl), run on 10% SDS-PAGE in NuPAGE MOPS/SDS running buffer (Invitrogen, Carlsbad, CA), and transferred to nitrocellulose membranes that were immunoblotted with primary antibody detected with peroxidase-conjugated secondary antibody (16). For the detection of cytochrome *c*, a membrane fraction and a cytosolic fraction were prepared after treatment. Briefly, 2×10^6 cell were collected, washed twice in ice cold phosphate-buffered saline, and resuspended in buffer A (250 mM sucrose, 20 mM Hepes-KOH, 10 mM KCl, 1.5 mM EGTA, 1.5 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors). Cells were then passed through a fine gauge needle several times and allowed to swell for 20 min on ice. The cell homogenates were centrifuged at 3,000 rpm for 10 min at 4 °C to clear the debris. The supernatants were further centrifuged at 40,000 rpm for 20 min. The pellet was used as a heavy membrane fraction, whereas the supernatant was used as a cytosolic fraction. The fractions were run on 10% SDS-PAGE as described above. Detection of signals was by chemiluminescence (ECL, Amersham Biosciences).

Confocal Microscopy—JE6.1 cells were treated with medium alone or with 1F7 (5 μg/ml) or CH-11 (100 ng/ml) for 3 h and allowed to adhere on polylysine-coated coverslips. The coverslips were fixed with 2% *p*-HCHO followed by permeabilization with n-α-D-glucopyranoside (7 mg/ml). Cells were stained with rabbit anti-CD95 and 2D3 anti-CD47 mouse mAb for 45 min followed by staining with TRITC- and FITC-conjugated secondary antibodies. The cells were washed in phosphate-buffered saline and mounted on freshly cleaned slides with anti-quencher (Molecular Probes). Images were obtained with a Bio-Rad MRC 1024 confocal system on a Zeiss microscope with a 63× objective.

Immunoprecipitation of CD47-Fas Complexes—JE6.1, JinB8, or Fas-null Jurkat cells (2.5×10^6) were incubated with magnetic beads (M-450, sheep anti-mouse IgG or rat anti-mouse IgM (Dynal, Lake Success, NY)) coated with CH11, 1F7, or other mAb for 1 h at 37 °C, and then lysed with 10 mM CHAPS in phosphate-buffered saline, pH 7.2, with protease inhibitor mixture (complete mini, Roche Applied Science). In some cases, 100 ng/ml CH11 mAb or 10 μg/ml $F(ab')_2$ of anti-CD47 mAb was added in soluble form. Beads were separated with a magnet, washed, and subjected to SDS-PAGE with (Fas blots) or without (CD47 blots) reduction. Transfers were blotted with anti-CD47 mAb B6H12 or anti-Fas C-terminal antibody (Santa Cruz Biotechnology). Detection of appropriate secondary antibodies was done with chemiluminescence (SuperSignal, Pierce).

RESULTS

Expression of CD47 Promotes Apoptosis—We had previously reported that a CD47-null mutant of Jurkat cells (JinB8) was much less sensitive to killing by the anti-Fas IgM CH11 (17). Using derivatives of the Jurkat JE6.1 cell line lacking CD47 or β1 integrin (primarily $\alpha4\beta1$ in Jurkat cells), we have now examined this finding in more detail. We assessed the induction of apoptosis in two ways. After apoptotic treatments, cells were stained with fluorescent annexin V to detect exposure of phosphatidylserine on their surface (Fig. 1*A*, *bottom*). In addition to this rapid assay, a clonogenic survival assay was also employed to assure that we were detecting a true commitment to die and not just early, potentially reversible, changes (Fig. 1*A*, *top*). To be sure that a previously unknown mutation in the JinB8 cells was not responsible for our observations, we re-expressed WT CD47 in the JinB8 line (JinB8-315 cells). A shown in Fig. 1*A*, lack of CD47 strongly inhibited the ability of CH11 to kill Jurkat cells as detected in both the short term and clonogenic assays. All killing was blocked by the pancaspase inhibitor (PCI) Z-VAD-fmk. The anti-CD47 mAb 1F7 killed all of the Jurkat lines that expressed CD47. Re-expression of CD47 (in JinB8-315) restored sensitivity to CH11 killing, indicating that lack of CD47, and not another mutation in JinB8, was responsible for resistance to CH11. In contrast to the effect of CH11, JinB8 cells were not resistant to apoptosis initiated by C_6-ceramide in either the short term annexin V assay or in the clonogenic assay. Treatment with C_6-ceramide resulted in death of all cell lines whether or not CD47 was expressed. (Fig. 1*A*). In Jurkat cells, CD47 has been shown to associate with $\alpha4\beta1$ integrin (10). However, Jurkat cells deficient in β1 integrins were just as sensitive to killing by CH11 or 1F7 as WT Jurkat cells (Fig. 1*A*), indicating that these effects of CD47 did not depend on its association with $\alpha4\beta1$ or $\alpha5\beta1$ integrin.

To determine whether the physiological ligand of Fas gave the same results as mAb CH11, we exposed JE6.1, JinB8, and JinB8–315 cells (expressing identical levels of Fas, Fig. 1*B*, *right*) to Fas ligand expressed on cells, the form in which it is usually encountered in a physiological setting. L cells expressing $FASL^+$ effectively killed JE6.1 cells and Jin B8–315 cells, but the JinB8 cells were much less sensitive to cell-bound Fas ligand (Fig. 1*B*, *top*). Control L cell transfectants lacking FASL did not kill any of the cell types (Fig. 1*B*, *top*). As mentioned above, killing was prevented by the pancaspase inhibitor Z-

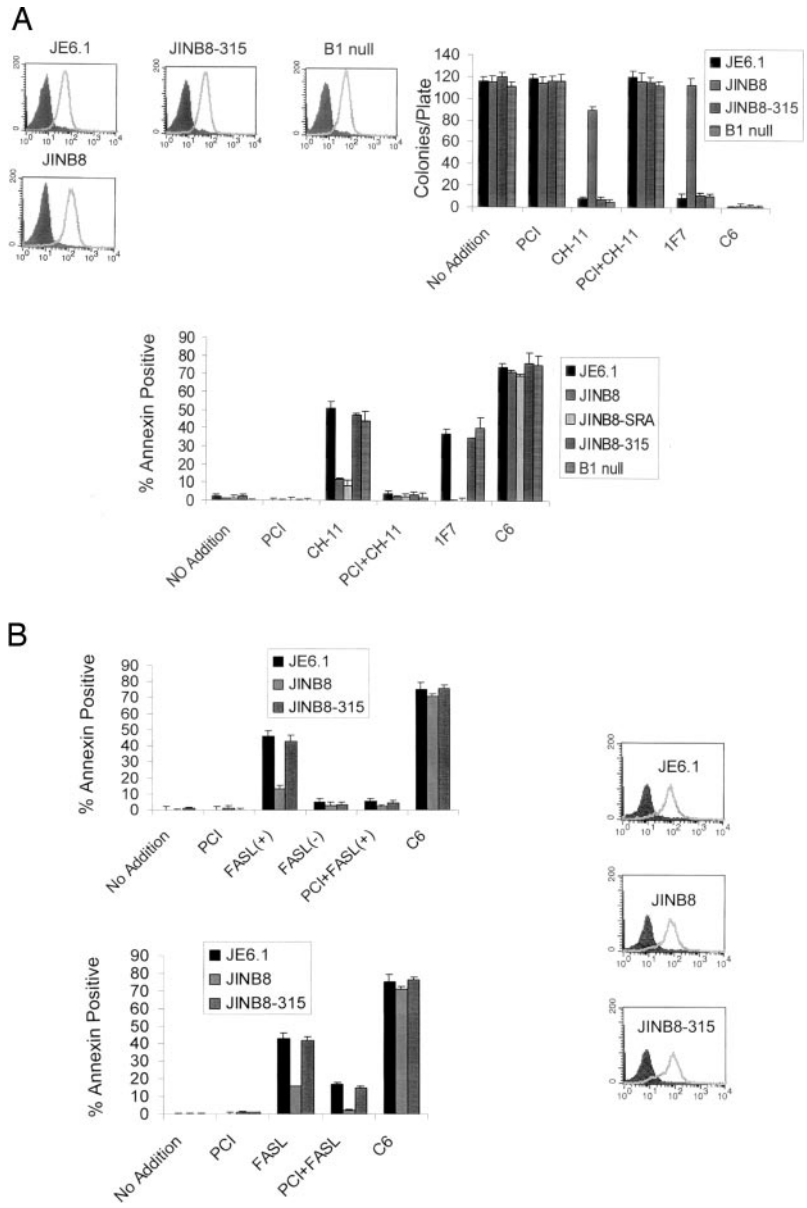

FIG. 1. **CD47 enables apoptosis stimulated by anti-Fas IgM and by Fas ligand.** *A*, JE6.1 (WT), JinB8 (CD47-null), JINB8-SRA (vector control), JINB8−315 (transfected with WT CD47), and β1-null Jurkat JE6.1 T cells (5×10^5) were stained with anti-human CD95 antibody and FITC-conjugated secondary antibody. The *dark histogram* in each case is the isotype control, and the *gray line* indicates CD95 staining (*upper right*). For the clonogenic assay (*upper right*), cells (10 μl from a stock of 1×10^5 cells/ml) were treated with various apoptotic stimuli and plated in triplicate in soft agar. The pancaspase inhibitor Z-VAD-fmk (100 μM) was added as indicated. After 12 days, the total number of colonies/plate was counted. Data were calculated as mean ± S.D. of triplicate determination and are representative of three individual experiments with similar results. Jurkat cells were also challenged (*bottom*) with CH-11 (in the presence or absence of Z-VAD-fmk (*PCI*)), the anti-CD47 mAb 1F7, or C_6-ceramide (*C6*) for 6 h in complete medium. Apoptosis was determined by staining with FITC-conjugated annexin V. *B*, FAS ligand, expressed on L cells or in soluble form, requires CD47 for optimal cell killing. FASL-transfected and mock-transfected L cells (10×10^3) were cultured overnight. After removal of unattached cells by repeated washing, JE6.1, JINB8, and JINB8–315 cells were cultured on the L cells for 8–10 h. The loose cells were collected after gentle shaking and analyzed for annexin V staining (*top*) and were then stained for human HLA (mAb W6/32) to ensure that the apoptotic cells were indeed human Jurkat cells (*right*). The three Jurkat lines were also treated with 10 ng/ml trimeric FASL or C_6-ceramide for 6 h and then analyzed for apoptosis as described in *A* (*bottom*).

VAD-fmk. Trimeric recombinant Fas ligand was also used to induce cell death (Fig. 1*B*, *bottom*) with results very similar to Fas ligand presented on the L cells. Again, C_6-ceramide killed the cells independently of CD47 expression.

Fas Is Not Necessary for Killing by CD47 mAb 1F7—To determine whether Fas played a role in CD47-dependent cell killing, we selected several clones of Jurkat cells that grew in the presence of CH11 and were found to lack Fas/CD95 expression (Fig. 2*A*). These Fas-negative cells were efficiently killed by 1F7 but not by CH11 (Fig. 2*B*), indicating that CD47-mediated killing was not Fas-dependent as suggested previously by the failure of caspase inhibitors to block 1F7 killing of Jurkat cells (17). The Fas-null Jurkat cells were efficiently killed by ceramide (Fig. 2*B*). DX-2 and SC-21730 are anti-Fas antibodies that block the effect of CH11 and prevent its killing of Jurkat cells (Fig. 2*C*). DX-2 or SC-21730 had no effect on killing of Jurkat cells by 1F7 or ceramide (Fig. 2*C*).

CD47-deficient JinB8 Cells Are Unable to Activate Pathways Downstream of FAS—The following events were defective in CH11-treated CD47-null cells: cleavage/activation of executioner caspase-7 and initiator caspase-8 (Fig. 3*A*), cleavage of the caspase substrate PARP (Fig. 3*B*), cytochrome *c* release (Fig. 3*C*), collapse of mitochondrial membrane potential (Fig. 3*D*), and DNA laddering (Fig. 3*E*). Transfection of JinB8 cells with a full-length CD47 expression plasmid (JinB8-315 cells) restored all of these downstream responses to CH11 (Fig. 3, *A–E*). This suggests that CD47 affects Fas function at a step upstream of caspase activation.

The IgV Domain of CD47 Is Sufficient to Enable Fas-mediated Apoptosis—To determine whether the transmembrane signaling domain of CD47 was necessary to enable Fas-mediated killing, we reconstituted CD47-deficient JinB8 cells with CD47 constructs in which the IgV domain is linked to the single transmembrane peptide of CD7 (pIAP323) or to a GPI anchor sequence (pIAP148) (24). The CD47-GPI construct has been shown to concentrate in GEMS or raft domains, whereas the CD47-CD7 construct is completely excluded from these domains in Jurkat cells (24). We chose clones of transfected JinB8 cells that expressed the same level of CD47 IgV domain (Fig. 4, *top*) and made sure that the transfected lines expressed the same level of Fas (not shown). To our surprise, both the CD47-GPI and the CD47-CD7 chimeras reconstituted CH11 killing of the Jurkat cells to the same extent as transfection of WT, full-length CD47. Further, both chimeras reconstituted Fas killing to the same extent, indicating that the ability of CD47 to concentrate in GEMS or rafts was not essential to enable Fas

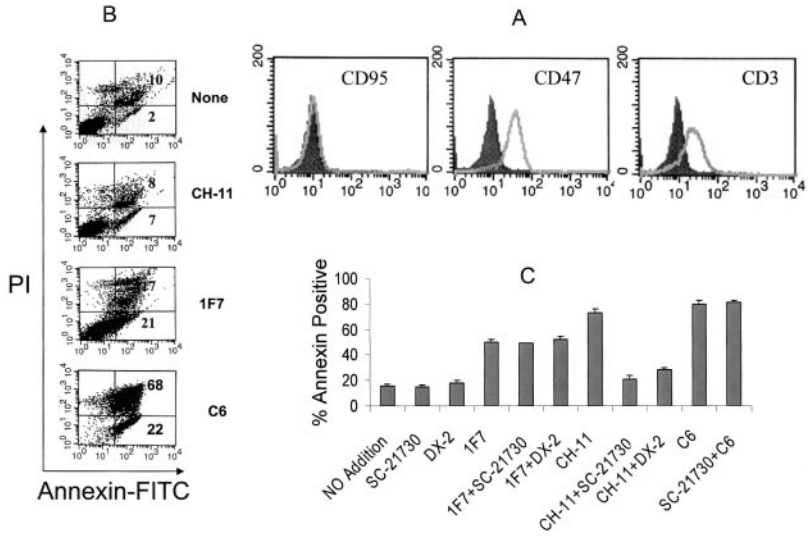

FIG. 2. **Killing of Jurkat cells by anti-CD47 mAb 1F7 is independent of Fas.** *A*, several clones of CD95-deficient CD47-positive Jurkat T cells were selected from JE6.1 by limiting dilution and continuous treatment with CH-11 (20 ng/ml). The clones were positive for CD47 and CD3 and deficient in CD95 expression, as shown in the FACS histograms. *B*, the Fas/CD95-deficient JE6.1 cells were treated with CH-11, 1F7, or C_6-ceramide (*C6*) for 8 h in complete medium, and percent apoptosis was determined by staining with FITC-conjugated annexin. The experiment was repeated five times with similar results. *C*, wild type Jurkat cells were treated with either CH11 or 1F7 to induce apoptosis in the presence or absence of anti-Fas antibodies DX-2 or SC-21730, both of which inhibit the binding of CH11 to Fas. Apoptosis was determined by annexin V binding.

apoptosis (Fig. 4, *bottom*). Killing by ceramide was equivalent in all cases (Fig. 4). Thus only the IgV domain tethered to the plasma membrane via a heterologous anchor is sufficient to restore Fas-dependent killing. Both of these chimeric forms of CD47 lack the ability to couple to G_i (14, 15). Further, pertussis toxin does not inhibit CH11 killing (17). Thus CD47 signaling via transmembrane interactions with G_i or other intracellular signaling molecules is unnecessary for Fas killing. Rather, a *cis* interaction between CD47 and Fas or a Fas-associated protein on the plasma membrane is likely to be required, and the association of CD47 with GEMS or rafts is not essential.

CD47 Associates with Fas—Because of the above mentioned data, we investigated whether CD47 and Fas could associate with one another. We reasoned that if such an association within the plane of the membrane is necessary for CD47 to enable Fas function, then there might exist monoclonal antibodies directed against the IgV domain of CD47 that could block this interaction. 1F7 and Ad22 are the only mAbs of a large panel of anti-CD47 mAbs that efficiently induced apoptosis of Jurkat cells (17, 19). The epitopes of 1F7 and Ad22 on the CD47 IgV domain are coincident and are spatially separated from epitopes of mAbs such as 2D3, which also recognize the IgV domain of CD47 (25).[2] We tested a number of anti-CD47 mAbs for their effect on CH11 killing of JE6.1 cells in both the short term (annexin V) and clonogenic assays. Two of these nonlethal anti-CD47 mAbs (2D3 and 2B7.1) protected Jurkat cells against CH11 killing to the extent of about 50% (Fig. 5), whereas anti-CD3 or anti-HLA (W6/32) mAbs, both of which bind well to Jurkat cells, had no effect. This suggests that mAbs such as 2D3 and 2B7.1 block the association of CD47 with Fas or a Fas-associated protein.

To further investigate the possible association of CD47 and Fas, we employed confocal fluorescence microscopy of Jurkat cells. With no prior antibody treatment, CD47 and Fas are both expressed on the surface of Jurkat cells but do not appear to colocalize (Fig. 6, *top row*). However, upon treatment with CH11, CD47 and Fas become strongly colocalized on T cells (Fig. 6, *bottom row*). The apparent increase in intensity in the presence of CH11 is indicative of clustering of the antigens. In contrast, treatment with the lethal anti-CD47 mAb 1F7 does not lead to colocalization of CD47 and Fas (Fig. 6, *middle row*). This result corroborates our observation that Fas-mediated apoptosis requires the presence of CD47, whereas CD47-mediated killing is independent of Fas.

The association of CD47 and Fas was also examined using co-immunoisolation approaches. Magnetic beads coated with CH11 were incubated with WT Jurkat cells or JinB8 cells. The cells were lysed, and the bound, Fas-associated proteins were detected by Western blotting of SDS gels. CD47 was associated with Fas in WT but not in JinB8 Jurkat cells (Fig. 7*A*). Because JinB8 cells do not express CD47, this serves as a control for the identity of the band illuminated in the anti-CD47 blot. In the converse approach, beads coated with the anti-CD47 mAb 1F7 were bound to JE6.1, JinB8, or the Fas-null Jurkat cells and analyzed for the association of Fas. As expected, no Fas band was detected in immunoprecipitates from JinB8 (CD47-null) or Fas-null Jurkat cells (not shown). Fas was detected in CD47 immunoprecipitates from wild type JE6.1 cells (Fig. 7*B*) and from JinB8 cells reconstituted with CD47 (not shown). When a small amount (100 ng/ml) of soluble CH11 anti-Fas mAb (sufficient to induce apoptosis in above experiments) was added during the incubation of cells with the anti-CD47-coated beads, the amount of Fas detected in association with CD47 was increased. The extent of the increase varied from one experiment to the next because the amount of Fas associated with CD47 in the absence of CH11 varied from undetectable to rather substantial amounts (as shown in Fig. 7*B*). This suggests that this basal association of CD47 and Fas is quite weak and easily perturbed upon cell lysis. This notion is further supported by the observation that adding the 1F7-coated beads to cells after detergent lysis resulted in no detectable association of Fas with CD47 (not shown). These experiments again suggest that activation or ligation of Fas promotes its association with CD47, as indicated in Fig. 6. Thus both confocal microscopy and immunoisolation support the activation-dependent association of Fas with CD47.

Because mAbs 2D3 and 2B7.1 directed against the IgV domain of CD47 were seen to inhibit Fas killing (Fig. 5) we tested

[2] A. Zheleznyak and W. A. Frazier, unpublished data.

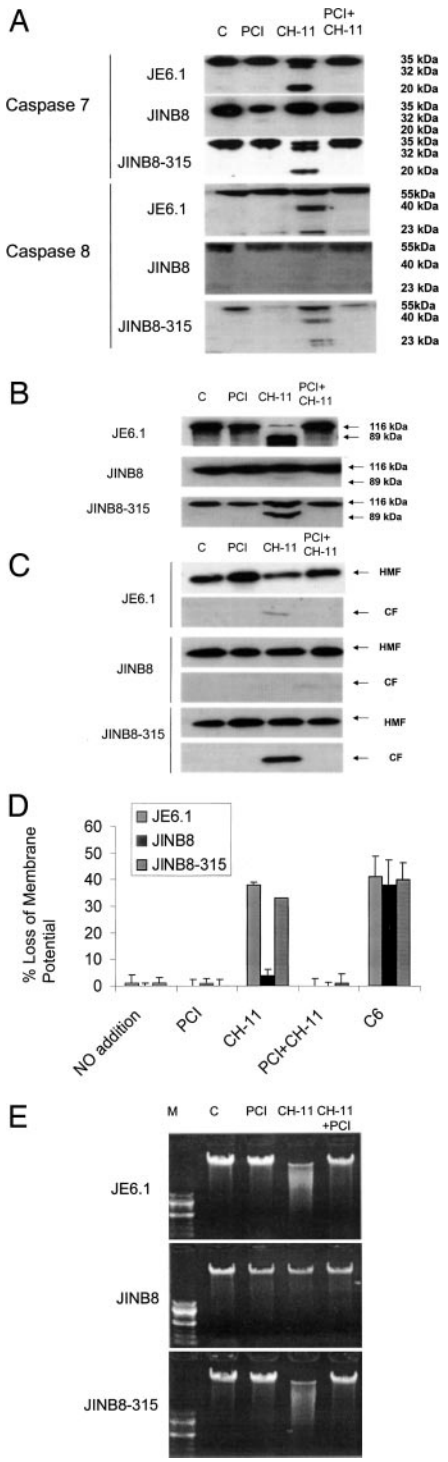

FIG. 3. **Downstream responses to Fas ligation are blunted in the absence of CD47.** *A*, Jurkat JE6.1 (WT), CD47-deficient JinB8 cells, and CD47-transfected JinB8 (JinB8-315) were treated with buffer control (*C*), Z-VAD-fmk alone (*PCI*), CH-11 alone, or CH-11 plus PCI. Cleavage/activation of the indicated caspases was determined by Western blotting. The experiments were repeated four times with similar results. Cleavage of PARP (*B*) was determined by Western blotting. In *C*, the heavy membrane fraction (*HMF*) containing mitochondria and the cytosolic fractions (*CF*) were analyzed by Western blot for the presence of cytochrome *c*. In *D*, mitochondrial membrane potential was determined using MitoTracker dye by FACS. DNA laddering is shown in *E*. *M* indicates the marker lane. The experiments were repeated four times with similar results.

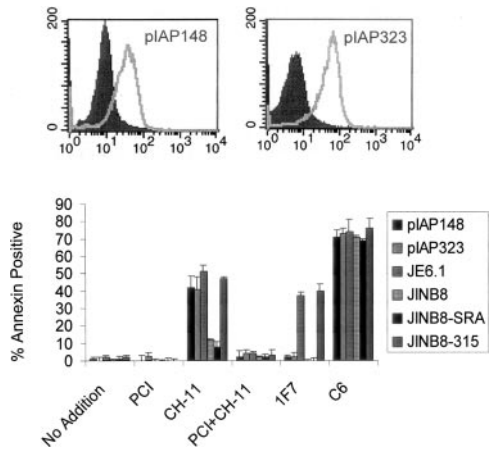

FIG. 4. **The extracellular IgV domain of CD47 is sufficient to enable Fas-mediated apoptosis.** JinB8 cells expressing the IgV domain of CD47 on a GPI (*pIAP148*, raft-localized) or a CD7-derived transmembrane polypeptide anchor (*pIAP323*, excluded from rafts) were analyzed by FACS for surface expression of CD95 (*upper panel*). The two cell lines along with JE6.1, JinB8, JinB8SRA (vector control), and JinB8-315 (CD47) lines were treated with CH11 (± Z-VAD-fmk (*PCI*)), the anti-CD47 mAb 1F7, or C_6-ceramide (*C6*). Apoptosis is expressed as percent annexin V-positive cells (*lower panel*).

these mAbs and two others to see if they could block the association of CD47 with Fas detected in these co-immunoprecipitation experiments. This was done by treating JE6.1 cells with $F(ab')_2$ fragments of anti-CD47 mAbs prior to the addition of soluble CH11 to induce the association of Fas with CD47. As seen in Fig. 7*B*, the $F(ab')_2$ fragments of 2D3 and 2B7.1 as well as 2E11, a mAb with an epitope that coincides with that of 2D3, significantly block the co-isolation of CD47 with Fas. Anti-CD47 mAb B6H12 binds to an epitope that overlaps that of 1F7, the immunoprecipitating mAb, and thus inhibits association of 1F7 with cell surface CD47. DX-2 is an anti-Fas IgG. Thus the amount of Fas recovered with this mAb suggests that a substantial portion of the cellular complement of Fas (about a third to a half) is capable of associating with CD47.

CD47 Enables Apoptosis in Normal Mouse T Cells—To determine whether normal T cells behaved like the Jurkat T cells, we isolated splenic T cells from WT and CD47-null mice and treated them with the anti-murine Fas mAb Jo-2, which kills Fas-bearing mouse cells (6). The $CD47^{-/-}$ T cells expressed the same level of Fas as WT murine T cells, as determined by FACS analysis (Fig. 8, *upper panels*). The WT T cells were killed by Jo-2, but the T cells from CD47-null mice were significantly protected from the effect of the antibody (Fig. 8, *bottom left*). As noted by others, Jo-2, being an IgG mAb, is not as effective at inducing apoptosis as the anti-human Fas CH11, which is a pentavalent IgM. The murine T cells were killed by C_6-ceramide whether or not CD47 was expressed. As in the case of Jurkat T cells, the killing of normal mouse T cells by Jo-2 mAb was blocked by Z-VAD-fmk (Fig. 8, *PCI*). The dependence of apoptosis on the concentration of Jo-2 revealed that at every concentration, the CD47-null mouse T cells are more resistant to killing than the WT T cells (Fig. 8, *bottom right*).

DISCUSSION

To our knowledge CD47 is the first example of a *cis*-interacting receptor that augments the normal, *ligand-dependent* activity of Fas, and in some experimental settings, it appears to be nearly essential for Fas-mediated killing of cells. The CD47 augmentation of Fas function is seen with antibody ligands of Fas (CH11 and Jo-2) and with the physiological Fas ligand presented in the form of the recombinant trimer or when expressed on the surface of L cells, a more physiologically relevant route of presentation (Fig. 1*B*).

Many intracellular pathways and components downstream of Fas activation have been found to modulate the response of

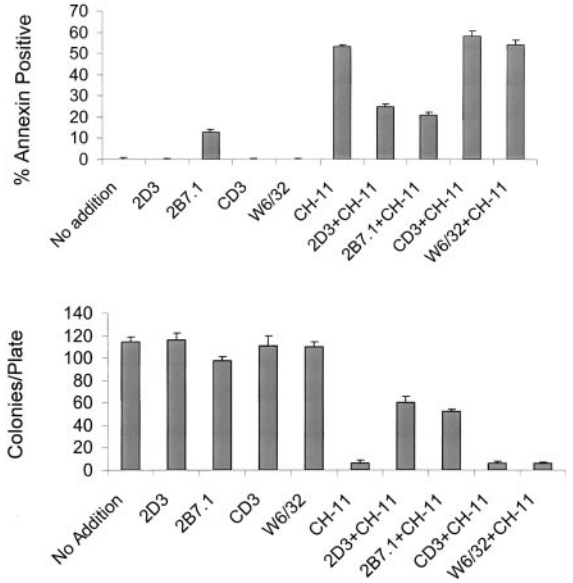

FIG. 5. **Anti-CD47 mAbs can inhibit CH11-initiated cell death.** The anti-CD47 mAbs 2D3 and 2B7.1 were added to wild type Jurkat cells simultaneously with CH11. Anti-CD3 and W6/32 mAbs were used as controls. Annexin-positive cells were determined after 6 h of incubation by FACS analysis (*upper panel*). The *lower panel* shows the results of the clonogenic survival assay (number of surviving clones after 12 days) under each of the same conditions.

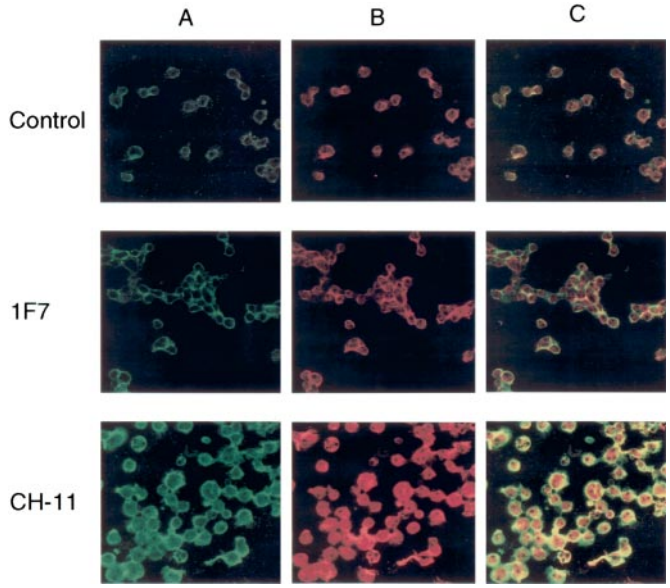

FIG. 6. **Co-localization of Fas and CD47 is seen upon ligation of Fas.** JE6.1 cells were treated with medium alone (*Control, top row*) with 1F7 (5 μg/ml, *middle row*), or CH-11 (100 ng/ml, *bottom row*) for 3 h and allowed to adhere on polylysine-coated coverslips. The cells were stained with rabbit anti-CD95/Fas (*A*) and mouse anti-CD47 (mAb 2D3) (*B*) for 45 min followed by staining with TRITC- and FITC-conjugated second antibodies. *Column C* is the merge of *A* and *B*. Confocal micrographs of a single plane show co-localization of FAS with CD47 after treatment with CH-11. Treatment with 1F7 shows much less co-localization, similar to the cells treated with control media alone. All images were obtained with the same laser and microscope parameters. The results shown are representative of five different experiments.

cells to Fas cross-linking by Fas ligand or anti-Fas IgM antibodies such as CH11 (1, 3, 26). However there are few reports of other cell surface proteins that interact with Fas to facilitate its activity. The epidermal growth factor receptor has been reported to associate with Fas after stimulation of hepatocytes with certain bile acids (27). This requires generation of reactive oxygen species, which leads to c-Jun NH_2-terminal kinase and protein kinase C activation prior to the association of the epidermal growth factor receptor with Fas. In the case of CD47 enabling of Fas killing, even membrane-tethered chimeras containing the IgV domain of CD47 are effective; thus, no CD47-initiated signaling is required to enable Fas signaling. Moorman *et al.* (7) have reported that the hepatitis C core protein is inserted into the membrane and causes Fas oligomerization leading to apoptosis, but this effect is independent of Fas ligands. A particular antibody *versus* CD99, a T cell membrane protein of unknown function, enhances Fas-mediated apoptosis, and again this appears to be because of ligand-independent aggregation of Fas (28).

The Fas-enabling role of CD47 appears to be a unidirectional effect. CD47-mediated cell death initiated by 1F7, TSP1, or 4N1K peptide does not require Fas and appears to proceed without the activation of caspases (17, 19). In contrast, Fas-mediated cell killing is strongly augmented by CD47 in that many if not all of the downstream effects of Fas activation are absent or severely blunted in Jurkat cells lacking CD47 (Fig. 3). This enabling effect of CD47 expression is not limited to the transformed Jurkat cell line but is also apparent in T cells isolated from CD47-null mice treated *ex vivo* with the anti-Fas mAb Jo-2 (Fig. 8).

What can we deduce about the mechanism of the CD47 effect? First, we have shown that the penta-spanning transmembrane domain that connects CD47 to G_i signaling, is not necessary for CD47 to enable Fas killing. The IgV domain of CD47 linked to either a heterologous transmembrane peptide from CD7 or to a GPI anchor is sufficient to promote Fas function (Fig. 4). The fact that either CD47 chimera is as efficient as full-length CD47 in restoring Fas function to JinB8 cells indicates that localization of CD47 within raft membrane domains is not critical for its interaction with Fas. Rebres *et al.* (24) determined that the same CD47-CD7 chimera used here is completely excluded from rafts or GEMS when it is expressed in Jurkat cells, whereas the vast majority of the CD47-GPI chimera is located in raft domains in Jurkat cells. Full-length CD47 can exist both in and out of raft domains, but G_i signaling by CD47 appears to require its localization within rafts (14–16). As shown previously (17), cell death initiated by the anti-CD47 mAb 1F7 depends, at least partially, on G_i signaling from CD47, but, as seen here, the augmentation of Fas-mediated death by CD47 does not. Thus neither the penta-spanning signaling domain of CD47 nor its localization in rafts is required for CD47 to enable Fas-mediated apoptosis. This suggests a cell surface interaction of CD47 with Fas.

Additional support for a *cis* interaction of CD47 and Fas is found in our observation that two mAbs against the CD47 IgV domain that do not in themselves kill cells partially block the killing by CH11 mAb, suggesting that they interfere with the association between CD47 and Fas (Fig. 5) This interpretation is confirmed by the observation that $F(ab')_2$ fragments of these two anti-CD47 mAbs can reduce the amount of Fas associated with CD47 in co-immunoprecipitations (Fig. 7*B*). In the confocal experiments (Fig. 6) we noted that strong colocalization of CD47 and Fas was not seen unless the cells were first treated with CH11 mAb. This conclusion was strengthened by the co-immunoprecipitation experiments in which CH11 could pull down Fas along with CD47, but anti-CD47 mAbs pulled down varying amounts of Fas unless the cells were first treated with CH11 (Fig. 7). Thus it appears likely that a direct protein-protein interaction between the extracellular domain of Fas and the IgV domain of CD47 is sufficient to promote Fas activity. However, at present we cannot rule out a model in which CD47 associates with another membrane protein that is itself in direct contact with Fas.

The most likely explanation for these results is that the

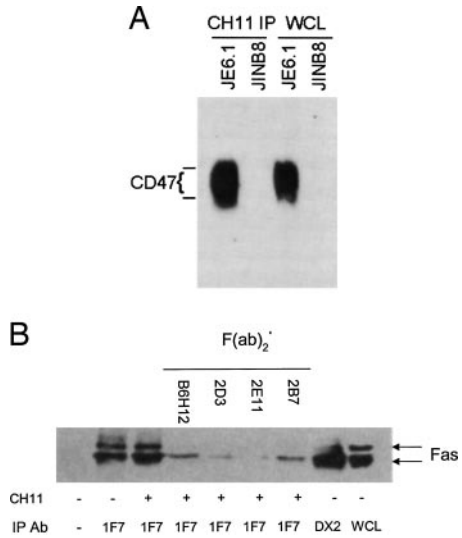

FIG. 7. **Co-immunoprecipitation of CD47 and Fas is disrupted by the anti-CD47 mAbs that inhibit CH11-initiated cell death.** *A*, JE6.1 or JinB8 cells were incubated with CH11 (anti-Fas)-coated magnetic beads and lysed with CHAPS. After washing, bead-bound material was solubilized in SDS sample buffer and run on SDS-PAGE. Western blotting was with anti-CD47 mAb B6H12. *WCL*, whole cell lysate. The Fas-null cells were also used in this experiment to control for nonspecific binding and failed to bind to the CH11-coated beads (not shown). *B*, magnetic beads coated with anti-CD47 mAb 1F7 were incubated with JE6.1 cells with or without additional soluble CH11 anti-Fas mAb or with $F(ab')_2$ fragments of the anti-CD47 mAbs 2D3, 2E11, and 2B7.1. The cells were lysed as described in *A*, and the bound material was Western blotted for Fas. The amount of Fas present was estimated by immunoprecipitation (*IP*) with the anti-Fas IgG DX-2. *WCL*, whole cell lysate. The results shown here are representative of five experiments. As noted in the text, the amount of Fas associated with CD47 in the absence of CH11 stimulation is variable.

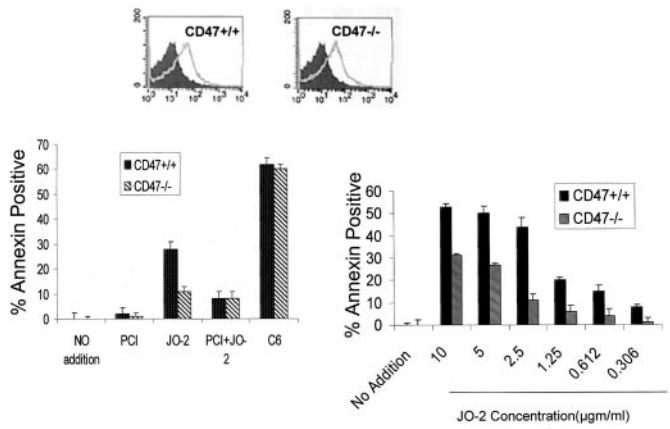

FIG. 8. **Lack of CD47 protects normal T cells against Fas-mediated killing.** In the *upper two panels*, T cells isolated from WT (*left*) or CD47-null (*right*) mice were analyzed for expression of FAS using flow cytometry with Jo-2. The *filled histogram* was obtained with control antibody and the *open histogram* with Jo-2. In the *lower right panel*, T cells from $CD47^{+/+}$ (*solid bars*) or $CD47^{-/-}$ (*hatched bars*) were treated with various concentrations of Jo-2 anti-Fas mAb for 6 h, and annexin V-positive cells were determined by FACS analysis. In the *bottom left panel*, the WT and CD47-null T cells were treated with Jo-2 (2 μg/ml) or C_6-ceramide (*C6*) for 6 h. Z-VAD-fmk (*PCI*) was present in some experiments. The experiment was repeated three times.

activation of Fas promotes its association with CD47, which, in turn, promotes an increased aggregation or clustering of Fas. Chemical cross-linking experiments[3] are consistent with this notion. It is well established that clustering or cross-linking of Fas is an important feature of its activation of downstream death pathways (1, 29). For example, soluble Fas ligand, even though it is trimeric, does not deliver a potent death signal until it has been further clustered with antibody or by immobilization on a surface (4, 30). It has been reported that ceramide, which is rapidly generated upon Fas ligation, acts to promote the aggregation and capping of Fas in the Jurkat membrane, thus facilitating apoptosis. However, at higher doses such as used here, ceramide can kill Jurkat cells independently of the Fas pathway (Fig. 2*B*) (31).

The effect of CD47 on Fas aggregation and activation is analogous to the effect of CD47 on the aggregation and increased avidity of the integrin $\alpha v\beta 3$, the best studied integrin partner of CD47 (16). In that study, we found that CD47-GPI is sufficient to promote a conformational change in $\alpha v\beta 3$, which increases its clustering, resulting in more avid binding of the integrin to its ligands. It is well established that clustering enhances intracellular downstream signaling from integrins (32), and in this sense, the roles of CD47 in promoting signaling from both integrins and Fas may be analogous. It should be noted that, although CD47 has been reported to associate with $\alpha 4\beta 1$ integrin on Jurkat cells (33), the effect of CD47 on Fas-mediated apoptosis does not appear to require the $\beta 1$ integrin, because cells lacking the integrin are killed by Fas ligands to the same extent as normal Jurkat cells (Fig. 1*A*). However, given that the mechanism of CD47 association with integrins is still incompletely understood and the full list of integrins with which CD47 can interact is likely not complete, we cannot rule out the participation of an integrin in the Fas-CD47 complex.

A number of physiological settings exist in which Fas-mediated apoptosis may be augmented by the presence of CD47, and emerging data suggest that CD47-null mice share some features with mice deficient in either Fas (*lpr*) or Fas ligand (*gld*). When either of these strains is bred onto a background such as MLR, prone to autoimmune disease, the severity of the autoimmune response is exacerbated (2). NOD (non-obese diabetic) mice develop multiple autoimmune diseases. In addition to type 1 diabetes, they also develop Sjogren syndrome (34) and a mild form of autoimmune hemolytic anemia (35). When CD47-null mice are crossed with NOD mice, mice of the resulting strain die of severe autoimmune hemolytic anemia before they can develop diabetes (13).

Another situation in which CD47 may augment Fas action is the pruning and remodeling of the retinal vasculature that occurs during the immediate postnatal period in rodents (36). Shortly after birth, the retina becomes vascularized as blood vessels grow from the optic disc in the center toward the periphery of the retina. These vessels are dense and highly branched. Over the next few weeks, the vessels are remodeled in a Fas-dependent process into a sparser network of proper capillaries (36). We have observed in the CD47-null mice that this remodeling fails to take place, leaving a persistent, neonatal pattern of dense and highly branched retinal vascularization.[4] Together these emerging results indicate that the role of CD47 in Fas-mediated apoptosis may be of broad physiological significance.

The regulation of Fas-mediated apoptosis is one of the least understood aspects of Fas function. Clearly, the simple expression of Fas or its ligand cannot alone determine whether Fas-bearing cells are killed (1–5). We report here that expression of CD47 can enable Fas-mediated cell death, apparently by enhancing Fas clustering. Although the expression of CD47 has been said to be ubiquitous, it is not expressed on all cells all the time. For example, endothelial cells express high levels of CD47 in culture, but under laminar flow, CD47 expression

[3] J. Dimitry and W. A. Frazier, unpublished data.

[4] S. Wang, W. A. Frazier, and N. Sheibani, manuscript in preparation.

disappears. Expression of CD47 at sites of turbulent flow correlates with apoptosis of the endothelial cells (37). Staining of tissue sections reveals that there are wide variations in the level of CD47 expression *in vivo*[5] (22) that may contribute to the differential susceptibility of tissues and cell types to Fas-mediated apoptosis. Thus CD47 expression and association with Fas represents a new role for CD47 in enabling Fas-mediated apoptosis and raises the possibility that some properties and functions attributed to CD47 may in fact be dependent on Fas.

In conclusion, CD47 can affect apoptosis in two ways. It can directly disable mitochondrial function via a G_i-dependent process (17, 18), and it can augment the function of Fas, thus promoting a classical, caspase-dependent mode of apoptosis.

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REFERENCES

1. Strasser, A., O'Connor, L., and Dixit, V. M. (2000) *Annu. Rev. Biochem.* **69,** 217–245
2. Nagata, S. (1999) *Annu. Rev. Genet.* **33,** 29–55
3. Peter, M. E., and Krammer, P. H. (2003) *Cell Death Differ.* **10,** 26–35
4. Siegel, R. M., Chan, F. K-M., Chun, H. J., and Lenardo, M. J. (2000) *Nat. Immunol.* **1,** 469–474
5. Rathmell, J. C., and Thompson, C. B. (2002) *Cell* **109,** (suppl.) S97–S107
6. Kolalovich, K., Li, W., DeAngelis, R., Greenbaum, L. E., Ciliberto, G., and Taub, R. (2001) *J. Biol. Chem.* **276,** 26605–26613
7. Moorman, J. P., Praythers, D., McVay, D, Hahn, Y. S., and Hahn, C. S. (2003) *Virology* **312,** 320–329
8. Brown, E. J., and Frazier, W. A. (2001) *Trends Cell Biol.* **11,** 130–135
9. Ticchioni, M., Raimondi, V., Lamy, L., Wijdenes, J., Lindberg, F. P., Brown, E. J., and Bernard, A. (2001) *FASEB J.* **15,** 341–350
10. Li, Z., Calzada, M. J., Sipes, J. M., Cashel, J. A., Krutzsch, H. C., Annis, D. S., Mosher, D. F., and Roberts, D. D. (2002) *J. Cell Biol.* **157,** 509–519
11. Oldenborg, P.-A., Zheleznyak, A., Fang, Y.-F., Lagenaur, C. F., Gresham, H. D., and Lindberg, F. P. (2000) *Science* **288,** 2051–2054
12. Blazar, B. R., Lindberg, F. P., Ingulli, E., Panoskaltsis-Mortari, A., Oldenborg, P.-A., Iizuka, K., Yokoyama, W. M., and Taylor, P. A. (2001) *J. Exp. Med.* **194,** 541–549
13. Oldenborg, P-A., Gresham, H. D., Chen, Y., Izui, S., and Lindberg, F. P. (2002) *Blood* **99,** 3500–3504
14. Frazier, W. A., Gao, A.-G., Dimitry, J., Chung, J., Brown, E. J., Lindberg, F. P., and Linder, M. E. (1999) *J. Biol. Chem.* **274,** 8554–8560
15. Green, J. M., Zhelesnyak, A. M., Chung, J., Lindberg, F. P., Sarfati, M., Frazier, W. A., and Brown, E. J. (1999) *J. Cell Biol.* **14,** 673–682
16. McDonald, J. F., Zheleznyak, A., and Frazier, W. A. (2004) *J. Biol. Chem.* **279,** 17301–17311
17. Manna, P. P., and Frazier, W. A. (2003) *J. Immunol.* **170,** 3544–3553
18. Manna, P. P., and Frazier, W. A. (2004) *Cancer Res.* **64,** 1026–1036
19. Pettersen, R. D., Hestdal, K., Olafsen, M. K., Lie, S. O., and Lindberg, F. P. (1999) *J. Immunol.* **162,** 7031–7040
20. Mateo, V., Brown, E. J., Biron, G., Rubio, M., Fischer, A., Le Deist, F., and Sarfati, M. (2002) *Blood* **100,** 2882–2898
21. Johansson, U., Higginbottom, K., and Londei, M. (2004) *Scand. J. Immunol.* **59,** 40–49
22. Reinhold, M. I., Lindberg, F. P., Plas, D., Reynolds, S., Peters, M. G., and Brown, E. J. (1995) *J. Cell Sci.* **108,** 3419–3425
23. Johnstone, R. W. Cretney, E., and Smyth, M. J. (1999) *Blood* **93,** 1075–1085
24. Rebres, R. A., Green, J. M., Reinhold, M. I., Ticchioni, M., and Brown, E. J. (2001) *J. Biol. Chem.* **276,** 7672–7680
25. Brown, E. J., Hopper, L., Ho, T., and Gresham, H. D. (1990) *J. Cell Biol.* **111,** 2785–2794
26. Brunner, T., Mogil, R. J., LaFace, D., Yoo, N. J., Mahboubl, A., Echeverrl, F., Martin, S. J., Force, W. R., Lynch, D. H., Ware, C. F., and Green, D. R. (1995) *Nature* **373,** 441–444
27. Reinehr, R., Graf, D, and Haussinger, D. (2003) *Gastroenterology* **125,** 839–853
28. Jung, K. C., Kim, N. H., Park, W. S., Park, S. H., and Bae, Y. (2003) *FEBS Lett.* **554,** 478–484
29. Tibbetts, M. D., Zhen, L., and Lenardo, M. J. (2003) *Nat. Immunol.* **4,** 404–409
30. Holler, N., Tardivel, A., Kovacsovics-Bankowski, M., Hertig, S., Gaide, O., Martinon, F., Tinel, A., Departhes, D., Calerara, S., Schulthess, T., Engel, J., Schneider, P., and Tschopp, J. (2003) *Mol. Cell. Biol.* **23,** 1428–1440
31. Cremesti, A., Paris, F., Grassmé, H., Holler, N., Tschopp, J., Fuks, Z., Gulbins, E., and Kolesnick, R. (2001) *J. Biol. Chem.* **276,** 23954–23961
32. Carman, C. V., and Springer, T. A. (2003) *Curr. Opin. Cell Biol.* **15,** 547–556
33. Barazi, H. O., Li, Z., Cashel, J. A., Krutzsch, H. C., Annis, D. D., Mosher, D. F., and Roberts, D. D. (2002) *J. Biol. Chem.* **277,** 42859–42866
34. Hu, Y., Nakagawa, Y., Purushotham, K. R., and Humphreys-Beher, M. G. (1992) *Am. J. Physiol.* **263,** E607–E614
35. Baxter, A. G., and Mandel, T. E. (1991) *Eur. J. Immunol.* **21,** 2051–2055
36. Ishida, S., Yamashiro, K., Usui, T., Kaji, Y., Ogura, Y., Hida, T., Honda, Y., Oguchi, Y., and Adamis A. P. (2003) *Nat. Med.* **9,** 781–788
37. Graf, R., Apenberg, S., Freyberg, M., and Friedl, P. (2003) *Apoptosis* **8,** 531–538

[5] P.-A. Oldenborg and W. A. Frazier, unpublished data.